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IMMUNOCHEMISTRY
OF
CREATINE-KINASE

A Thesis submitted
to the
University of Glasgow
for the degree of
Doctor of Philosophy
in the
Faculty of Science
by
SAFI CHERIF

September 1979

Department of Biochemistry,
University of Glasgow.

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Summary

Rabbit muscle creatine kinase has been found to be weakly immunogenic in sheep. Of twelve sheep immunized with the enzyme only one animal gave an immune response. Of six chickens tested with the same enzyme, antiserum could be detected in four but the solubility properties of the antibodies isolated rendered them less suitable for experimental work. Further work was pursued using antiserum from the single responsive sheep. The animal was killed in order to obtain large amounts of antisera. This work therefore in part represents an attempt to investigate the immunological properties of a weak immunogen.

Creatine kinase was covalently attached to sepharose 4B gel by the cyanogen bromide technique. This immobilized enzyme was used to investigate the stoichiometry of binding of the antibodies.

Two bleedings of the secondary response of the responsive sheep were made at an interval of $3\frac{1}{2}$ days. It was observed that the antibodies of the first bleeding precipitated CK more readily either in gel media or liquid media than did the antibodies of the second bleeding. The immobilized dimeric enzyme was found to bind 3.5 antibody molecules of the first bleeding and 2.1 - 2.9 antibody molecules of the second bleeding. Work with other and, in all cases, stronger immunogens suggests that values of 5 or 6 might be expected for a protein of this size.

Most of the antibody protein bound could be eluted by a buffer of pH 2.2 from the immobilized immune complex using antibodies of the second bleeding; with the first bleeding elution was only partial. Most of the antigen dimer remained associated under these conditions. The antibody protein eluted was

found to have lost most of its antigen-binding capacity.

Immobilized monomeric creatine kinase was prepared; using the second bleeding a valency of 1.2 was found.

The two essential thiol groups of the creatine kinase dimer were alkylated by reaction with iodoacetamide. Single radial immunodiffusion studies using the first bleeding were carried out to compare the antigenicity of the native and modified enzyme; there was an unusual cross-reaction pattern which could be interpreted as a higher affinity for the antibody population by the modified enzyme. The alkylated enzyme was found to bind 3.8 antibody molecules; none of them could be eluted with pH 2.2 buffer. With the second bleeding the modified enzyme behaved like the native enzyme.

Both antibody populations were found to inhibit the enzyme activity of soluble creatine kinase. Those of the second bleeding appeared to inhibit completely in excess but those of the first bleeding did not.

With the immobilized enzyme inhibition by an excess of either of the antibody populations, especially with those of the second bleeding, was much less.

The unretarded antibodies resulting from immunoabsorption of antibodies of the second bleeding inhibited creatine kinase almost as effectively as the unabsorbed antibodies; those of the first bleeding showed much less inhibition.

Some differences of the antigenicity of various liganded forms of creatine kinase were found using antibodies of the first bleeding; with those of the second bleeding the antigenicity was mostly the same as for the native enzyme. When the enzyme was present as the tightly-bound creatine- NO_3^- - MgADP complex, which is believed to be an analogue of the transition state of the enzyme

catalysed reaction, it showed a significant decrease of immune precipitate and bound only 1.6 antibody molecules of the first bleeding; these could not be eluted using a pH 2.2 buffer.

The unretarded antibodies resulting from this immunoabsorption were found to inhibit the enzyme activity of soluble creatine kinase. In the presence of Mg ADP and creatine but without NO_3^- ions the antigenic valency was the same as for the native enzyme but none of the antibodies were found to be eluted at pH 2.2

The experiments described indicate that in immunoabsorption only high affinity antibodies are detected whereas in solution both high and low affinity antibodies are likely to be detected due to their higher avidity in the immuno-complex.

These results have been interpreted in terms of a model system where each subunit of the antigen has two determinants, one with high affinity for homologous antibodies, the other with a lower affinity. Some or all of the low affinity antibodies have the property of inhibiting enzymic activity. The affinities of all the corresponding antibodies of the first bleeding appear to be higher than those of the second bleeding.

Abbreviations

The abbreviations used in this thesis are as laid down in the Biochemical Journal, Instructions to Authors (revised form, 1978) with the following additions.

B.S.A.	Bovine serum albumin
CK	Creatine kinase
CK, MM	Muscle type creatine kinase
CK, MB	Hybrid of muscle and brain type creatine kinase
CK, BB	Brain type creatine kinase
D.E.C.	Dead end complex (creatine kinase + MgADP + creatine)
D. T. T.	Dithioerythritol
DTNB	Di-thio bis-(2-nitrobenzoic acid)
H	Heavy chain
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgA	Immunoglobulin A
L	Light chain
M.N.P.	Mercuri-nitrophenol
SDS	Sodium dodecyl sulphate
T.E.M.E.D.	NNN' ¹ N ¹ -Tetramethylethylene diamine
TSA	Transition state analogue complex (creatine kinase + MgADP + creatine + NO ₃ ⁻)

INTRODUCTION

Chapter 1: INTRODUCTION

1) Review of Literature

a) Nomenclature and definition

Creatine kinase (adenosine 5^g → triphosphate creatine phosphotransferase, EC 2.7.3.2) is a dimeric enzyme of molecular weight 81,000 Daltons which catalyses the reaction:



The reaction proceeding from left to right is arbitrarily designated as the forward reaction.

b) Structure

i) Primary structure amino acid sequence

Of the 360 or so amino acids comprising each subunit only a few short lengths representing less than 10% of the total sequence are known: for a better review see Watts (1973). Attention has also been drawn to the similarity of the amino acid composition of all phosphagen kinases and the relative constancy of the ratios of the sum of the amino acids (acidic + basic)/ hydrophobic found per mass of protein (Morison, 1973).

Thomson et al. (1964) and Atherton et al. (1970) showed that the sequence around the single cysteine residue which is readily and specifically labelled with alkylating agents is identical in the brain and muscle type enzymes except that valine is replaced by isoleucine in brain type CK. The sequences reported for the other phosphagen kinases (arginine kinase) are also very similar (Morison, 1973).

A further striking feature of all the sequences is the presence of proline adjacent to the essential cysteine residue. Markham et al. (1977) by using the method of calculations of Chou and Fasman (1974) found that the position of this thiol is predicted at a β turn separating two portions of β sheet. It seems reasonable to assume that, since this amino acid is a "misfit" in this region of the protein structure, its occurrence in this position is of considerable significance. One of the characteristics of creatine kinase and several other sulphhydryl enzymes is the unexpected finding that the reactivity of their essential thiol groups to iodoacetate or iodoacetamide does not vary between pH 6 and 10. This had led to the suggestion that these thiol groups are hydrogen bonded to histidine (Watts & Rabin, 1962).

Thomson et al. (1964) found a histidine residue in the sequence around the reactive SH group of creatine kinase which in the folded polypeptide chain might well exist as a thiol-imidazole pair.

ii) Secondary and tertiary structure: subunit shape and organisation

Thomson et al. (1964) showed that native CK consists of two freely dissociable subunits, each consisting of a single polypeptide chain containing no disulfide bonds. Sedimentation studies by Yue et al. (1967) under various conditions confirm this picture. A useful finding was that, while guanidinium hydrochloride dissociates and unfolds the subunits into the random coil configuration, SDS causes them to dissociate without any apparent loss of structural organisation. It is worth recalling that SDS is a mild denaturant (trypsin for example retains a part of its enzymic activity in SDS (1% solution)).

As the native enzyme is resistant to tryptic cleavage, the molecule can be envisaged as globular and compact in shape.

c) Thiol content

i) Total thiol content

The literature is controversial over the thiol group content of creatine kinase. Bayley and Thomson (1967) showed that the number of thiol groups reacting depends on the nature and severity of the denaturing conditions; they found that the titration of the thiol groups by Ellman's reagent DTNB is maximum at about 1 h in urea (8M) or SDS (1%), whereas the reaction is complete after 2 min in guanidinium hydrochloride (6M). This effect showed the accessibility of the DTNB only after complete unfolding of the molecule and recalls the finding of Yue et al. (1967). However (Rybina et al., 1971; Rybina & Chetverikova, 1974), by using amperometric titration in the absence of sulfite found one disulfide bond per subunit the denatured form of CK. These workers claimed that they had thereby demonstrated the presence of a disulfide bond in an intracellular protein. However, it should be pointed out that this result may have been due to rapid oxidation of exposed sulphhydryl groups in the denatured protein. In summary, muscle type CK contains 8 cysteine residues per dimer. All mammalian enzymes investigated so far (Kuby et al., 1970; Keutel et al., 1968, 1972) have similar thiol content and also similar specific activities.

ii) The "Essential thiol group"

The investigations of the cysteine residue near the catalytic site are reviewed by Watts (1973). Since then the picture has been slightly modified.

Rabbit skeletal muscle creatine kinase has a single fast reacting cysteine residue per active site; Watts et al. (1961) showed, using an iodide-sensitive electrode to estimate the extent of the chemical reaction, that the reaction with iodoacetate is proportional to the loss of enzymic activity. Complete reaction resulted in almost complete activity loss. Watts and Rabin (1962) originally observed that there was protection by an equilibrium mixture of substrates against iodoacetamide inhibition. Nitrate was present in their experiment so their equilibrium mixture protection must have been due to creatine- NO_3^- -MgADP formation. Milner-White and Kelly (1976) measured the chemical reaction by incorporating ^{14}C labelled iodoacetamide into the enzyme and studied more closely the effect of the substrate equilibrium mixture.

This thiol group was judged to be essential because its chemical reaction was found to obliterate enzymic activity. However, Kenyon et al. (1974, 1975) reported that the degree of inhibition of creatine kinase by a variety of common thiol reagents was determined by the chemical properties of the blocking group introduced such as bulk, charge, hydrophobicity and hydrogen bonding ability. Thus when the active thiol groups are fully substituted with the relatively small neutral non-hydrogen-bonding methane thiol group (CH_3S) using methyl-methane thiosulfonate, the enzyme retained 18% residual activity. The physical studies of Markham and Reed (1977) indicated that the reaction with the CH_3S group greatly diminished the catalytic efficiency of the enzyme with little effect on substrate binding. However, Reddy & Watts (1978) by using a much smaller alkylating reagent (iodomethane) showed that the methylated CK formed retained only 2% of residual activity. It may be concluded that whatever the explanation

for the residual activity observed by Kenyon et al. after attachment of the CH_3S group to the essential thiol groups of rabbit muscle CK the answer does not reside in the small size of the agent used.

Recently Der Terrossian & Kassab, (1976) prepared S-cyano derivatives of creatine kinase by reacting the two accessible thiols with Ellman's reagent. The resulting inactive mixed disulfide derivative was then subsequently substituted with ^{14}C cyanide, a small uncharged thiol-blocking group. The modified enzyme contained 1.6 mole label/mole protein and showed by titration with Ellman's reagent and amino acid analysis a concomitant loss of about 0.8 – 0.9 SH group per subunit. This mono-S-cyano derivative of CK was found to be 73% as active as the native unmodified protein. It was still able to react in the native state with a variety of thiol reagents with the further blocking of another pair of thiol groups; their substitution once more with cyanide resulted in the bis-S-cyano derivative of CK, which had lost 2 thiols per subunit and had about 50% of the original catalytic activity.

This work suggests that the four cyanylated thiol groups are not required for the catalytic activity of creatine kinase. These ^{14}C cyano protein derivatives may serve in radioimmunological studies needing a catalytically active enzyme; on the other hand the preparation of this derivative labelled with (^{13}C) cyanide opens up the possibility of probing the environment of the presumed essential thiol group by means of ^{13}C nuclear magnetic resonance spectroscopy. Partial peptide bond cleavage of the S-cyano derivatives of CK by the method of Jacobson et al. (1973) which promotes specific chemical peptide bond cleavage at the thiocyanolanyl residues, generate fragments which may be helpful in

amino acid sequence determination of the protein. (By this method they found that the mono-S-cyano derivative of CK is split into a large fragment of MW > 37000 daltons and a small peptide; this suggests that the presumed essential thiol is located near one end of the polypeptide chain.)

Quioco et al. (1973, 1977) have shown that the 2 mercuri-4 nitrophenol group can be introduced into the enzyme from chicken breast muscle at a thiol group distinct from that which is reactive towards iodoacetamide. They also showed that the spectrum of the 2 mercuri-4 nitrophenol group changes on addition of various substrate and ligand combinations.

d) Anion effect. Hypothesis of the transition state analogue complex.

The first attempts to study the effect of anions on creatine kinase led to the conclusion that small anions interacted with a site on the enzyme that normally bound the transferable phosphoryl group of the substrate. But unfortunately interpretation of this work was ambiguous because so much of the research on CK was carried out using chloride buffers or solutions containing other anions that materially affect the interpretation of the results.

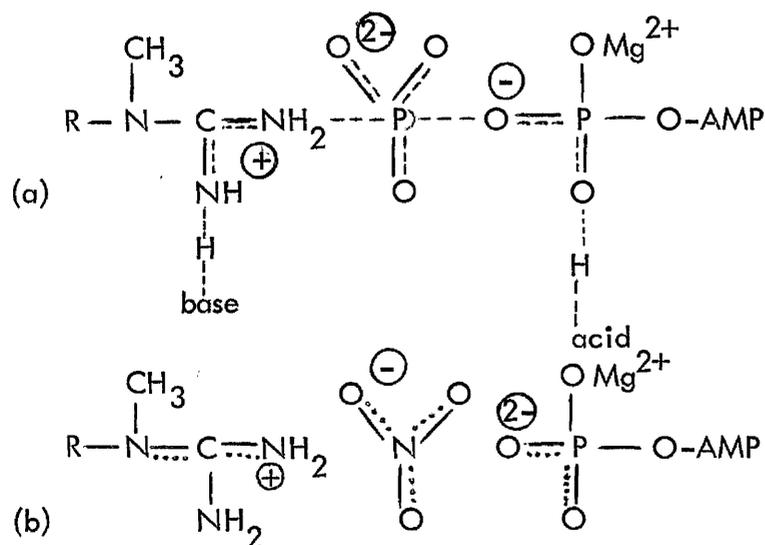
The problem was seriously tackled by Milner-White and Watts (1971). They showed that small anions can be divided into three groups according to the way in which they affect creatine kinase.

I. Acetate reversibly increases enzyme activity in the forward reaction but does not affect the rate of inhibition by iodoacetamide in the presence of creatine + MgADP.

II. Planar anions and some halides (HCO_3^- , HCO_2^- , NO_3^- , NO_2^- , Cl^- ,

Br^- , F^-) in the presence of creatine plus MgADP protect the enzyme against inhibition by iodoacetamide.

III. Tetrahedral anions (SO_4^{2-} , HPO_4^{2-} , ClO_4^- , BF_4^-) and iodide do not affect the rate of inhibition by iodoacetamide in the presence of creatine plus MgADP but may decrease the protection by class II anions under these conditions. Hence class II anions form an extremely stable and inactive quaternary enzyme - creatine-MgADP - anion complex and this is responsible for the effect attributed by previous workers to the ternary complex lacking anion. Small, planar anions such as NO_3^- are particularly effective in this respect and the hypothesis has been presented that the anion acts by simulating a planar phosphoryl group in a stable intermediate complex inferred to occur in the course of the reaction.



Scheme 1. Diagrammatic representation of (a) the postulated transition state of the creatine kinase reaction and (b) the quaternary dead end complex showing how this can simulate the transition state.

It appears as illustrated in scheme 1 that the NO_3^- ions occupy the site of the phosphoryl group transferred between the substrates in the working enzyme and mimic the planar phosphoryl group that is expected to be a feature of the reaction mechanism. The NO_3^- ions greatly enhance the binding of the components of the dead end complex creatine -MgADP to the enzyme. The strong binding suggests that this complex behaves as a transition-state analogue (it will be referred to below as such, although the postulated planar phosphoryl group may be an intermediate rather than a transition state). Certain other anions of suitable shape may replace NO_3^- (Chegwidden & Watts, 1976).

e) Substrate-induced conformational changes

There is now compelling evidence to believe that the trans-phosphorylation process involves a conformational change in creatine kinase.

The first evidence for a conformational change came from immunological experiments. Samuels (1961) found that inhibition of creatine kinase activity by chicken anti-rabbit muscle enzyme antibodies was prevented by creatine plus MgATP but not by either substrate alone. This observation was later confirmed by other immunological experiments. Benjamin and Robin (1975) indeed observed a decrease of immunoprecipitate when creatine kinase was incubated with creatine plus MgATP prior to the addition of specific antibodies (goat anti-rabbit enzyme). They also demonstrated that the transition state analogue complex was more effective than the working enzyme in decreasing the antigenic reactivity of creatine kinase towards its specific antibodies. These observations suggest that the working combination of substrates or substrates analogue altered the conformation of the enzyme so that the antibody would

not bind.

On addition of NO_3^- ions to the dead-end complex, various changes occur in the native enzyme. A spectral change in the tryptophan side chains, indicating transfer of the residues to a more polar environment (Watts, 1973), occurs. Also there is a change in an attached chromophoric probe (Quioco & Thomson, 1973), the reactivity of a thiol group is diminished by a factor of 200 (Milner-White & Watts, 1971; Quioco & Olson, 1974). Working substrates have a similar effect on the thiol, but only decrease its reactivity by a factor of 5 (Milner-White & Kelly, 1976). These authors showed that protection from iodoacetamide inhibition is due to the "working" enzyme complexes, either the forward "working" substrates creatine and MgATP or the backward "working" substrates phosphocreatine and MgADP or CaADP.

These findings suggest that the conformation of the enzyme in the TSA complex is similar to that of the working enzyme, as judged by the quite similar reactivity of the thiol group enzyme towards iodoacetamide, and the different quaternary complex (enzyme creatine MgADP plus NO_3^-). These findings strongly suggest that these thiols are located near the catalytic site.

Keighren and Price (1977) used the 2-mercuri-4 nitrophenol reporter group to examine the ligand induced conformational changes of various MNP derivatives of the rabbit muscle enzyme and in particular to study those conformational changes that are associated with formation of the TSA "complex". Their results support the hypothesis that the integrity of the reactive thiol group is required for conformational changes in the enzyme that are associated with

formation of the catalytically active complex, but is not apparently required for formation of the binary (enzyme + MgADP) or ternary (enzyme + MgADP + creatine) complexes.

E.p.r. (electron paramagnetic resonance) work on the creatine- NO_3^- -Mn ADP complex by Reed and Cohn (1972) has shown that the coordination sphere of the metal ion becomes much less accessible to solvent water. McLaughlin et al., (1976) have used the creatine formate - Mn ADP complex for a nuclear magnetic - resonance mapping study on the conformation of the bound ligands. McLaughlin (1974), Price and Hunter (1976) have shown that the affinity of binding of MgADP, only when it is binding as the creatine- NO_3^- - MgADP complex, appears to be different for the two sites of the dimeric enzyme.

The effect of this complex on the proteolytic digestion of the enzyme is noteworthy. It was found, by using pig trypsin (Milner-White & Young, 1975) that addition of NO_3^- ions to the dead-end complex did not cause any additional effect on the nature or rate of digestion of creatine kinase. The effects found with proteinase K are presented in our paper (Williamson et al., 1977). The transition state analogue complex appeared to protect the enzyme completely against proteolytic digestion.

f) Isoenzyme distribution

In vertebrates the soluble creatine kinase occurs as three forms, readily distinguishable by their electrophoretic mobility. Dawson et al. (1965) showed that the form with intermediate mobility was the hybrid of the fast and the slow form; these were respectively found in the brain and muscle and subsequently

10a
Fig. 1 Scan of CK stained gel electrophoresis of different muscle extracts.

A: cytoplasmic skeletal muscle

B: mitochondrial, muscle

C: cytoplasmic heart muscle.

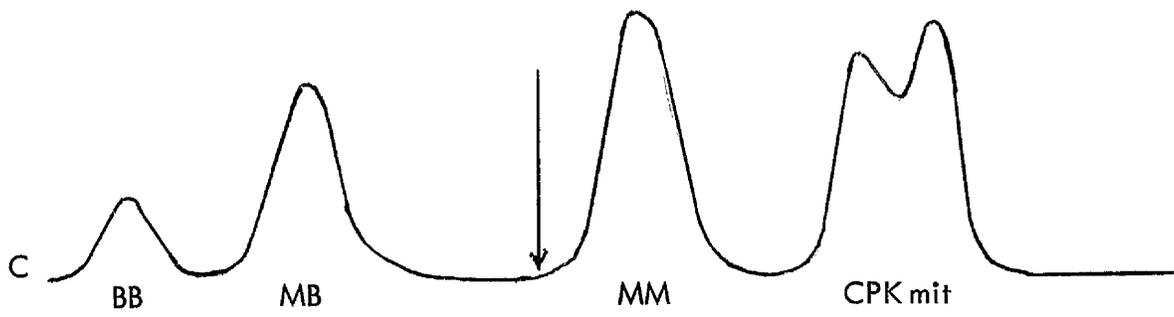
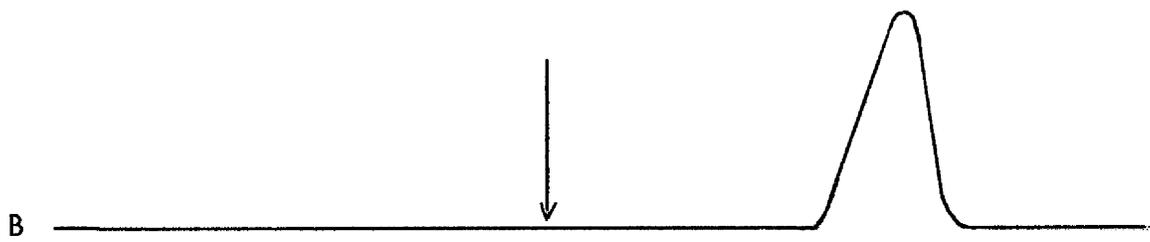
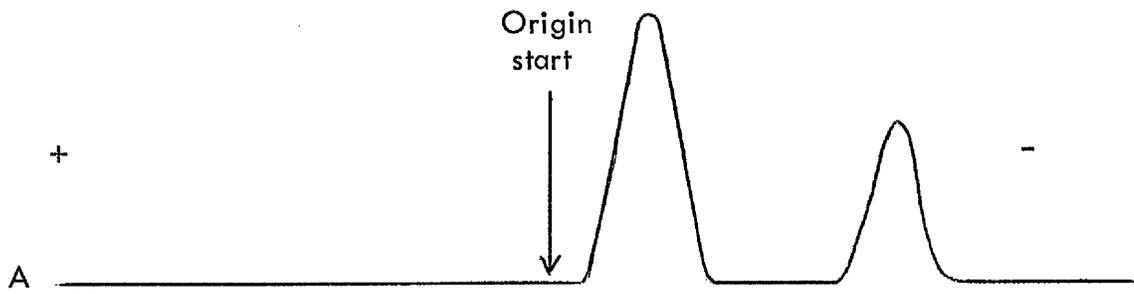
BB refers to the brain type CK

MM refers to the muscle type CK

MB refers to the hybrid CK

CPK mit refers to the mitochondrial CK.

After Saks et al. (1977)



called BB and MM. The hybrid was called MB.

i) Changing pattern during development

In brain, only the BB form exists (Dawson et al., 1968; Kuby et al., 1970) but recently Lindsey & Diamond (1978) claimed that they have isolated significant quantities of MM in the brain (basal ganglia).

During ontogeny the changing isoenzyme pattern in skeletal and cardiac muscles is similar among various mammalian species. Dawson et al. (1968) observed that only the BB form exists in the very young foetus. As development proceeds MB activity gradually appears followed by MM activity. BB activity diminishes and shortly after birth the adult pattern in which MM activity predominates together with lesser amounts of MB and only a trace of BB. The transition is less complete in heart than in skeletal muscle.

ii) Isoenzyme spectrum

Saks et al. (1977) found that the isoenzymes of CK contained in the skeletal and myocardium have very similar kinetic properties. However, the isoenzyme spectra of skeletal and heart muscles differ both qualitatively and quantitatively; the skeletal muscles contain mainly isoenzyme MM and a small amount of the mitochondrial isoenzyme, in myocardium the mitochondrial isoenzyme constitutes a considerably larger proportion of the total CK activity (30%) see Fig. 1.

g) Molecular forms of muscle type CK

Graig and Truagott (1973) resolved purified rabbit muscle CK MM isoenzyme into a pattern of six sub-bands by prolonged starch gel electrophoresis at pH 6.3 in a Tris-citrate buffer system. By conventional agar gel electro-

phoresis, Dawson et al. (1965) and Saks et al. (1977) found the same isoenzyme appearing as single homogeneous band. However in other stabilizing media sub-banding (existence of a major isoenzyme in multiple bands of different electrophoretic mobility) of the MM has been reported. The molecular basis of mammalian CK sub-banding is unknown. In the work of Graig and Truaugott (1973) this may be due to the unusual electrophoresis conditions (starch gel electrophoresis for 18 h at 150 V in low buffer ionic strength pH 6.3). Cattan et al. (1978) also resolved a sub-banding pattern by polyacrylamide gel isoelectric-focusing. They found a pI of value around 6.3 for the three different main species; this work may explain the unusual sub-banding pattern reported by Graig et al. Indeed at pH 6.3 the species with different pI values have a small net charge difference and hence slightly different mobility which in turn increases the electrophoretic resolution at lower ionic strength for a longer run.

Turner (1973), by using immunological and electrophoretic techniques, identified a protein that binds specifically to the M-line of skeletal muscle as the muscle form of creatine kinase. Studies of the interactions of MM CK with myosin and myosin fragments may lead to a better understanding of the structure of the M-line protein. The catalytic activity of creatine kinase may well be affected in important ways when it is bound to the myofibril.

h) Non-identical behaviour of CK subunits

Haughland (1975) first observed difference in reactivity of the two subunits with the covalent hydrophobic fluorescent probe 2- [4-(2-iodo acetamido) phenyl] aminonaphthalene-6-sulfonic acid. However there is no clear

indication from this work on the location of the binding site of this probe.

Hence it is difficult to draw any conclusive evidence on the biphasic behaviour of the enzyme which was originally reported by the author.

Price and Hunter (1976) showed that under conditions in which rabbit muscle CK forms the postulated TSA complex the two subunits of the enzyme behave non-identically both in terms of ADP binding and thiol group reactivity. This is in accord with our finding (Williamson et al., 1977). The time course for the proteolytic inactivation by proteinase K in the presence of this complex, but not in its absence, was biphasic.

i) Physiological role

The enzyme has a wide tissue distribution; quantitative data on the iso-enzyme spectrum in human tissue are given by Wretou and Pfeleiderer (1975). This can generally be associated with the physiological role of ATP regeneration in conjunction with contractile or transport systems. Saks et al. (1977) investigated the role of CK isoenzymes in the energy metabolism of skeletal muscles and myocardium; they found identical mechanisms of transport of energy from the mitochondria to the cytosol with the participation of the mitochondrial CK isoenzyme; this provides complete conversion of the energy of mitochondrial ATP (resulting from oxidative phosphorylation) to the energy of creatine phosphate. This is shown by the dependence of the rate of creatine phosphate synthesis in skeletal muscle mitochondria on the rate of oxidative phosphorylation or on the rate of ATP transport from the mitochondrial matrix.

j) Duchenne Muscular Dystrophy

Duchenne Muscular Dystrophy is an x-linked progressive muscular dystrophy. The phenotype expression of an abnormal genotype shows in young males who die before the age of 19; the females are carriers of the disease. For a better review on the subject, see Das et al. (1976) and Smith (1977). The exact role of CK in this disease has yet to be evaluated. However, recently Kuby et al. (1977) isolated three human CK isoenzymes from tissues of patients with Duchenne Muscular Dystrophy. These direct isolation studies have led to the remarkable observation that the isoenzymic distribution pattern of CK in the atrophied musculature of the terminal human progressive muscular dystrophic organism appears strikingly similar to that of the human fetal muscle. Work is now in progress to compare their kinetic and physico-chemical properties and to detect any differences between the dystrophic isoenzymes and their normal human counterparts with the eventual goal of determining whether a structural alteration of this enzyme has taken place which might be characteristic of Duchenne Muscular Dystrophy.

k) Clinical interest

Creatine kinase has been of special interest to those engaged in research in the muscular dystrophies since elevated enzymatic activities in the serum have proved to be of great assistance in the early diagnosis of these disorders; Smith (1977) and Thomson et al. (1975) reviewed its clinical application for detecting the female carrier in the sex-linked Duchenne type muscular dystrophy.

Roses et al. (1976) describe a new method for detecting a maternal carrier; they measured the endogenous phosphorylation of peak 11 of the erythrocyte membrane and were thus able to identify some mother carriers who did not show abnormal levels of CK.

Roberts and Painter (1977), Roberts et al. (1978) introduced a radioimmunoassay to measure micro-gramme amounts of the MB form of CK. Sobel et al. (1976) used the determination of the MB isoenzyme in serum to the diagnosis of cardiac disease. For this purpose Witteven et al. (1978) applied both the chromatographic and the immunologic method but found that none of these assays were entirely satisfactory in clinical diagnosis of cardiac disease. Among other clinical tests used in daily practice are the automated fluorometric CK assay of Armstrong et al. (1974), the estimation of CK B subunit activity by immunologic inhibition of M activity of Gerhard et al. (1977), a modified version of the method described by Wretou and Pfiederer (1975). Markhu et al. (1977) claimed that an estimate of brain type CK activity in serum was useful for monitoring neurological damage. Bell et al. (1978) used a radioimmunoassay to measure BB in serum and cerebrospinal fluid for diagnosis of neurological disorders.

2) Aims of the Thesis

a) General Approach

Enzyme-anti enzyme systems can serve as a valuable tool in the study and localisation of antigenic specificity determinants. The use of enzymes as antigens rather than other proteins in this type of investigation offer an

advantage because the antigens possess biologic activity that resides in a limited area of the molecule and antibodies specific toward this or related regions may have an effect on the catalytic activity. Consequently the contribution of different antigenic determinants (defined by Goodman in his review, 1975) to the immunologic reactivity of an enzyme can be evaluated in the light of their relationship to its catalytic site. Conversely, elucidation of the immunologic behaviour could provide a sensitive probe of the surface conformation of enzymes. While this point is discussed in detail by Arnon (1975), let us first consider two important immunologic concepts: immunogenicity and antigenicity.

Crumpton (1975) used the term immunogenicity to describe the capacity of a protein to stimulate antibody production and antigenicity as the capacity to interact with antibody produced. The capacity of a protein to elicit an immune response reflects a complex series of interacting parameters such as its physical state, the degree of dissimilarity between its structure and the homologous protein of the immunised animal, its rate of catabolism, the amount injected, the species and strain of immunised animal, etc. With regard to the role of foreignness in eliciting an immune response the results of various studies indicate that the immunogenicity of a protein is related to the degree of phylogenetic difference between the source of the immunogen and the animal species used for immunization. This phenomenon is most probably an expression of the general principle that antibodies are formed against only those areas of a foreign protein which are not shared by the animal's own proteins; then antigenic determinants should be primarily associated with the non-conserved portions of the amino acid sequence and conversely the regions of sequence

Fig. 2 Phylogenetic tree showing the evolution of cytochrome c.

It was constructed by computer from the amino acid sequences of the cytochrome c's of many species. Each circle represents the sequence of a cytochrome c deduced to be ancestral to all species higher in the branches leading from that circle. The small figures alongside each branch indicate the number of amino acid residue differences, per 100 residues, from the ancestor. Thus, mung bean cytochrome c is different from its ancestor by 5 residues per 100, whereas sesame cytochrome c is 2 residues per 100 different.

(Adapted from Dayhoff et al., 1972)

identity should be immunologically silent.

Hence immunological methods are used to follow biochemical evolution of enzymes. The extent of cross-reaction and cross-inhibition of homologous enzymes by their specific antibodies seems to be dependent on the phylogenetic distance between the species compared and on the similarity in their amino acid sequence; but the latter was reported for lysozymes by Arnon in his review (1975) to be a more decisive factor than zoological relationship. Margoliash and his colleagues (1972) have carried out a comprehensive study of amino acid sequence variations in the cytochrome *c* isolated from over a wide range of different species. The cytochrome *c*'s from many species have been used to construct a phylogenetic tree showing the number of amino acid residue differences. Although the tree of Fig. 2 concerns cytochrome *c*, the general rule emerging from it is that the more homologous proteins (e.g. equifunctional enzymes) of different species are unrelated, the better is the immune response.

In this work the immunogenicity of rabbit muscle CK in chicken and sheep is studied.

b) Outlines of the Project

The main purpose of this thesis is to produce specific anti-rabbit muscle creatine kinase antibodies and to use them as probes for conformational changes in the enzyme upon binding of ligands, substrates and the transition-state analogue complex. The antibodies are also used to study these conformational changes in the immobilized enzyme. On the other hand, the antigenicity of immobilized CK dimer and CK monomer is studied; the role of conformation in antigenicity is assessed by comparing the immunologic behaviour of the native

enzyme; the chemically modified enzyme, the proteinase K cleaved CK fragment and the denatured enzyme.

MATERIALS AND METHODS

Chapter 2:

MATERIALS AND METHODS1) Materialsa) Animals

All experiments on animals were carried out under licence from the Home Office.

Chickens: Six chickens (fowl and hen) of the same age = 3 months, were kept at Yorkhill Hospital Animal House for the period of the experiment.

Sheep: Twelve sheep were constituted into 2 lots, each group of sheep was from the same breed. The sheep (18 months old) of about the same weight were kept at Cochno Experimental Farm, Glasgow for the period of the experiments.

b) Chemicals

Freund's complete adjuvant and Freund's incomplete adjuvant were from Difco Laboratories (Detroit, Michigan). DEAE cellulose (DE 52) precycled was from Whatman (Springfield Mill, Maidstone, Kent, U.K.). Hexokinase, glucose 6 phosphate dehydrogenase, proteinase K from tritirachium album Limber and rabbit muscle aldolase were obtained from Boehringer Corp., Lewes, Sussex, U.K. Hen egg ovalbumin (grade V), pig stomach pepsin, papain, horse heart cytochrome c and sperm whale myoglobin were from Sigma Chemical Co., Kingston, Surrey, U.K. Bovine serum albumin was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Rabbit muscle aldolase, cross-linked by dimethyl suberimidate, was prepared by Dr. J. Lumsden and Dr. J.R. Coggins using their method (Lumsden & Coggins, 1977).

Coomassie Brilliant Blue was from Serva Feinbiochemica, Heidelberg, West Germany. ATP and ADP were from Boehringer, creatine phosphate from Sigma. Creatine and Bicine [NN-bis-(2 hydroxyethyl) glycine] from BDH were recrystallized once from 1 mM EDTA, pH 9.0, and once from water. Iodoacetamide (BDH Chemicals) was recrystallized five times from aq 50% (V/V) ethanol.

Agarose and tris (hydroxy methyl amino-methane) were from Sigma. Sephadex, Sepharose 4B and CN Br-activated Sepharose 4B were from Pharmacia (GB) Ltd., Paramount House, 75 Uxbridge Road, London. Dialysis tubing from BDH were boiled for 5 min in 1 mM EDTA, pH 9 before use. Ampholine carrier ampholytes of pH ranges 3.5 - 10 and 5 - 8 were from LKB Ltd., 232 Addington Road, South Croydon, Surrey. All other chemicals were analytical reagent grade from BDH Chemicals.

c) Sheep antibodies

In the present work, these have mainly been used, either as the original antiserum or as the IgG fractions; the latter were purified and concentrated as described in the Methods section (h). These two forms may derive from either the first or the second bleedings as described in Methods section (g).

d) Creatine kinase

The enzyme was purified from rabbit back and leg muscle and stored as previously described (Kuby *et al.*, 1954; Kelly & Milner-White, 1976; Williamson *et al.*, 1977). It was 95% pure by the criteria of SDS gel electrophoresis and had a specific activity in the forward direction of 140 $\mu\text{moles H}^+/\text{mm}/\text{mg}$.

2) Methods

a) Buffers

Phosphate EDTA buffer: The pH of a solution containing: Na_2HPO_4 , 10 mM, NaH_2PO_4 , 10 mM, EDTA, 1 mM was adjusted to pH 8 with NaOH (2 N).

Tris-acetate or Tris HCl buffer: Solutions containing Tris (10 mM or 20 mM or 100 mM) were made to pH 8.6 with acetic acid. Solution containing Tris 0.375 M was made to pH 8.8 with HCl (N).

Glycine-HCl buffer: A solution containing glycine (0.2 M) and NaCl (0.5 M) was made to pH 3.0 with HCl.

Glycine-NaOH buffer: Solutions containing glycine (0.1 M) were made to pH 8 with NaOH (2 M).

Bicarbonate buffer: Solutions containing NaHCO_3 (0.1 M) were made to pH 9 with NaOH (2 M).

Veronal buffer: One litre of the buffer for immunoelectrophoresis contained sodium barbitone (sodium diethyl barbiturate) 10.3 g, acid barbituric, 1.84 g, and made to pH 8.6 with NaOH (2 M).

Electrode buffer: The buffer contained: Tris, 0.025 M; glycine, 0.192 M and SDS, 0.1%.

b) Anion exchange chromatography

A further purification on DEAE cellulose was achieved by the method of Sasa and Noda (1964) but using a linear gradient of Tris (0.01 M, 0.1 M) acetate buffer pH 8.6.

c) Gel filtration

Gel filtration of proteins was performed according to the procedure described by Andrew (1965).

d) Polyacrylamide gel electrophoresis

i) For soluble proteins

Electrophoresis in 1% polyacrylamide gels in the presence of SDS was carried out as described by Weber and Osborn (1969). To the reduced and detergent treated protein samples was added 10 μ l of 0.05% (W/V) Bromophenol Blue; after electrophoresis the position of the dye was marked by a thin wire. Gels were stained for protein by incubation for 1 h at 4^oC in 0.1% Coomassie Brilliant Blue G 250 in methanol/acetic acid/water (5 : 1 : 4, by vol.). Gels were scanned at 660 nm in a Gilford spectrophotometer fitted with a model 252 gel scanner; the measured absorbance did not exceed 1 unit. This was quantified by cutting out the peak areas on the chart recorder papers and weighing them. Proteins containing disulfide bonds were previously reduced and alkylated before the run. Sometimes gels containing 2-Mercaptoethanol (5 mM) in the well buffers were run similarly.

ii) For gel immunoprecipitates

The gel plates were washed for 48 h in 1% NaCl (frequent changes) to remove unprecipitated proteins; the ring precipitates were sliced out from the gel and dissolved in 1% SDS to dissociate and solubilise the antigen-antibody precipitate; the mixture was left overnight at room temperature. The sample was dialysed overnight against distilled water at room temperature to prevent

precipitation of SDS and subsequently overnight at 4°C, then freeze-dried before running polyacrylamide gels as described in (i).

e) Protein concentration

Proteins were concentrated by ultrafiltration through Ultrathimble apparatus UH 100 and Ultrathimble cells purchased from Anderson & Co., Central Avenue, East Molesey, Surrey, U.K. Otherwise they were concentrated by vacuum dialysis.

f) Protein estimation

Protein concentration was determined spectrophotometrically at 280 nm (Milner-White & Watts, 1971) assuming a molecular extinction coefficient of 7.45×10^4 for CK and 2.02×10^5 for immunoglobulin (Benyamin *et al.*, 1976). It was also determined by the method of Lowry *et al.* (1951); a modification of this method by Havekes *et al.* (1974) was used to estimate protein in the gel. A standard with BSA was used, the curve was linear up to 50 µg of protein.

g) Immunisation procedure

i) Chickens: A solution containing 1 mg of CK was mixed well with an equal volume of Freund's complete adjuvant. At 7 days interval this material was injected intramuscularly into the thighs of each rooster. A total of 4 injections was administered initially followed by booster injection one month later.

ii) Sheep: A preliminary experiment on a single sheep showed that antibodies were detectable only after administration of a high dose of CK.

There was no precipitation with the antigen using double or single diffusion

systems but the antiserum was found to inhibit the enzymic activity.

In a first antiserum production six sheep were used. The emulsions of antigen, oil and adjuvant were made by brief sonication (10 seconds) using an MSE sonicator. Each animal received intramuscularly one injection 1: V/V emulsion of CK (10 mg) and Freund's complete adjuvant. After three weeks the sheep were bled; the antisera collected were then tested by double diffusion precipitin test. By this method one sensitive sheep, giving a better primary immune response, was found amid the other ones. Six weeks after the primary injection, the sheep selected for the best immune response was boosted with an injection of CK (10 mg) in Freund's incomplete adjuvant and bled nine days later. As the secondary response was positive, the animal was sacrificed $3\frac{1}{2}$ days later. The operation was carried out with the assistance of technicians of the Department of Animal Husbandry at the Veterinary School, according to the procedure described by Stewart Tull et al. (1975). The antiserum collected (800 ml) was divided into two fractions. One fraction (100 ml) was stored in the deep freeze; the second (700 ml) was fractionated by sodium sulphate.

The same procedure was tried on another group of six sheep but the immune response was not as successful as the previous one, being negative according to the immunodiffusion test.

h) Immunoglobulin fractionation

The fractionation of pure Ig was performed with sodium sulphate (18%, W/W) solution according to the method of Heide and Schwick (1973). A 2-fold

concentrated immunoglobulin fraction was achieved from the initial antiserum (of the first bleeding) with a protein content of 43 mg/ml and a 3-fold concentrated immunoglobulin fraction was achieved from the second bleeding antiserum with a protein content of 42 mg/ml.

i) Immunodiffusion

i) Single radial immunodiffusion

For the native enzyme, the method of Mancini (1965), as improved by Vaerman et al. (1969) was used for single diffusion precipitin reactions of CK and antibodies through agarose (1%). There is a linear relationship between the area of the precipitate and the CK concentration. CK was dialysed either against Tris (0.02 M, acetate) buffer or cysteine (0.01 M) buffer made to pH 8.6 with NaOH.

For the complex of the TSA bound to the enzyme, the same experimental conditions were applied except that both CK and the agarose gel medium contained: ADP, 0.001 M; Mg acetate 0.01 M; Creatine 0.04 M; NaNO_3 0.1 M.

ii) Double diffusion

Double diffusion in 1% agarose was carried out according to the method of Ouchterlony (1949). CK was dialysed either against Tris (0.02 M, acetate) buffer pH 8.6 or cysteine 0.01 M buffer pH 8.6 before use. Immuno-precipitin lines were well developed after 48 h at room temperature in a moist environment. These lines were sometimes stained for protein. Before staining the plates were washed for 48 h in 1% NaCl (frequent changes) to remove unprecipitated protein and subsequently in water to remove salt. The agar plates free of soluble protein

and salt, were dried at room temperature and stained for 10 min with Coomassie Brilliant Blue 5% in ethanol : acetic acid : distilled water (4 : 1 : 4). Then they were destained for 30 min in ethanol : acetic acid : distilled water (4 : 1 : 4).

j) Immuno-electrophoresis

The immuno-electrophoresis in agarose (1%) gel veronal buffer, pH 8.6, was carried out on a microscale on glass slides according to Scheidegger, (1973).

k) Quantitative immuno-electrophoresis: fused rocket immuno-electrophoresis.

Rocket immuno-electrophoresis through agarose in veronal buffer, pH 8.6, was carried out on glass plate (10 cm x 10 cm) as described in detail in the "Manual of Quantitative Immuno-electrophoresis" by Axelsen et al. (1976).

l) Quantitative precipitin test

The procedure was adapted from the method of Dean and Webb, reported by William and Chase (1968). Increasing amounts of creatine kinase in Tris (0.02 M) acetate buffer, pH 8, were added to 1 ml of antiserum and the mixtures were kept for 2 h at 37°C followed by 60 h at 4°C. The washed precipitates were dissolved in 1 ml of NaOH (0.1 M) and the absorbance at 280 nm was determined. From the equivalence zone the antibody titre of the antiserum was determined.

m) Immunoabsorption

i) Synthesis of immunoabsorbent antigen column : The matrix bound CK dimer and monomer were prepared by the method of Bickerstaff and Price (1976). Their method was also used to estimate protein concentration in the gel. CK was

always purified by using ion-exchange chromatography with DEAE cellulose before immobilisation.

The method used (Cherif & Milner-White, 1978) is described as follows and in Fig. 3. The immunoglobulin fraction was filtered through a millipore filter (pore size 0.22μ) then recycled or percolated at a flow rate of 20 ml/h for 2 h at 4°C through the creatine kinase immunoabsorbent column. The unretarded antibodies were dialysed against water (frequent change) and concentrated to the maximum by ultrafiltration. The protein concentration in the gel was determined after washing off the unretarded antibodies, and after elution of the specific antibodies. The columns were eluted with either urea (8 M) or glycine HCl buffer, pH3, or KSCN (2 M). Care was taken to avoid vigorous shaking or stirring of the Sepharose 4B gel which generates fines appearing in the effluent.

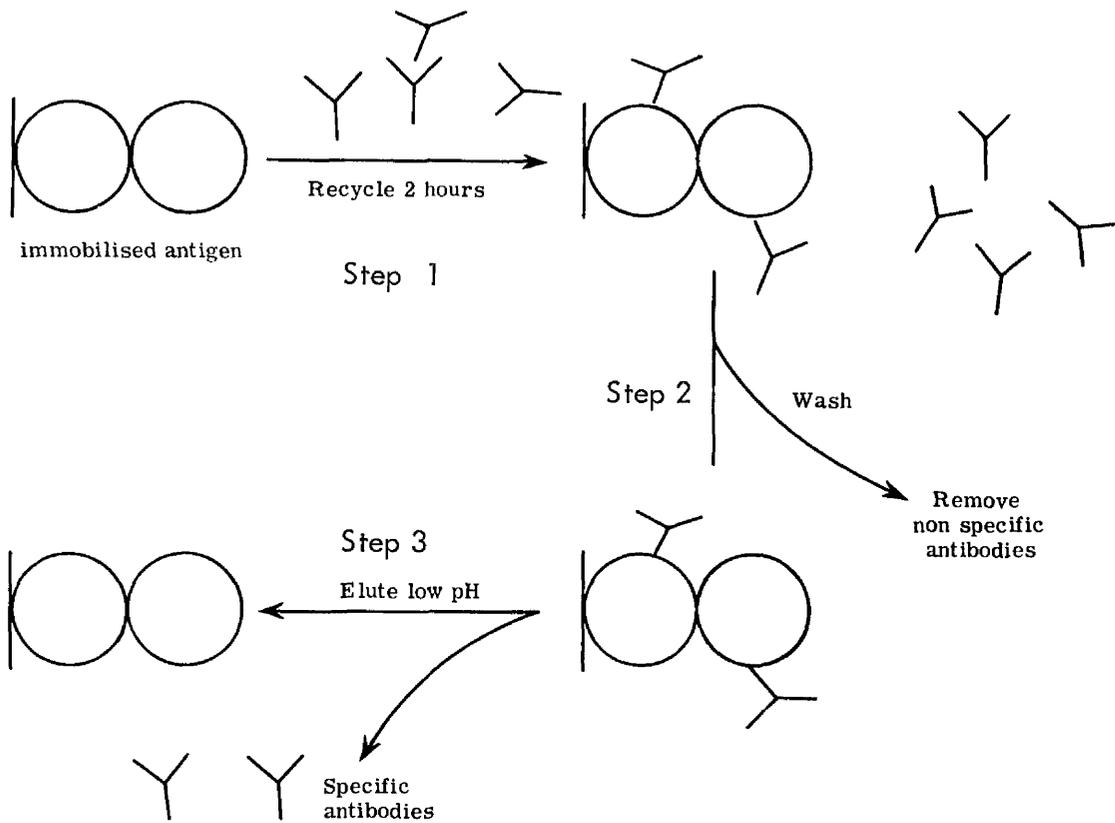
ii) Immunoabsorption of the iodoacetamide treated CK: The alkylation of soluble CK was carried out as described by Kelly and Milner-White (1976); the iodoacetamide treated enzyme immunoabsorbent was synthesised as described earlier. Otherwise the alkylation was carried out on the column by washing the matrix-bound enzyme (2 ml gel bed volume) with iodoacetamide (50 ml, 0.01 M) at room temperature for 30 min. Then the same experimental conditions as described in Fig.3 were applied.

n) Immunoabsorption of CK by immobilized antibodies

The antibody immunoabsorbent column was synthesised either by a modified version of the technique of Bickerstaff and Price (1976) or by the procedure described by Walker and Mayer (1977) under conditions recommended for moderate

Fig. 3. Immunoabsorption procedure

- Step 1 refers to the adsorption of antibodies by the gel containing matrix-bound CK.
- Step 2 refers to the washing and removal of the unbound antibodies.
- Step 3 refers to the elution of specific antibodies from the gel containing matrix-bound CK + specific antibodies.



activation; or by direct coupling of antibodies to CNBr-activated sepharose 4B using the method of the manufacturer. 10 mg of antibodies were coupled to 1 ml of gel in phosphate buffer pH 8. This time the antigen (a dilute solution of CK was slowly pumped (5 ml/h) through the immunoabsorbent antibody column; then the column was washed with 20 ml phosphate buffer, pH 8, and re-equilibrated in this buffer before eluting the enzyme by urea (8 M). The eluted CK was dialysed against 1 mM DTT Tris-acetate buffer, pH 8.6.

o) Isoelectric focusing

ii) Analytical electrofocusing: The protein solutions were dialysed against double distilled deionised water to remove salt and freeze dried before running the sample. Thin layer polyacrylamide gel electrofocusing was performed by the method of Awdeh et al. (1968) using their design of apparatus. After the samples had been applied as small drops (10 - 20 μ l) on top of the polyacrylamide gel layer (2 mm), the plate was turned upside down and put on to two carbon electrodes. The electrodes were coated lightly beforehand with the electrode solutions: a dilute acid solution (5% H_3PO_4) for the anode (+) and a dilute alkali solution (5% T.E.M.E.D) for the cathode (-). The pH gradient is established across the whole gel plate and all the samples were then separated and focused 18 h at 4°C under the same conditions. This makes evaluations and comparisons more accurate. Protein staining was carried out as previously described (Methods, d). The enzyme activity stain was performed according to the method of Dawson et al. (1965).

ii) Preparative flat-bed electrofocusing in a granulated gel: The separation

of CK was performed in a tray across the length of the LKB multiphor 2117 according to the manufacturer's instructions (Winter et al., 1975).

p) Digestion procedure

Limited proteolysis of CK by proteinase K was carried out as described in our paper (Williamson et al., 1977).

Proteolytic cleavage of antibodies by papain was carried out according to the original method of Porter reported by Weir (1973). The F ab fragments were freed from undigested antibodies and from the small peptides by gel filtration on Sephadex G 150 or G 200.

q) Creatine kinase assays

i) pH stat assay: A pH stat assembly (SBR 2C) TTT b/SBU a from Radiometer, Copenhagen, Denmark, was used to monitor the forward reaction at 30°C and pH 9.0 under a stream of nitrogen gas (Kelly & Milner-White, 1976). The assay mixture contained finally: creatine, 40 mM; ATP, 4 mM; magnesium acetate, 5 mM; sodium acetate, 0.1 M; cysteine, 1 mM. The reaction was started by adding the creatine kinase solution and followed by automatic addition of NaOH (5 mM) to maintain the pH.

ii) Phosphate assay: The assay on a microscale (1 ml) was adapted from the original method of Kuby et al. (1954). The absorbance at 660 nm was determined after centrifugation; a standard using creatine phosphate was used. Assays of immobilized CK were carried out in the same manner as for the soluble enzyme.

iii) Inhibition assay of creatine kinase activity by specific antibodies:

Increasing amounts of antibodies were incubated with a constant amount of CK; the enzymic activity was determined either by the pH stat method or by the phosphate assay. CK activity of the immobilized immunocomplex was similarly determined after suitable dilution of the washed gel containing matrix-bound CK+ antibodies.

RESULTS

Chapter 3:

RESULTS1) Chromatography Results

Chromatography of CK on DEAE cellulose resulted in a single peak with considerable tailing (Fig. 4) and only 50% yield. A similar yield was obtained for the mouse muscle enzyme by Hooton and Watts (1966). An additional 10% of enzymically active protein was recovered from the column after elution at high ionic strength (Tris 0.1 M, NaCl 2 M, pH 8.6). The purified enzyme was run on SDS gel electrophoresis.

2) SDS polyacrylamide gel results

Fig. 5B shows that CK was purified free from any trace of protein contaminant. Only one band was observed when the electrophoresis was performed, either with the addition of Mercaptoethanol (5 mM) to the electrode buffer or when the protein sample was previously fully reduced and alkylated. Otherwise two bands were visualised as reported in our paper (Williamson et al., 1977). The position of the top band (Fig. 5) corresponds to a molecular weight of 41,000. This value is used in the present work for the molecular weight of CK subunit.

3) Isoelectric focusing results

Samples from various parts of the DEAE-cellulose elution profile of CK (Fig. 4) were analyzed by isoelectric focusing on thin layer polyacrylamide gels. Fig. 6 shows that the samples corresponding to the peak of elution of CK contains three bands. The enzyme before chromatography contains three other minor bands. This finding is in agreement with the results of Cattani et al. (1977). A similar pattern was observed with the proteinase K cleaved enzyme, but the iodoacetate or iodoacetamide treated enzyme shows only a single band.

Fig. 4 Additional purification of CK using ion exchange chromatography

Chromatography of CK on a DEAE cellulose column (9 x 4.4 cm). The enzyme was dialysed against Tris (0.01 M) acetic acid buffer (pH 8.6) and added to the column (10 ml, 20 mg/ml). Elution was by a linear gradient formed by mixing equal volumes of Tris (0.01 M) acetic acid (pH 8.6) and Tris (0.1 M) acetic acid (pH 8.6) in a total volume of 500 ml. Flow rate was set for 15 ml/h and fractions of 5 ml were collected. Eluates were concentrated by ultrafiltration. The enzyme in the main peak had a specific activity of 150 $\mu\text{moles H}^+/\text{min}/\text{mg}$ of protein by the pH stat assay.

The arrows indicate the fractions that were taken for isoelectric focusing.

The enzyme in the fractions A, B and C have also a specific activity of 150 $\mu\text{moles H}^+/\text{min}/\text{mg}$ of protein.

Fig. 4

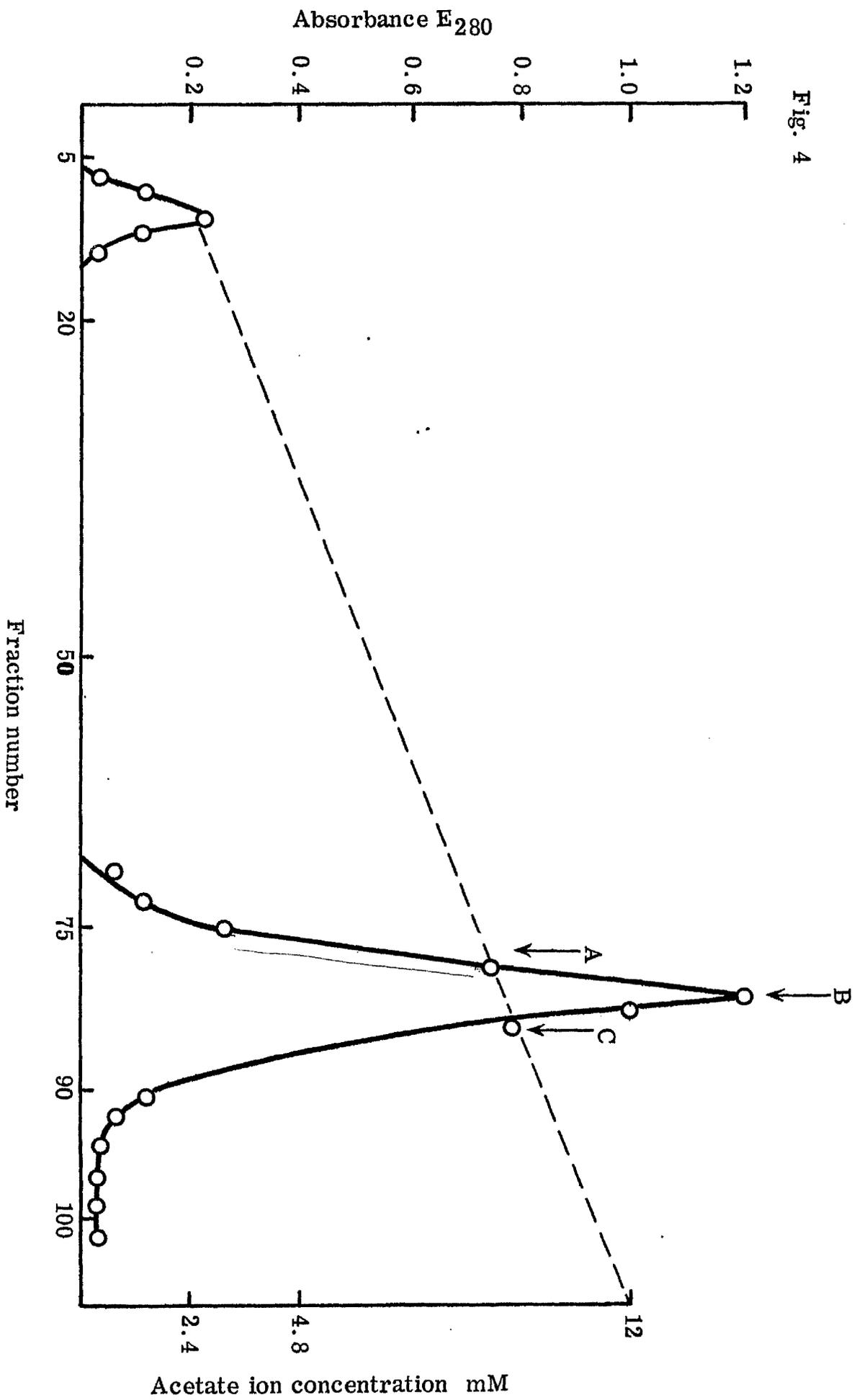


Fig. 5 SDS polyacrylamide gel electrophoresis of CK;
mercaptoethanol 5 mM was present in the electrode buffer.

The electrophoresis in the presence of SDS and mercaptoethanol (5 mM) was performed for 4 h at 4 mA per tube until bromophenol blue marker reached the end of the gel tube. The position of the dye is indicated.

- A. photograph of blue stained SDS/polyacrylamide gel of CK before purification on DEAE cellulose.
- B. photograph of blue stained SDS/polyacrylamide gel of CK after purification on DEAE cellulose.

This observation has allowed A. Burgess (unpublished work) to show that there is about one unit of charge difference between each molecular form. Hence three bands are well resolved by thin layer gel electrofocusing. However, isoelectric focusing of CK reduced and fully alkylated in the presence of urea shows only one single band (Jamieson & Milner-White, unpublished results). Attempts to isolate those bands on preparative granulated gel electrofocusing resulted in the separation of four fractions (Milner-White, unpublished work); the fractions labelled P, Q, R, S with respective pI 6.2, 6.6, 6.8 and 7 were in turn refocused on thin layer polyacrylamide gels; they each showed three bands similar to that found before this treatment.

4) Anti-creatine kinase antibodies

a) Antibodies raised in chickens

Double diffusion of chicken antibodies versus CK showed two precipitin lines indicative of the presence of at least two antigen-antibody reactions. As the immunisation was carried out with CK not purified on DEAE cellulose, one immune precipitate might correspond to the protein trace contaminant of the CK preparation. However, it was difficult to perform definitive immunoelectrophoretic studies on this system because the precipitation of immuno-complexes with chicken antibodies requires the presence of NaCl (2%).

b) Antibodies raised in sheep

Double diffusion and immunoelectrophoresis of sheep antibodies versus CK further purified on DEAE cellulose showed very faint precipitin lines indicative of a weak immune response. The reaction is slightly improved by using the concentrated Ig fraction of the original antisera. A more

Fig. 6. Thin layer polyacrylamide gel electrofocusing of CK taken from regions of the elution profile of the ion-exchange chromatography.

The gel contained Ampholine of pH range 5 to 8. Electrofocusing was performed for 18 h at 200 V and 4°C.

A, B and C refer to the fractions that were taken from the DEAE cellulose elution profile of Fig. 4.

CK refers to the enzyme before purification on DEAE cellulose.

A



B



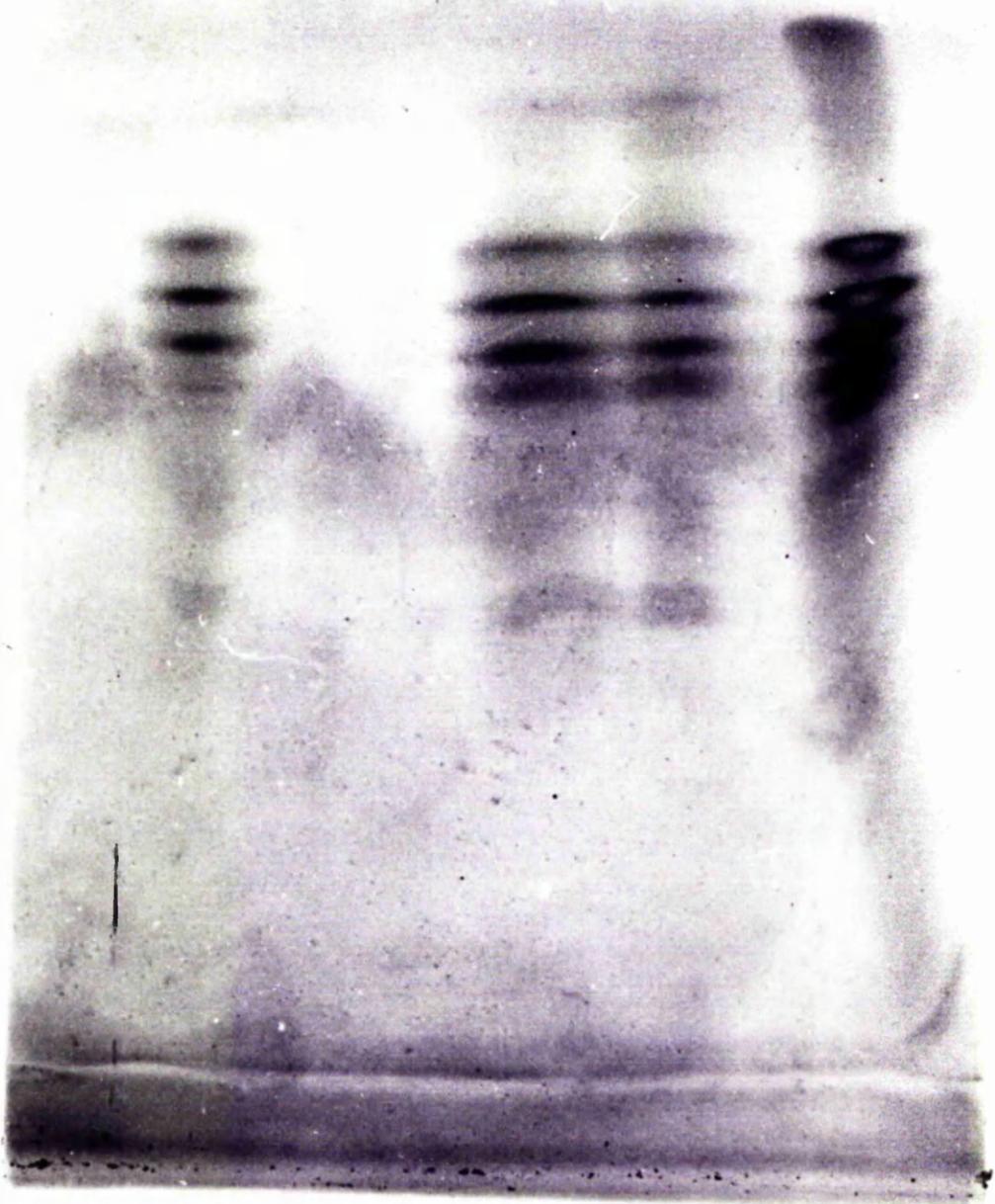
C



CK



⊕



⊖

sensitive method, single radial immunodiffusion, was used to detect these weak antibodies.

With the less highly purified enzyme, two precipitates were observed which are referred to as the inner and outer rings; they may be seen in Fig. 7 iii and iv. With the enzyme purified by DEAE cellulose, the outer ring, but not the inner ring, is found; the identity of all the outer ring precipitates is established by their coalescence observed in Fig. 7. Figs. 8 and 9 also show that the outer ring precipitates were rather faint and diffuse; these characteristics were always found with the antibodies of the second bleeding. With the fractions of CK separated by preparative gel isoelectric focusing, as in Fig. 7, the outer ring, but not the inner, is found (Fig. 9). Table 1 gives the diameters of the outer ring precipitates using IgG of the first and second bleedings. Controls carried out with IgG of a non immunised sheep showed no precipitate.

Experiments were carried out to demonstrate the presence or absence of cross reaction between inner and outer ring precipitates. An example is shown in Fig. 10. It was found that there was again fusion between the outer ring precipitates but none between the outer and inner rings, while the precipitates of adjacent inner rings do show coalescence.

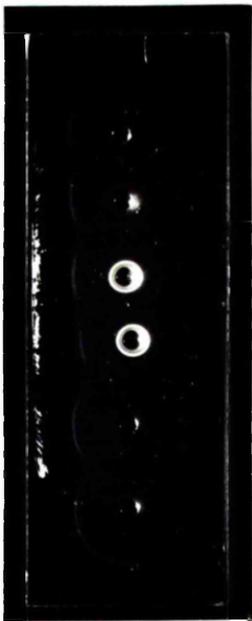
In order to firmly establish the identity of the antigens in the immuno precipitates observed part of the gels were excised; the proteins were extracted as described in the Methods section, and run on SDS electrophoresis gels.

Fig. 11 shows a band of 41,000 which resembles the band observed on SDS polyacrylamide gels of CK (Fig. 5). On the other hand, a major protein band of molecular weight 55,000 and minor bands of 60,000 and 70,000 are seen

Fig. 7. Single radial immunodiffusion of CK before and after purification on DEAE cellulose in agarose gel containing the Ig fraction of the first bleeding of the secondary response.

The antigen wells were filled with CK (4 μ l, 10 mg/ml): before purification on DEAE cellulose column (iii, iv) and after this purification (i, ii, v, vi).

The plate was photographed on a dark background using indirect light. This picture was taken after 48 h of diffusion in a moist atmosphere. The agarose contained 0.5 ml antibodies per slide in a total volume of 2 ml.



- i
- ii
- iii
- iv
- v
- vi

Fig. 8. Single radial immunodiffusion of CK in agarose gel containing the Ig fraction of the second bleeding of the secondary response.

The antigen wells were filled with CK before purification:

4 μ l, 10 mg/ml i, ii and iii.

The plate was photographed on a dark background using indirect light after 48 h of diffusion in a moist atmosphere.

A, photograph

B, diagram showing the outer ring CK immune precipitate and the inner rings (impurities).

A



B

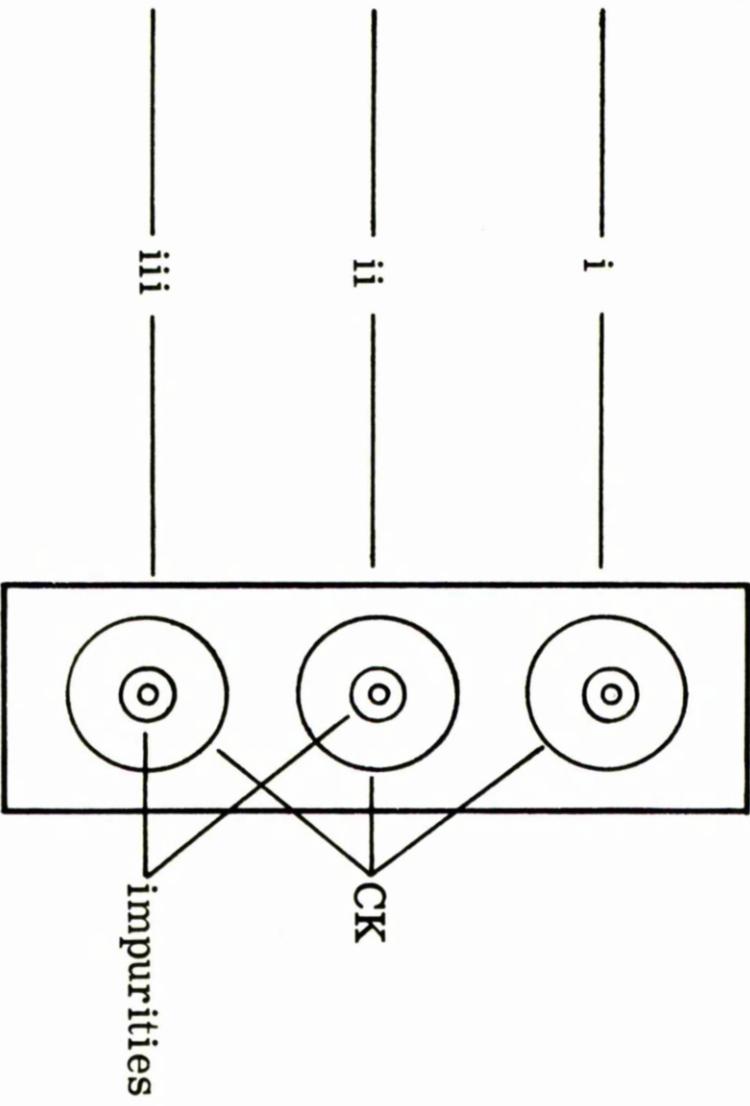


Fig. 9 Single radial immunodiffusion of fractions of CK separated by preparative gel isoelectricfocusing.

The agarose gel contained IgG fraction (0.5 ml) of the second bleeding per microslide in a total volume of 2 ml.

The antigen wells were filled with CK (4 μ l) before separation on preparative gel isoelectricfocusing (11 mg/ml, ii) and after this separation (P \approx 10 mg/ml, i; Q \approx 4 mg/ml, iii). The photograph was taken as previously described in Figs. 7 and 8.

P and Q refer to the fractions isolated by flat bed gel isoelectric-focusing (Milner-White, unpublished results).

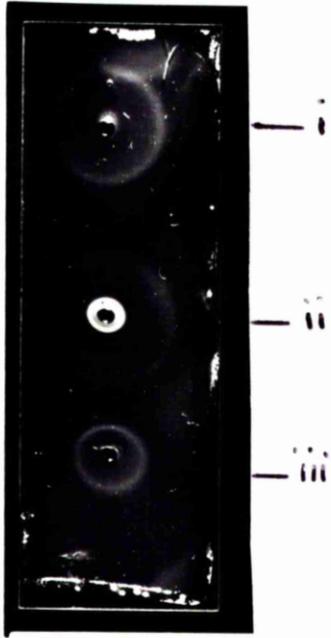


Table 1. Comparative single radial immunodiffusion of CK in agarose gel containing Ig_G of the first and second bleedings.

The diameters of the outer ring precipitates shown in Fig. 7, 8 and 9 were measured with a Boehringer made rule specially designed to measure small distances, with (± 0.1 mm) accuracy.

Bleeding	Ig _G (0.5 ml) mg/ml	CK (4 μ l) mg/ml	Diameter of outer rings (mm)
First	43	10	12.9
		10	13.0
		10	13.1
Second	42	4	7.5
		10	13.0
		11	14.0
		12	15.0

The ring precipitates were inserted between two divergent lines drawn on the rule, the diameter is given by reading the tangent point on the graduated horizontal line.

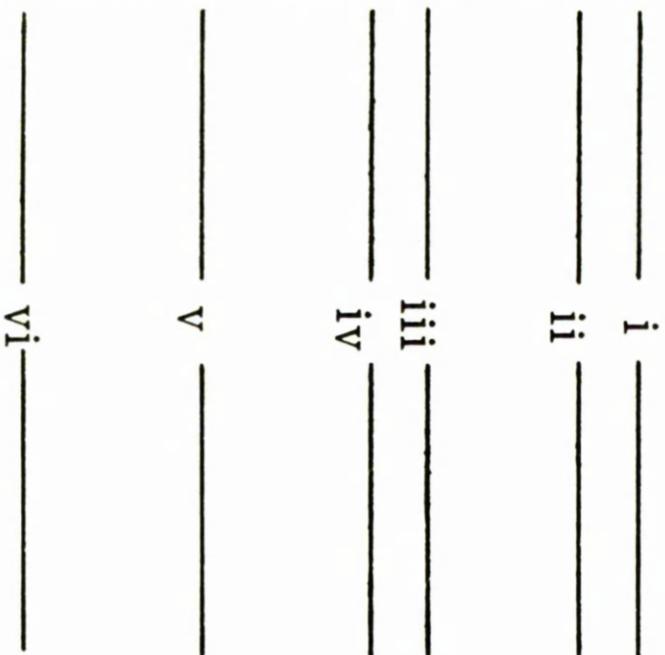
Fig. 10 Single radial immunodiffusion of CK with and without additional purification on DEAE cellulose in agarose gel containing IgG fractions (secondary response, second bleeding).

The agarose contained 0.5 ml sheep IgG per microslide (2 ml of total volume). The antigen wells were filled with CK (4 μ l) before passage on a DEAE cellulose column (20 mg/ml i, ii, iii; 10 mg/ml, v) and after this treatment (20 mg/ml, iv; 10 mg/ml, vi).

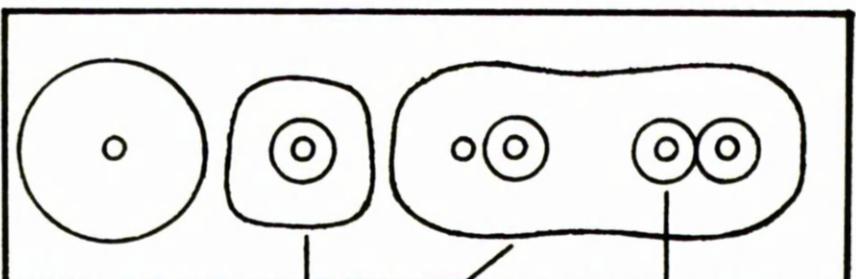
A, the plate was photographed on a dark background using indirect light after 48 h of diffusion in a moist atmosphere.

B, diagram showing the outer ring precipitate (faint) and the inner ring (strong) precipitate.

A



B



impurities

CK

on the top of the gel; at the bottom of the gel, a major protein band of molecular weight 23,000 is visualized. This low molecular weight band corresponds to the light chain of all classes of immunoglobulins (IgG, IgA, IgM ...). The protein bands of 55,000, 60,000 and 70,000 Daltons molecular weight correspond respectively to the heavy chain of IgG, IgA and IgM antibodies. It is known (Pillot and Peltier 1973; Nisonoff et al., (1975, etc) that an immunoglobulin molecule contains the same number of H and L chains. The proportion of each class of antibodies is calculated as follows: IgM/IgG for example:-

$$\frac{\text{weight of peak area of } \mu \text{ chain}}{\text{weight of peak area of } \gamma \text{ chain}} \times \frac{55,000}{70,000}$$

$$= \frac{27 \text{ mg}}{82.1 \text{ mg}} \times \frac{55,000}{70,000} = 0.25$$

μ and γ are respectively the heavy chains of IgM and IgG. The molar ratio of IgG antibodies over CK is calculated from the molar ratio of their respective subunits.

$$\frac{\text{weight of peak area of } \gamma \text{ chain}}{\text{weight of peak area of CK}} \times \frac{41,000}{55,000}$$

$$= \frac{82.1}{14} \times \frac{41,000}{55,000} = 4.2$$

The amounts of IgA and IgM antibodies are quite small. The molar ratio of IgG antibodies to CK is well over 4 whereas, for a standard precipitin reaction, this value is expected to approach 2 at the equivalence zone. Samples of the gel taken from outside the region of the outer ring precipitate treated and run similarly on SDS polyacrylamide gel electrophoresis showed no protein bands,

Fig. 11 SDS polyacrylamide gel electrophoresis of the protein of the outer ring precipitate.

The precipitates obtained by single radial immunodiffusion (Fig. 8) were treated as described earlier and run on SDS polyacrylamide gel electrophoresis. The electrophoresis in the presence of SDS was performed for 4 h at 4 mA until the bromophenol blue marker reached the end of the gel tube. The position of the dye is indicated.

A, photograph of the Coomassie blue stained SDS polyacrylamide gels.

B, corresponding scan at 600 nm and OD = 1 unit of the Coomassie blue stained gel.

either of CK or of antibody subunits; these results demonstrate the presence of CK in the outer ring precipitate.

5) Measurement of antibody titre

A quantitative precipitin test was difficult to achieve in liquid media. However, precipitation of CK with antiserum of the first bleeding gave a standard precipitin curve whereas for the antiserum of the second bleeding an unusual pattern was observed; at the equivalence zone the amount of protein in the immune precipitate was less than the corresponding amount of antigen added. This shows the presence of soluble immune complexes in the supernatant. For the antiserum of the first bleeding the quantitative precipitin reaction was used as described in the excellent reference book "Methods in Immunology and Immunochemistry" (William & Chase, 1968) to calculate the antibody/antigen molar ratio. If the molecular weights of both the antigen and the specific antibody are known, and also the amount of each component in the precipitate, then it is possible to calculate the molecular ratio of the components in the specific precipitate. In the region of antibody excess (left part of the graph of Fig. 12) it is possible to estimate the number of antigenic determinants (e.g. Valency) of the antigen molecule by plotting the number of antibody molecules bound per molecule of antigen versus the antigen concentration. This gives a straight line with an intercept (initial combining ratio) of 4.0, which is shown in Fig. 13. This value is the theoretical valency of the CK molecule which represents the maximum number of antibody molecules that can bind an antigen molecule.

Fig. 12 Immuno precipitation of sheep antiserum, 1 ml (secondary response, first bleeding) with increasing amounts of CK further purified using DEAE cellulose.

Increasing amounts of CK were added to 1 ml of antiserum to give a final value of 1.5 μ l in Tris (20 mM) acetate buffer, pH 8; the mixtures were kept for 2 h at 37°C followed by 60 h at 4°C. The washed precipitates were dissolved in NaOH (1 ml, 0.1 N) and the absorbance at 280 nm was determined.

The antibody titre of the antiserum =

$$\frac{\text{Absorbance of ppt} - \text{absorbance of antigen}}{1.35} \\ = 0.38 \text{ mg antibodies/ml antiserum.}$$

Fig. 13 Plot of the valence of CK versus increasing amounts of antigen (CK)

This figure represents the left hand part of the previous graph, Fig. 12, differently plotted.

It is assumed that the amount of antigen in the precipitate is known, i.e. all the added antigen is found precipitated in the region of antibody excess.;

The concentration of antibody in the precipitate was calculated from the precipitin curve (antibody excess region, left hand part of Fig. 12) 25% of the antibodies were assumed to belong to the IgM class. Molecular weights of 150,000 for IgG, 900,000 for IgM and 82,000 for CK were taken.

Fig. 12

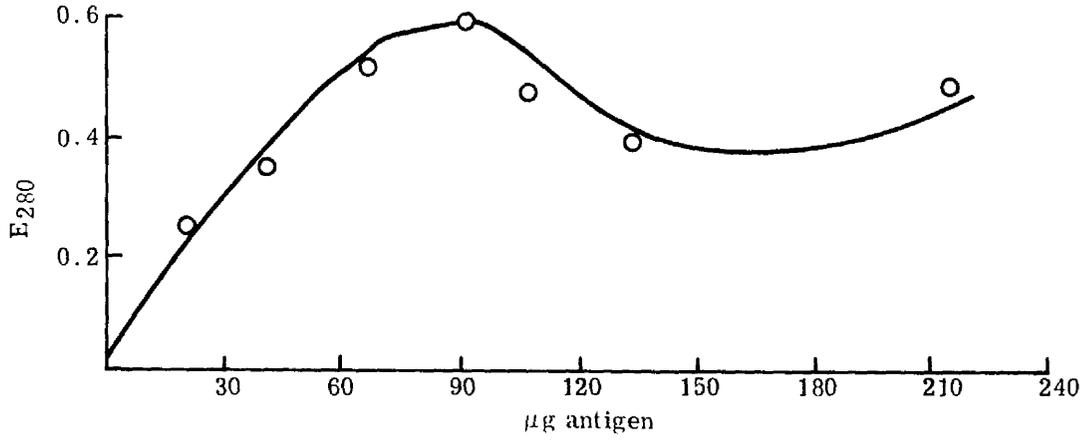
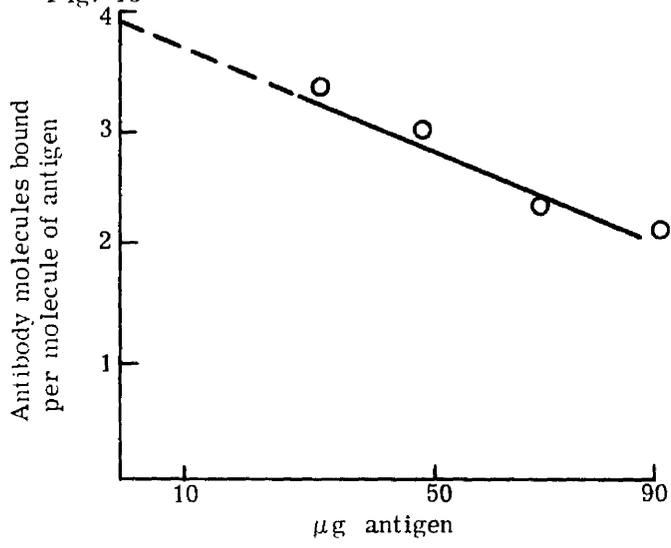


Fig. 13



6) Immunoabsorption

The immunoabsorption was quantitatively followed by measuring the protein concentration of the gel at every stage of the experiment. The column after immunoabsorption (the gel containing matrix bound CK + specific antibodies, i.e. immobilised immune complex) was washed with phosphate, EDTA buffer at pH 8 until OD = 0.001 before estimating the protein concentration of the gel. The immunoabsorbent column (2 ml bed volume) was completely free of unretarded IgG after a wash of 70 ml phosphate, EDTA buffer (Fig. 14). The valence, i.e. the number of antigenic determinants per molecule of antigen, was calculated from the antibody/antigen molar ratio at saturation of the immunoabsorbent column with the antibodies.

a) Immunoabsorption of IgG fraction from the first bleeding

A graph of the valence of CK versus antibody concentration is plotted, with only a few experimental points because of the limited volume of antiserum available from the first bleeding.

From Fig. 15 it appears that the number of antibody molecules bound per molecule of CK reaches a plateau at high antibody concentration. The valence maximum of CK determined by immunoabsorption is 3.5.

From the left part of Fig. 15, the antibody titre of the IgG fraction could be evaluated. In this region of antigen excess all or most of the specific anti-CK antibodies bound to their homologous antigenic site; the antibody titre of 1ml of the IgG fraction is given by the amount of antibodies adsorbed per 2ml of packed gel = 0.67 mg antibodies per ml of IgG fraction. This value is in agreement with the antibody titre found by the precipitin test (the concentration of the Ig

Fig. 14 Washing profile of the column after immunoabsorption of IgG (4 ml) fraction from the first and second bleeding.

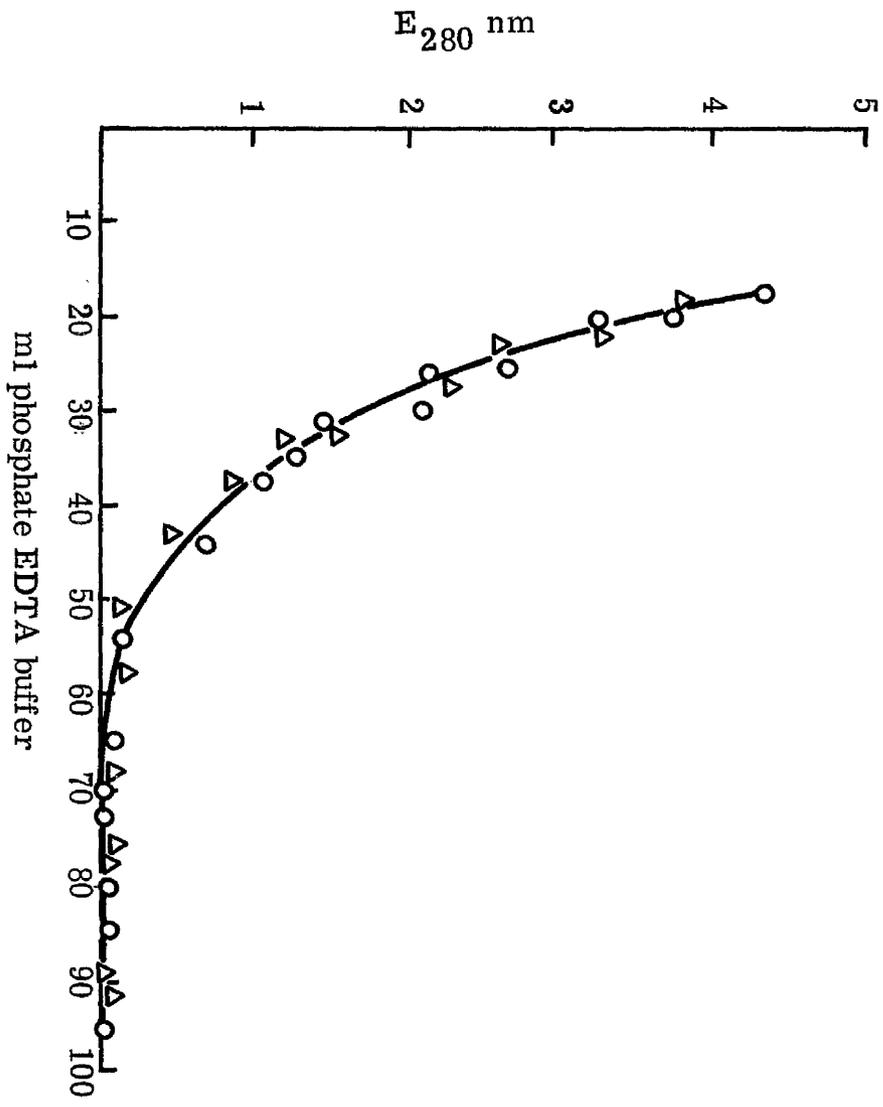
The immunoabsorbent column (2 ml bed volume) was washed with phosphate EDTA buffer, pH 8 (100 ml) at 4°C. The flow rate was set at 60 ml/h and fractions of 10 ml were collected.

The first fractions contained most of the unretarded IgG fraction.

O, washing profile of the column after immunoabsorption of IgG (4 ml) fraction from the first bleeding.

A, washing profile of the column after immunoabsorption of IgG (4 ml) fraction from the second bleeding.

Fig. 14



fraction is 1.9 fold that of the original antiserum). The antibodies eluted from the column by low pH or KSCN (2.5 M) represent only 53% of the total antibodies adsorbed. This was confirmed by measuring the protein concentration of the gel after elution of specific antibodies. 47% of antibodies still remained attached to the immobilized CK; this suggests that some antibody populations bind more strongly to the antigen. Controls with normal sheep Ig were carried out similarly. There were no antibodies adsorbed and none eluted. Moreover, the elution of the immobilized CK column by low pH or KSCN did not dissociate the CK dimer. This was shown by the 100% recovery of the original protein concentration of the sepharose bound CK after elution by low pH.

b) Immunoabsorption of Ig fraction from the second bleeding

Results of immunoabsorption with the Ig fraction of the second bleeding are reported in our communication (Cherif & Milner-White, 1978). The graph of valence versus antibody concentration is given in Fig. 16. The valence of CK determined by the immunoabsorption of antibodies from the second bleeding was found to be dependent on other factors (duration of the washing step and conditions of storage of the antisera). The maximum valence obtained was 2.9; however, this value was found to slip down to 1.8 with longer washing (> 2 h) of the immunoabsorbent column. The recovery of antibodies eluted from the column was, respectively, 80% for a fresh Ig fraction stored at -20°C , and 100% for an old Ig fraction left a short time at room temperature (<30 min). As the antibody titre of the Ig fraction from the second bleeding was difficult to measure by immunoprecipitation, it was determined by immunoabsorption as

Fig 15: Saturation curve of immobilized CK with Ig fraction of the secondary response, first bleeding.

Ordinate: valence, i.e. number of antibody molecules bound per molecule of antigen.

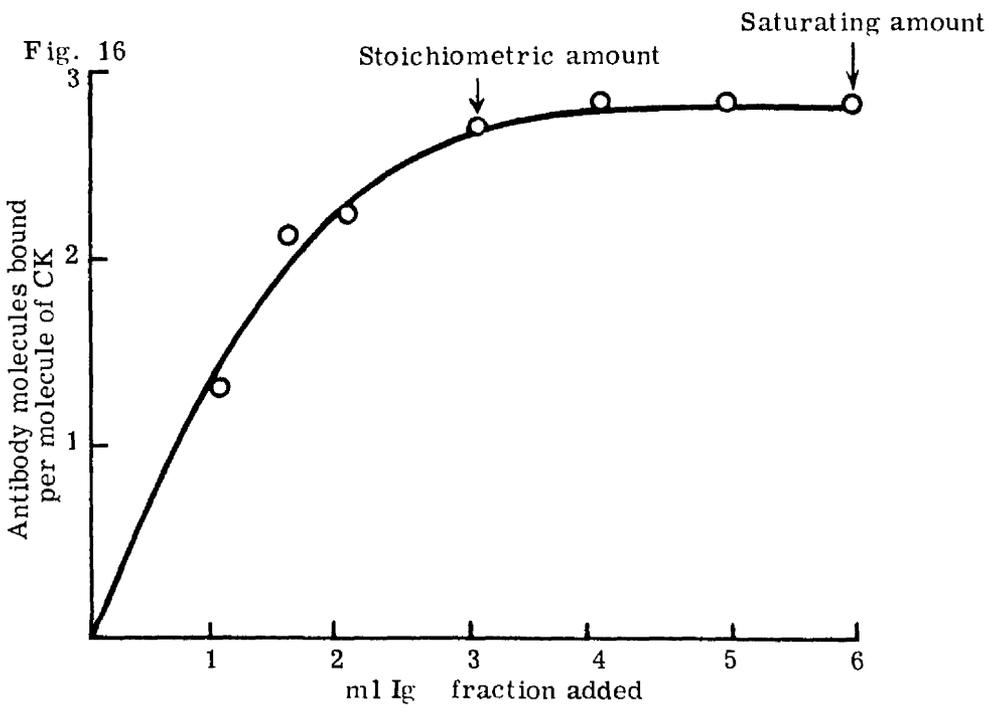
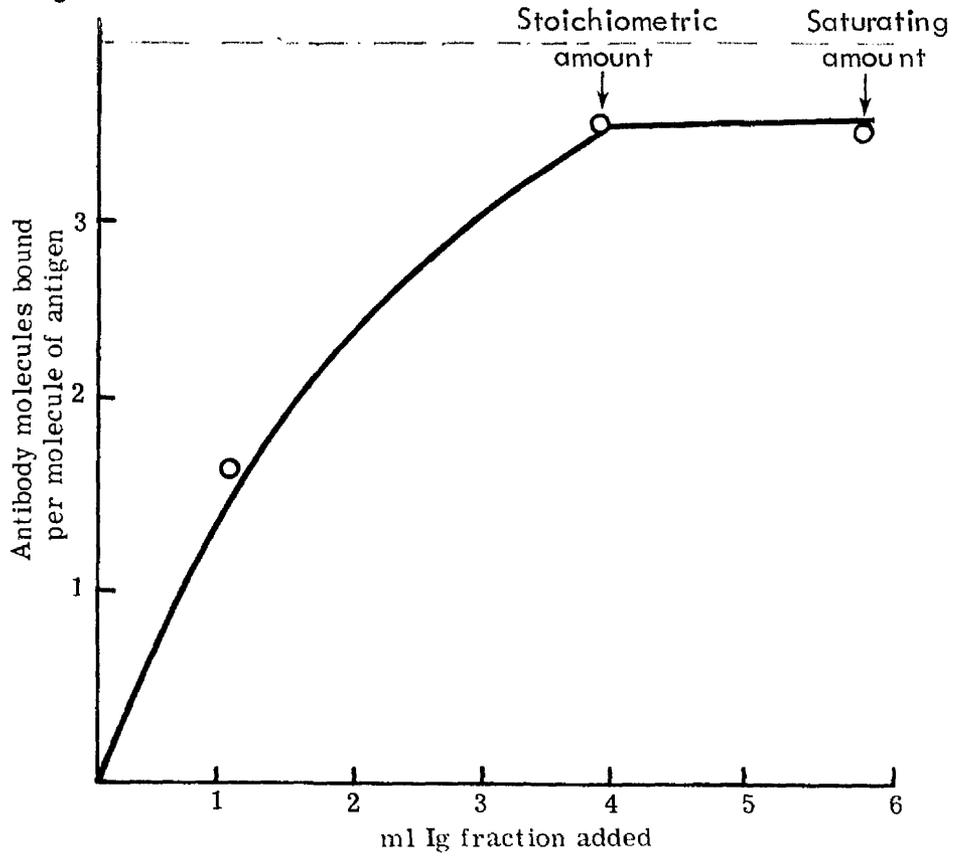
Abscissa : volume (ml) of Ig fraction (43 mg total protein/ml) added to the immunoabsorbent column (2 ml bed volume of packed gel). The major classes of antibodies were considered in the calculation (5% of IgM and 95% of IgG) according to the results of Fig. 17. The concentration of immobilized CK was 0.2 mg/ml packed gel. Molecular weights of 900,000, 150,000 and 82,000 Daltons were taken for IgM, IgG and CK respectively. The amount of antibodies absorbed per ml of packed gel was taken to be equal to the concentration of the gel after immunoabsorption minus the protein content of the gel before immunoabsorption. The dashed line (---) corresponds to the valence maximal expected from the immunoprecipitation test (see Fig. 13).

Fig. 16 Saturation curve of immobilized CK with Ig fraction of the secondary response, second bleeding.

Ordinate: valence, i.e. number of antibody molecules bound per molecule of antigen.

Abscissa : volume (ml) of Ig fraction (42 mg/ml) added to the immunoabsorbent column (2 ml bed volume of packed gel). Molar ratios were calculated as in Fig. 15. The concentration of immobilised CK was 0.2 mg/ml of packed gel. The arrows indicate the amount of antibodies which is stoichiometric (i.e. minimum amount needed to saturate the immunoabsorbent) and saturating (i.e. large excess which already saturate the immunoabsorbent).

Fig. 15



described earlier (from the left part of Fig. 16). A value of 0.92 mg antibodies per ml of Ig fraction was found.

Valence of CK subunit

The immunoabsorbent monomer was synthesized as described in the Methods section. Results of immunoabsorption are given in Table 2. The valence maximum for the CK monomer was determined as described for the dimer at saturation of the immunoabsorbent monomer column with anti CK antibodies (Ig fraction of the second bleeding). A value of 1.2 was found for the monomer. (given in Table 2).

c) Immunoabsorption by denatured CK of Ig from first and second bleeding

Less than 0.1% of the Ig fraction from both bleedings was found to be absorbed on columns of immobilized CK that had been denatured either by citric acid (pH 2.2) or carbonate buffer (pH 11). This was shown by the absence of antibodies eluted by low pH urea (8 M) and by the 100% recovery of the original protein concentration of the gel at the end of the immunoabsorption.

d) Isolation and properties of the specific anti-CK antibodies

The antibodies eluted from the immunoabsorbent column were renatured as described in the Methods section and tested for their immunologic activity. They showed no precipitate either by double diffusion or by single radial immunodiffusion; however, when added to a new immunoabsorbent column only 30 - 40% of the soluble antibodies were found to bind to the immobilized CK. Table 3 shows that the extent of recovery of the immunological activity depends on the nature of the Ig fraction (first or second bleeding) and the mode of elution

Table 2. Immunoadsorption of antibodies from the second bleeding by CK monomer and dimer.

Two columns containing immunoadsorbent (immobilized CK, 6 ml for the monomer and 3 ml for the dimer) were run simultaneously at 4°C. The Ig fraction (3 ml, second bleeding) was added to each column. Then they were washed with phosphate buffer (100 ml) to remove unbound antibodies. The specific antibodies were eluted by urea (8 M).

Immuno adsorbent CK	Volume of the column ml packed gel	Volume of Ig ml (42 mg/ml) added per column	Protein content of the gel before immuno adsorption mg/ml of gel	Protein content of the gel mg/ml of packed gel after absorption	Protein content of the gel mg/ml of packed gel after urea 8 M elution	Antibody absorbed mg/ml of packed gel	Protein eluted from the column mg/ml of packed gel	Valency	% of recovery Protein eluted/ antibodies absorbed
Monomer	6	3	0.1	0.54	0.20	0.44	0.30	1.2	68
Dimer	3	3	0.2	1.08	0.42	0.88	0.66	2.5	72

(low pH, KSCN, urea). In general urea eluted antibodies from the monomer column have more activity than KSCN or low pH-eluted antibodies. The latter strongly disrupt their structure (50% of the antibodies were soluble; the other 50% precipitated and were not soluble even at high ionic strength). Antibodies isolated from the Ig_U fraction of the first bleeding recovered more activity than those from the second bleeding. They were also run on SDS polyacrylamide gel electrophoresis to estimate the composition of different classes of antibody and to check if CK dissociated during elution of antibodies by low pH and urea (8 M); this is shown in Figs. 17 and 18.

Fig. 17B also gives the molar ratio of IgM/IgG calculated as described in Fig. 11. A value of 0.05 was found. A band of CK not visualized on the photograph is however shown on the scanned gel (Fig. 17B). A value of 0.06 was found for the molar ratio of CK/IgG.

A different SDS gel electrophoresis pattern was obtained with the same antibodies eluted from the same immunoabsorbent (dimer) column with urea (8 M). The urea (8 M) causes both the antibodies and the subunits of CK to dissociate and elute from the column (immobilized immunocomplex). The pattern is different from that of Fig. 17.

Fig. 18 shows that the molar ratio of H/L chains is well below 1 but the molar ratio of IgM/IgG molecules is higher than that of Fig. 17.

The molar ratio of CK/IgG equals 0.08 which is less than the expected ratio of 0.18.

The antibodies isolated after immunoabsorption were tested for heterogeneity by analytical electrofocusing. All exhibited great heterogeneity;

Table 3. Properties of the antibodies eluted from the immunoadsorbent column. The antibodies eluted from the experiments described in Figs. 15 and 16 and Table 2 were pooled. The solubility was determined by measuring the protein content of antibodies after neutralization and dialysis against distilled water.

Properties	Antibodies eluted from immunoadsorption of		
	First bleeding low pH	KSCN	Second bleeding low pH urea (only from monomer immunoadsorbent)
% of solubility of the eluted antibodies	50	60	40
immunoprecipitation	No	No	No
% of binding to a new immunoadsorbent column	35	35	30
			40

Fig. 17 SDS polyacrylamide gel electrophoresis of antibodies eluted at low pH.

The protein sample (2 μ g) was run on SDS polyacrylamide gel electrophoresis in the presence of mercaptoethanol (5 mM) as described previously. The antibodies were eluted by citric acid (0.1 M, pH 2.2) from an immobilised native CK column after immunoabsorption of the Ig fraction (secondary response, second bleeding). The eluted antibodies from the experiments described in Fig. 16 were pooled.

A, photograph of the Coomassie blue stained gel.

B, corresponding scan at 600 nm and OD = 1 unit.

Fig. 18 SDS polyacrylamide gel electrophoresis of antibodies eluted by urea (8 M).

The protein sample (2 μ g) was run on SDS polyacrylamide gel electrophoresis in the presence of mercaptoethanol (5 mM). The antibodies were eluted by urea (8 M) from the same immunoabsorbent column as used for Fig. 17, and were pooled as before.

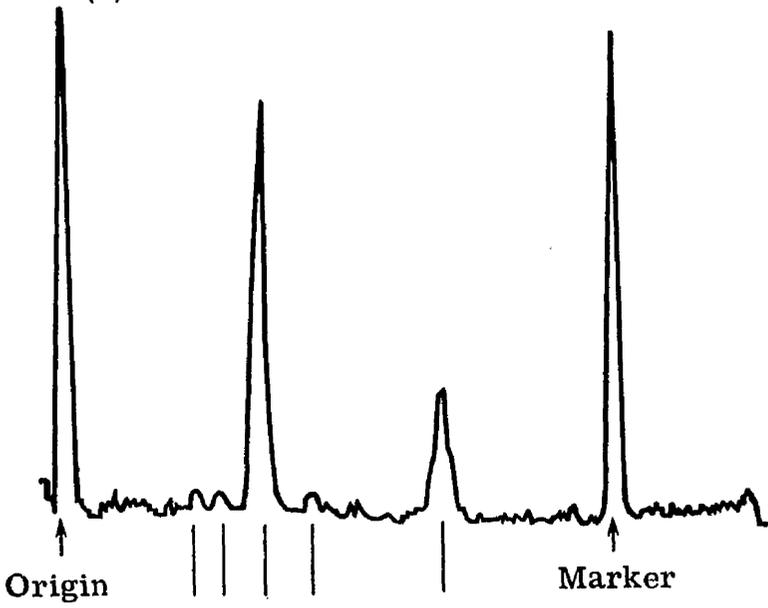
A, photograph of the Coomassie blue stained gel.

B, corresponding scan at 600 nm and OD = 1 unit.

The peaks of 70,000, 55,000 and 23,000 Daltons correspond to μ , γ and L chains of immunoglobulin; the peak of 41,000 Daltons corresponds to CK.

Fig. 17 (-)

(+)



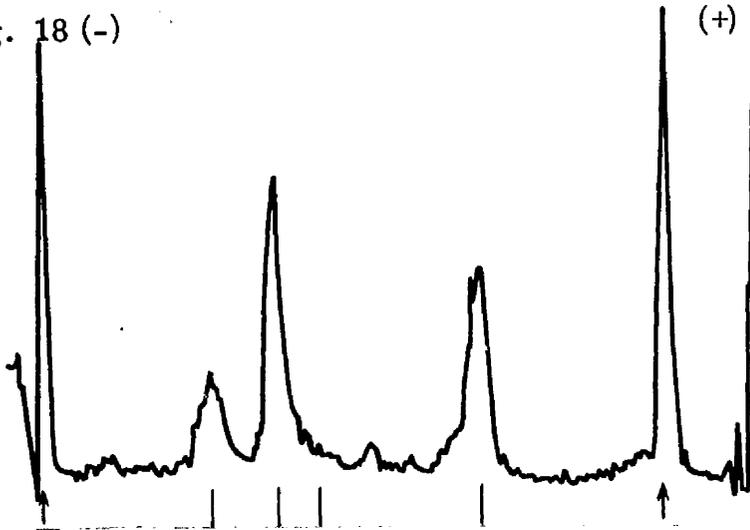
B

A

mol. wt. 70 60 55 41 23
(10^3)

Fig. 18 (-)

(+)



B

A

mol. wt. 63 55 41 23
(10^3)

individual antibodies were not resolved.

e) Properties of the unretarded antibodies

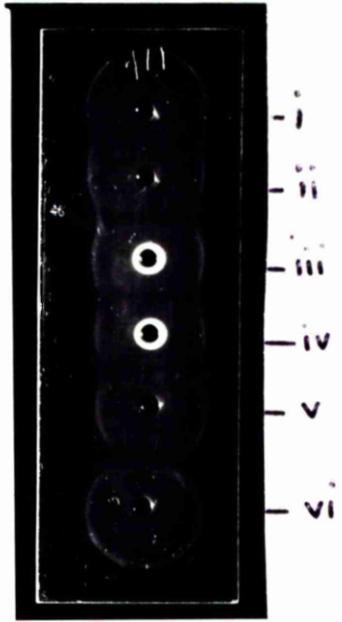
All the unbound antibodies were dialysed and freeze dried as described (p. 28). They were concentrated to their limit of solubility. In general, unretarded antibodies of the first bleeding were more soluble and could be concentrated more than antibodies of the second bleeding. The unretarded antibodies resulting from immunoabsorption of non saturating and stoichiometric amounts of the Ig fraction from both bleedings (at low concentration see left hand side of the graph before the plateau, Figs. 15, 16) did not appear to contain anti CK antibodies as shown by immunodiffusion tests; whereas those resulting from immunoabsorption of excess antibody (saturating amounts at the extreme right hand side of each graph in Figs. 15 and 16) contained some anti CK antibodies as shown by immunodiffusion.

The pattern for these antibodies from the first bleeding is shown in Fig. 19. It is similar to that in Fig. 17.

The unbound antibodies resulting from immunoabsorption with excess antibodies of the second bleeding were also tested by immunodiffusion using single radial or double diffusion. With CK in Tris buffer there was no precipitate with either method, but double diffusion of CK in cysteine showed one precipitate. A more sensitive method, fused rocket immunoelectrophoresis, was also used (Fig. 20). This technique is a variant of quantitative immunoelectrophoresis and combines qualitative and quantitative

Fig. 19 Single radial immunodiffusion of CK in agarose gel containing unretarded Ig fraction of the first bleeding.

The unbound antibodies resulting from immunoabsorption of an excess of the Ig fraction (secondary response, first bleeding) were dialysed and concentrated to a final concentration of 30 mg/ml. The agarose gel contained 0.5 ml of the concentrated unretarded Ig fraction. The antigen wells were filled (CK, 4 μ l, 10 mg/ml) before purification on DEAE cellulose (iii, iv) and after this treatment (i, ii, v, vi). Experimental conditions are the same as those described in Fig. 7.



sensitivity. It was used to detect anti CK antibodies.

Fig. 20 shows as expected total cross-reaction (complete identity of the antigen present in the wells) of the rocket shaped precipitates; nonetheless, the precipitates of antibodies against CK in cysteine are stronger than those in Tris alone. These results show that the unretarded Ig fraction contains some anti CK antibodies, although they do not easily precipitate. Their presence was also detected by immunoabsorption of CK. On this occasion, the Ig fraction was immobilized on a column and an excess of CK added (see Methods section, n). A column (5ml bed volume) of gel containing matrix bound unretarded Ig, 14 mg/ml (3 ml) absorbed 0.2 mg of CK. These results confirm that anti CK antibodies are present in the unretarded Ig fraction and that the immunoabsorbent was well saturated with the Ig fraction from the second bleeding.

7) Antigenic reactivity of the enzyme upon binding of ligands

The antibody binding capacities of CK, its TSA complex (CK + MgADP + creatine + NO_3^-) and its DE complex (CK + MgADP + creatine) were compared by single radial immunodiffusion and immunoabsorption, using Ig fractions of the first and second bleedings.

a) Antigenic reactivity of the TSA complex with anti-enzyme antibodies of the first bleeding

i) Comparative immunodiffusion of CK in the presence and absence of MgADP + creatine and MgADP + creatine + NO_3^- .

Fig. 20 Fused rocket immunoelectrophoresis of CK in agarose gel containing unretarded antibodies.

The unbound antibodies resulting from immunoabsorption of the Ig fraction (secondary response, second bleeding, corresponding to the point indicated by an arrow on the right-hand side of Fig. 16) were pooled, dialysed and concentrated to 14 mg/ml. The plate contained 1 ml of a concentrated unretarded antibodies in 10 ml agarose gel. The antigen wells were alternately filled with CK purified on DEAE cellulose 10 mg/ml dialysed versus cysteine 10 mM, pH 8.6 and CK purified on DEAE cellulose 5 mg/ml dialysed versus Tris 10 mM, pH 8.6. Electrophoresis was carried out overnight at 2 V/Cm in the LKB apparatus.

A, photograph

B diagram

Fig. 21A, B and C show that precipitin reactions of CK versus anti CK antibodies in the presence of $MgADP + NO_3^- + creatine$ were weaker than those in the absence of NO_3^- or substrates. The complete fusion of the outer ring precipitates shown in Fig. 21 (i, ii, iii, iv, v) suggests that the antigens added in these wells are identical; thus CK and its proteinase K cleaved fragments share the same antigenic determinants; but an unusual type of cross reaction takes place in Fig. 21A and C (v, vi), suggesting that the alkylation of the enzyme did affect the antigenicity of CK; this will be further investigated.

ii) Comparative immunoabsorption in the presence and absence of TSA complex and DE complex.

Immunoabsorption of immobilized CK by adding stoichiometric amounts of antibodies (the minimum amounts needed to saturate the unliganded enzyme, as described in Fig. 15) in the presence and absence of substrate analogues was carried out as previously described. Table 4A shows that fewer antibodies (48%) bound to the immobilized antigen in the presence of substrate analogues; none of these were subsequently eluted at low pH. In the absence of substrate analogue 52% were eluted and 48% remained bound to the immobilized antigen. It also shows that all antibodies bound to the immobilized antigen in the presence of $MgADP$ and creatine but none of these were subsequently eluted. The antibodies which remained attached on the column after elution by low pH were partially eluted by urea (8 M) (see Table 4B).

b) Antigenic reactivity of the TSA complex with anti enzyme antibody of the second bleeding.

i) Single radial immunodiffusion of the TSA complex.

Fig. 21 Single radial immunodiffusion of CK and its derivatives with Ig from the first bleeding.

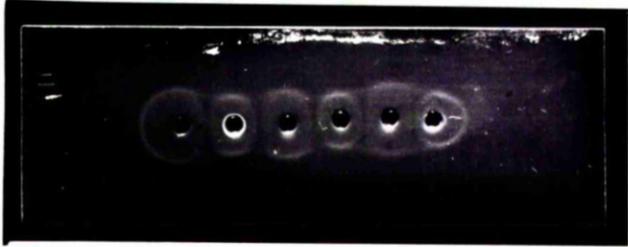
A, The agarose gel (2 ml) contained the Ig fraction (0.5 ml, 42 mg/ml). The antigen wells were filled with 4 μ l of CK purified on DEAE cellulose 2 mg/ml (i, iii, v); CK not purified on DEAE cellulose 2 mg/ml (ii); proteinase K cleaved CK 2 mg/ml (iv) and iodoacetamide treated CK 2 mg/ml (vi).

B, Same as A except that the agarose gel contained the ligand solution (ADP, 1 mM; Mg acetate, 10 mM; creatine, 40 mM; NaNO₃, 0.1 M). The antigen wells were similarly filled as above.

C, Same as A except that the agarose gel contained ADP, 1 mM; Mg acetate, 10 mM; creatine, 40 mM and no NO₃⁻. The antigen wells were filled with 4 μ l of CK purified on DEAE cellulose 20 mg/ml (i, iii, v); CK not purified on DEAE cellulose 10 mg/ml (ii); proteinase K cleaved CK 8 mg/ml (iv) and iodoacetamide treated CK 8 mg/ml (vi).

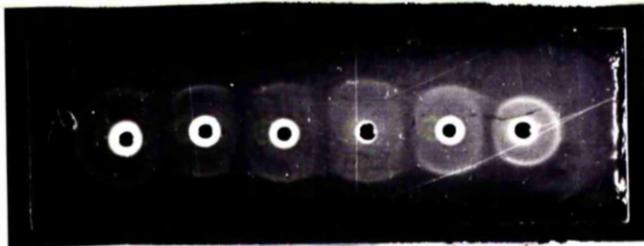
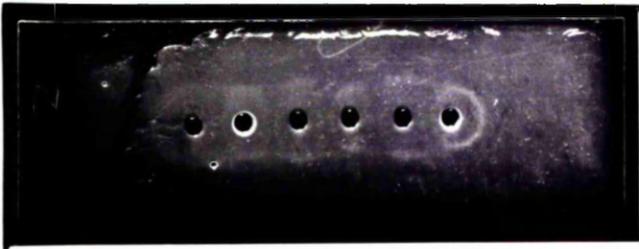
The white thick inner rings are not immune precipitates.

A



i ii iii iv v vi
| | | | | |

B



i ii iii iv v vi
| | | | | |

C

Table 4A Comparative binding capacity of immobilized CK in the presence and absence of substrates analogue with IgG fraction of the first bleeding.

Three columns containing immunoadsorbent (immobilized CK, 3 ml) were run simultaneously at 4°C. One was slowly washed with a solution (50 ml) containing: ADP, 1 mM; Mg acetate, 10 mM; creatine, 40 mM; NaNO₃, 100 mM, phosphate, 10 mM, EDTA, 1 mM, pH 8.0. The second column was only washed with phosphate buffer (50 ml). Then the IgG fraction (7 ml, first bleeding) dialysed against the same buffer in the presence of ligand were added, to each column. The third column was slowly washed with phosphate buffer (50 ml) containing only Mg ADP and creatine, before adding the IgG fraction (4 ml, first bleeding), dialysed against the same buffer in the presence of MgADP and creatine. Afterwards they were washed with phosphate buffer to remove unbound antibodies. The specific anti enzyme antibodies were eluted by citric acid, 0.1 M (10 ml), pH 2.2).

Immuno- absorbent	Volume of the column ml/gel	Volume of IgG (4.3mg/ml) ml added per column	Protein content of the gel before immuno- absorption mg/ml of gel	Protein content of the gel after		Antibodies absorbed mg/ml of gel	Antibody eluted from the immunoabsorbent column mg/ml of gel	Valency	% of recovery antibodies eluted/ antibodies absorbed
				absorption	low pH elution				
CK + Mg ADP + Creatine + NO ₃	3	7	0.28	1.09	1.09	0.81	0	1.6	0
CK - no ligand	3	7	0.28	1.99	1.09	1.71	0.9	3.4	52
CK + Mg ADP + Creatine	3	4	0.15	1.18	1.18	1.03	0	3.6	0

Table 4B. Elution of Antibodies in the Immunoadsorption Experiments of Table 4A.

Further details of the elution of specific antibodies from the immunoadsorbent columns.

Immunoadsorbent	Antibodies adsorbed (mg/ml of gel)	Antibodies adsorbed remaining bound to the column after low pH elution (mg/ml of gel)	% of antibodies adsorbed remaining bound to the column after low pH elution	% of Protein adsorbed remaining bound to the column after urea(8 M)elution
CK + MgADP + creatine + NO ₃ ⁻	0.81	0.81	100	52
CK + no ligand	1.71	0.81	48	25
CK + MgADP + creatine	1.03	1.03	100	51

Single radial immunodiffusion of CK in agarose gel containing the MgADP, creatine, NaNO_3 and the Ig fraction showed weak ring shaped precipitate (the outer ones in Fig. 22). The diameters of these precipitates were proportional to the antigen concentration and agree with the rule established by Mancini et al. (1965). The pattern was similar to that of Fig. 8. Control experiments using normal sheep antibodies showed no precipitate; controls using anti enzyme antibodies in the presence of NO_3^- alone were identical to gels in its absence (Fig. 22b).

ii) Immunoabsorption in the presence and absence of the TSA complex:

This is described in Table 5. Immunoabsorption of stoichiometric amounts of antibodies calculated from Fig. 16 was carried out as described before. Table 5 shows that almost the same amounts of antibodies were eluted from both columns (CK and TSA immunoabsorbent); more than 70% of the bound antibodies were recovered from each column. The unretarded antibodies were dialysed and concentrated as previously described.

Some of the properties of the unretarded antibodies recovered from the TSA immunoabsorbent columns are given in Table 6.

8) The effects of antibodies from the first and second bleedings on the enzymic activity.

a) Inhibition of the enzyme activity of immobilized CK

The enzyme activity of the gel was measured after immunoabsorption and washing off the unbound antibodies. These assays were directly carried out using the pH stat apparatus by the method described by Milner-White and

Fig. 22 Single radial immunodiffusion of CK in the presence of the TSA complex with IgG fraction of the second bleeding.

A, The agarose gel (2 ml) contained the ligand solution (ADP, 1 mM; Mg acetate, 10 mM; creatine, 40 mM; NaNO₃, 100 mM) and IgG fraction (0.5 ml) from the second bleeding. The antigen wells were filled with 4 μl of CK not purified on DEAE cellulose (10 mg/ml, i, ii ; 18 mg/ml, iii).

B, Control with NO₃⁻ (0.1 M) incorporated in the agarose containing the Ig fraction (0.5 ml, 42 mg/ml). The antigen wells were similarly filled as in A.

A

B



i

ii

iii

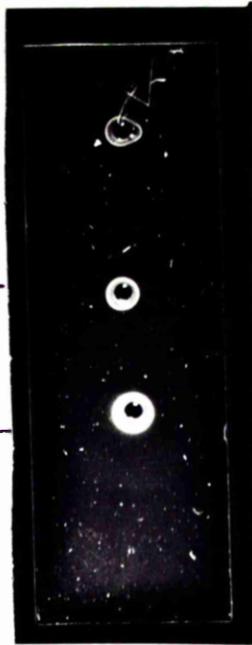


Table 5. Comparative binding capacity of immobilized CK in the presence and absence of the TSA complex with Ig fraction of the second bleeding.

Two columns containing immunoadsorbent (immobilized CK, 5 ml) were run simultaneously at 4°C. The experimental conditions are the same as those described in Table 4 except that 3 ml of Ig fraction of the second bleeding were added to each column.

Immuno- adsorbent	Volume of column ml of gel	Volume of IgG 43 mg/ml added ml	Protein content of the gel before immunoadsorption mg/ml of gel	Protein content of the gel after adsorption and low pH elution mg/ml of gel	Antibodies adsorbed by the immunoadsorbent mg/ml of gel	Antibodies eluted from the immunoadsorbent mg/ml of gel	% of recovery of antibodies eluted/ antibodies adsorbed
CK + TSA	5	3	0.15	0.18	0.60	0.45	75
CK - no ligands	5	3	0.15	0.16	0.65	0.50	77

The antibodies were eluted by citric acid (0.1M, pH 2.2)

Table 6. Comparative properties of the unbound antibodies recovered from the TSA immunoabsorption of both bleedings

The unbound antibodies from the experiments of Tables 4 and 5 were dialysed and concentrated to the limit of solubility. Immunodiffusion was carried out with the same amount of protein for each set of Ig fraction.

Unbound antibodies of	Solubility	Protein concentration (mg/ml)	Immunoprecipitation of CK in gel media
first bleeding	high	100	No
second bleeding	low	15	No

Kelly (1976).

The % enzymic activity of these gels are given in Table 7.

A graph of the enzymic activity of immobilized CK incubated with various amounts of the Ig fraction from the second bleeding is shown in Fig. 23.

b) Inhibition of the enzymic activity of soluble CK

i) by antibodies

An experiment was performed which indicated that there was no change of the degree of inhibition after incubating CK plus antibodies for 5, 10, 15 or 30 min.

In solution high concentrations of the Ig molecules of the second bleeding inhibited the enzyme almost completely, whereas for the first bleeding there was significant residual activity (20%). Fig. 23 shows the effects of various amounts of the Ig fractions from the first and second bleedings.

The results of inhibition of soluble CK by the unretarded antibodies are given in Table 8. It is notable that the unbound antibodies recovered from the TSA immunoabsorption of the Ig fraction from both bleedings inhibit the enzyme considerably.

ii) by the F(ab) fragment

The F(ab) fragments were prepared as described in the Methods section (a), the elution profile of the Ig₂ digestion mixture from the second bleeding is given in Fig. 24. CK, 2.5 µg, incubated with high concentrations of F(ab) fragments (0.5 ml, 20 mg/ml) showed 60% of the usual specific enzymic activity.

Table 7. Enzymic activity of immobilized CK after immunoabsorption of antibodies.

The gel containing 0.2 mg immobilized CK/ml of gel suspension was diluted 10-fold. The assays were carried out on 0.1 ml aliquot. Controls using the Ig fraction of a non-immunized sheep were run similarly, and did not inhibit the activity.

Stoichiometric and saturating amounts are indicated by the arrows in Figs. 15 and 16.

The specific activity of the immobilized CK under the conditions described by Milner-White & Kelly (1976) was found to be 145 $\mu\text{moles H}^+$ /mg protein/mm.

Bleeding	% Enzymic activity of the gel after immunoabsorption of antibodies	
	stoichiometric amount	saturating amount
First	60	55
Second	15	20
Control	100	100

Fig. 23 Inhibition of soluble and immobilized CK by antibodies of the first and second bleeding.

The immobilized CK was washed with Tris (0.01 M) acetic acid, pH 8.6 to remove the phosphate ions.

O, soluble CK + first bleeding; Δ , soluble CK + second bleeding; \blacktriangle , immobilized CK + second bleeding.

Soluble CK (2.5 μ g; O, Δ) and 0.1 ml of gel containing 2.5 μ g matrix bound CK (\blacktriangle) were incubated with increasing volumes

of the IgG fraction from O, first bleeding; Δ , \blacktriangle second bleeding. The final volume of the mixtures was made to 1 ml with Tris

(0.01 M) acetic acid, pH 8.6. Aliquots (0.2 ml) were taken after incubation for 5 min and assayed for enzymic activity by the phosphate

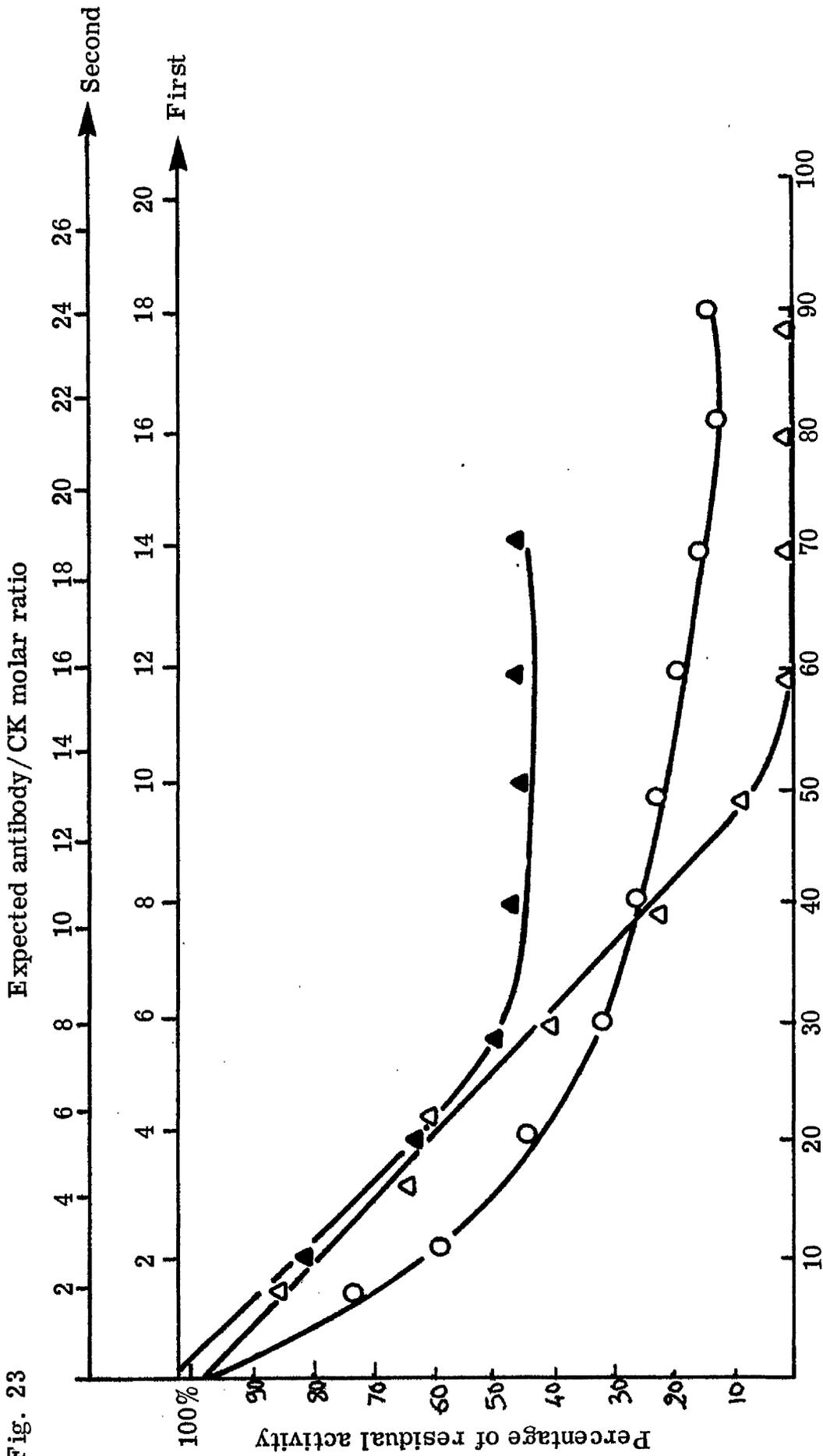
method. Control experiments using no antibodies and also using the IgG fraction of a non immunized sheep serum showed no inhibition.

The specific activity of the enzyme under the conditions described by Kuby et al. (1954) was found to be 56 μ moles phosphocreatine/mg/mn.

$$\% \text{ residual activity is referred as } \frac{\text{Activity of aliquots from the assay}}{\text{Activity of aliquots from the control}} \times 100$$

The expected antibody/CK molar ratio was calculated from the immunoabsorption results.

Fig. 23



μ l Ig fraction (42 mg/ml second or 43 mg/ml first)

Table 8. Inhibition of soluble CK by the unretarded antibodies from both bleedings.

These results refer to the unretarded antibodies from the experiments described in Tables 4, 5 and 6. Fig. 23 shows that maximal inhibition was found with 4.4 and 3.5 mg of the Ig fraction before immunoabsorption from the first and second bleedings respectively.

The unbound antibodies were dialysed to remove the phosphate buffer then concentrated before use. Soluble CK (2.5 µg) was incubated with the unretarded fraction. The final volume of the mixture was made to 1 ml with Tris (0.01 M) acetic acid, pH 8.6. Aliquots (0.2 ml) were taken after incubation for 5 min and assayed for enzymic activity by the phosphate method.

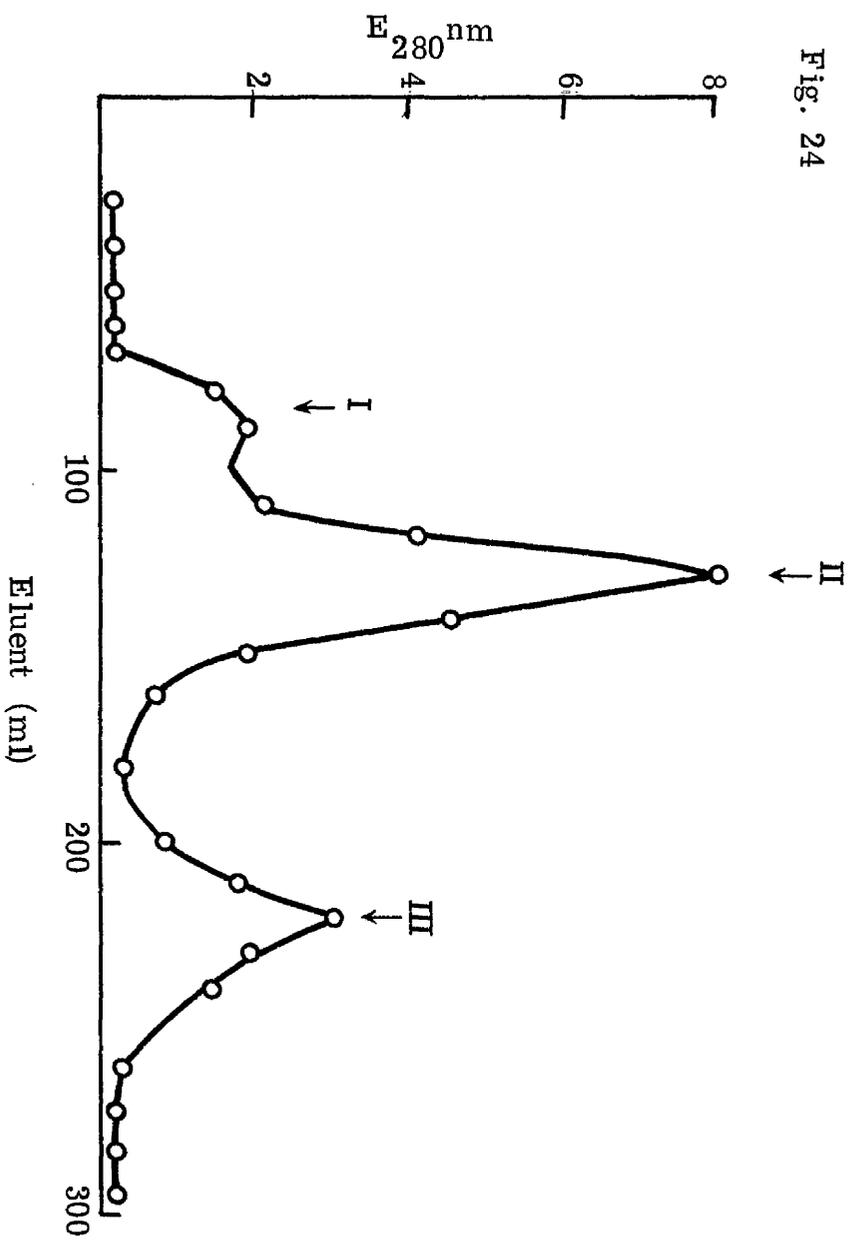
Bleeding	Immuno- absorbent	Amount of protein (mg) of the unretarded Ig fraction added to 2.5 ug CK mg	% of enzymic specific activity
First	CK	7	90
	DE complex	6	80
	TSA complex	5	10
		10	0
Second	CK	4.4	10
	TSA complex	2.2	40
		4.5	10

W41 f
Fig. 24 Elution profile of the IgG digestion mixture from the second bleeding on Sephadex G150.

10 ml of digested IgG (150 mg) containing 10% glycerol were layered on the top of a pre-equilibrated Sephadex G150 column (240 ml). Elution was carried out with Tris (0.01 M) acetic acid, pH 8.6 at a flow rate of 10 ml/h at 4°C.

Peak I corresponds to the uncleaved IgG, peak II to the F(ab) fragment and peak III to the small peptide released by papain digestion. Fractions of peak II were dialysed and concentrated up to 20 mg/ml of protein content.

Fig. 24



9) Antigenicity of proteinase K cleaved CK and the alkylated enzyme

The antibody binding capacity of the iodoacetamide treated enzyme was compared by single radial immunodiffusion and immunoabsorption with the Ig fraction of the first and second bleedings.

a) Single radial immunodiffusion

Figs. 21 and 25 shows that all these derivatives yielded a visible precipitate; the diameters of the outer ring precipitate are given in Table 9. The cleaved CK and the native enzyme show complete cross reactivity while the iodoacetamide treated CK and the unmodified enzyme show an unusual type of cross reaction.

b) Immunoabsorption

Immunoabsorption by the alkylated enzyme was performed as described in the Methods section (m). CK was modified at one single essential thiol per subunit.

The valency found for the alkylated CK was 3.8 which is slightly higher than that of the unmodified CK. Table 10 shows that the antibodies of the first bleeding bound to the alkylated enzyme immunoabsorbent are not eluted by low pH and only 48% of them elute by urea (8 M). This effect was seen only with the first bleeding. The alkylated CK binds the same number of antibodies from the second bleeding as the unmodified CK; however, it was found that CK in solution, containing cysteine (10 mM, buffer, pH 8.6) precipitated these antibodies more effectively than without cysteine according to all immunodiffusion methods used. This may be seen in Fig. 20.

Fig. 25 Single radial immunodiffusion of CK and its derivatives
in agarose gel containing antibodies of the first bleeding.

The agarose gel (2 ml) contained 0.5 ml of IgG fraction of the first bleeding antiserum. The antigen wells were filled with 4 μ l
i) iodoacetamide treated CK, 1.8 mg/ml; ii) CK purified on DEAE, 10 mg/ml, iii) proteinase K cleaved CK, 2 mg/ml.

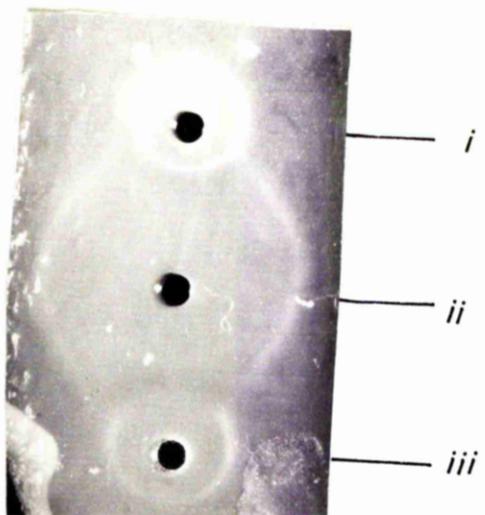


Table 9. Diameters of the outer ring precipitates shown in Fig. 25.

Experimental details are described in the legend of Fig. 25 and in Table 1.

Derivatives	Protein concentration of antigen added to well mg/ml	Diameter outer ring precipitate (mm)
CK	10	15
Proteinase K cleaved CK	2	7
Iodoacetamide treated CK	2	6.5

Table 10. Comparative immunoadsorption by the alkylated enzyme of Ig fraction from the first and second bleedings.

% antibodies eluted refers to the amount of antibodies eluted from the column/amount of antibodies adsorbed. Experimental details are given in Tables 4 and 5. The volume of gel used was 2 ml.

Bleeding	Volume of Ig fraction 42 mg/ml	Protein content before immuno adsorption mg/ml gel	mg/ml of gel after immuno adsorption	Antibodies adsorbed mg/ml gel	Valency of CK	% antibodies eluted by low pH	% Protein eluted by urea 8 M
First	3	0.10	0.8	0.7	3.8	0	48
Second	5	0.20	1.12	0.92	2.5	80	11

DISCUSSION

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DISCUSSION

Under normal circumstances, animals do not show an immune response against self components. The fact that many proteins of phylogenetically related species show considerable structural homology may provide an explanation for the low immune responses obtained when they are used for immunisation (Arnon and Geiger, 1977). Thus the phylogenetic tree showing the evolution of cytochrome C (Fig. 2) should be considered before raising antibodies against any protein.

1) Immunogenic Capacity of Creatine Kinase

It is generally admitted that CK is a weak immunogen; immune responses have only been obtained after challenging animals with high doses of CK (Bulcke and Sherwin, 1969; Viala et al., 1970; Benyamin and Robin 1975; 1976, Roberts and Painter, 1977; Armstrong, et al., 1977; Kuby et al., 1977) though the enzyme from rabbit muscle was more immunogenic in chickens than in mammals as shown in Fig. 2; the chicken enzyme is phylogenetically more distant than other mammalian enzymes.

In the present work twelve sheep were immunised with rabbit muscle CK and only one sheep was found to be a responder. However, the human enzyme was reported to be more immunogenic than the rabbit enzyme in sheep (Steirteghem et al., 1978.) This could also be explained by referring to Fig.2 in which the degree of dissimilarity of the human and sheep enzymes is more acute than that of the rabbit and sheep enzymes.

2) Relationship between Structure and Immunogenicity of Creatine Kinase

Bulcke and Sherwin (1969) found that homologous MM CK of several

mammalian species (rabbit, ox, cat, man) cross reacted with chicken anti rabbit MM antibodies; similar cross reactions between rabbit, ox and human enzymes against rat anti rabbit MM antibodies were reported by Viala et al. (1970). They also showed no cross reactions and no cross inhibition between the B and M type enzymes, observations which were confirmed later by many authors (Armstrong et al., 1977; Gerhardt et al., 1977; Roberts and Painter, 1977) These observations suggest that the catalytic site is not immunogenic as expected from the structural homology of the sequence near the catalytic centre reported for all creatine kinases (see Introduction, p. 2).

The differences in antigenicity between B and M isoenzymes reside in those domains on the surface of the molecule where differences in structure already exist (amino acid composition, pI, thiol content) (Kuby and Jacobs, 1970).

Antigenicity is preserved to some extent in homologous MM forms while BB forms show more species specificity (Viala et al., 1970; Armstrong et al., 1977). The first authors found that CK and arginine kinase do not cross react in their native form, but a few immunological relationships occur between the denatured forms of the two enzymes (Robin et al., 1976). This shows that conservative structures have been preserved in spite of the changes in the molecular size and substrate specificity and confirms the homology of the vertebrate and invertebrate muscle phosphagen kinase suggested by previous biophysical and biochemical studies (see p. 2 and Van Thoai, 1971). They also showed that the cross reactive structures are mostly of the sequential type, characteristic of unfolded proteins whereas the native enzymes possess conformational determinants.

Globular proteins mainly have conformational determinants rather than

sequential determinants. Antibodies to globular proteins cross react very poorly with the denatured protein; reciprocally immunisation of animals with denatured proteins by a variety of methods generally leads to antisera that are specific to the denatured protein and cross react poorly if at all with the native protein (Reichlein, 1975). CK denatured by alkaline pH or heat inactivation showed no cross reaction with the native CK using anti native enzyme antibodies. Benyamin et al. (1976) also showed that CK and the enzyme denatured by performic acid oxidation cross reacted poorly with goat anti rabbit muscle CK. These results suggest that the antigenic capacity of CK is solely determined by its molecular folding. The relation between protein conformation and antigenicity was recognised early in the development of immunochemistry.

3) Heterogeneity of Creatine Kinase

Reinterpretation of the results.

Fig. 5 shows that one band only was resolved by SDS gel electrophoresis in the presence of reducing reagent in the electrode buffer. We reported previously; (Williamson et al., 1977) that two bands of CK were found of slightly different molecular weight; these experiments were carried out in the absence of reducing agent in the electrode buffers. Thus the double band is postulated to arise from an intra chain air oxidation of the thiol groups which are accessible after denaturation; although mercaptoethanol was added to the protein samples before electrophoresis it is likely that the reducing reagent migrates ahead of the protein, leaving it susceptible to air oxidation.

The oxidised form may have one or two disulfide bonds; this might reduce

the size of the molecule and consequently increase the molecular sieving and the mobility of the oxidised form. This would explain why the final SDS gel pattern under the conditions prescribed by the original method (Weber and Osborn, 1969); give rise to two bands (the top band corresponding to the reduced form and the lower band to the oxidised form). These observations suggest that the denatured protein might contain thiols that rapidly oxidise to a disulfide bond. This would also explain why an unusual SDS gel pattern was observed in Fig. 18 for the protein eluted by urea (8 M) from an immunoabsorbent column. This shows that a part of CK and H chain of IgG appear to be missing. This loss may arise during dialysis of the antibodies and CK eluted from the immunoabsorbent dimer column by urea (8 M); the denatured CK and the IgG oxidise by a disulfide exchange mechanism, cross link and precipitate.

Isoelectric focusing of the reduced and fully carboxymethylated CK in the presence of urea (8 M) shows only one band. It is also found that electrofocusing of the enzyme alkylated at the essential thiol groups shows only one band. There is therefore a possibility that the heterogeneity observed with the native enzyme might be due to a partial oxidation of the thiol groups, especially due to persulphate in polyacrylamide gels. However, these three bands were also resolved in a preparative isoelectric focusing system; the isolated proteins had not lost their specific enzymic activity (Milner-White, unpublished work). Wevers *et al.* (1977) also resolved three enzymically active bands of human serum CK by column isoelectric-focusing.

Heterogeneity of CK could also account for the evidence showing a biphasic

association of substrates with CK when binding as the TSA complex (McLaughlin, 1974; Price and Hunter, 1976; Williamson et al., 1977). Recently McIntyre and Milner-White (unpublished results) reacted 90% of the essential thiol groups of the unliganded enzyme with iodoacetamide. After adding ligands to form the TSA complex, they found that the rate profile of the reaction of the remaining enzyme with iodoacetamide was biphasic. This may be interpreted as further evidence for heterogeneity.

No evidence for heterogeneity of CK was found by crystallographic studies (Burgess et al., 1978). Furthermore, the protein from each of the three bands isolated from preparative isoelectric focusing experiments was found to precipitate anti CK antibodies to the same extent (Fig. 9). Thus there is no immunological evidence for heterogeneity.

4) Antigenicity of Creatine Kinase

a) Immunoprecipitation

The secondary response containing mainly antibodies of the IgG class was used in the present work to probe the antigenic surface of the CK molecule. Antibodies to the secondary response collected from two bleedings carried out at an interval of $3\frac{1}{2}$ days on the same sheep showed marked differences in their capacity to precipitate CK purified on DEAE cellulose either in gel media or in liquid media. Table 11 shows that antibodies from the first bleeding give a stronger precipitate with CK than those from the second bleeding.

i) Mechanism of precipitation

The mechanism of precipitation proposed by several immunologists (Roitt,

Table 11. Immunoprecipitation of antibodies from immunised and control sheep antisera.

The immunological activity of the antiserum of the first and second bleeding tested with CK, purified on DEAE cellulose, was compared with a non-immunised sheep antiserum by single radial immunodiffusion and precipitin analysis. The immunodiffusion results are taken from six determinations for the first bleeding antiserum and normal sheep antiserum and twenty determinations for the second bleeding antiserum. These results are drawn from data in Figs.7, 8, 9, 10 and 11.

Nature of antiserum	Strength of immune precipitate in	
	gel media	liquid media
first bleeding	medium	medium
second bleeding	weak	weak
normal sheep	nil	nil

1974; Steward, 1977) may be summarised as follows. As increasing amounts of antigen are added to a fixed amount of antibody, the quantity of specific antibody precipitated increases. After addition of small amounts of antigen some precipitate is formed and free antibody can be detected in the supernatant. This is the antibody excess region where the ratio of antibody to antigen in the immune complex depends on the valency of the antigen since binding of antibodies to their homologous antigenic determinants occurs only via one F(ab) region of the molecule. The addition of larger amounts of antigen results in increased precipitation until a point where no free antibody or antigen is detectable in the supernatant. This is the equivalence zone where optimal proportions of antibody and antigen form a continuous stable antibody-antigen lattice which precipitates. At higher levels of antigen, free antigen appears in the supernatant but precipitate formation is still high; however, precipitation is reduced at very high levels of antigen.

The first bleeding antiserum gave a nearly standard precipitin curve, shown in Fig. 12. From this, both the valence of the CK molecule and the antibody titre have been calculated as described on p. 35. Both the quantity and quality of antibody are important in determining whether a precipitate will be formed. Antigenic valency is also a critical factor since the formation of a lattice is impossible if the antigen is monovalent. The antibody titre of the first bleeding is 0.38 mg/ml which is very low compared to that obtained (2 - 6 mg/ml) in secondary immune responses with most other protein antigens and confirms the weak immunogenicity of CK.

ii) Interpretation of the results

The fact that antibodies from the second bleeding antiserum precipitate less easily than do antibodies from the first bleeding may be ascribed to the quality rather than to the quantity of antibodies.

There is evidence that antibodies of differing affinities have different precipitating characteristics and that high affinity antibodies are more effective than low affinity antibodies in many biological reactions (Precipitation, agglutination, enzyme inactivation, complement fixation, immune elimination, etc.; Steward, 1977). The antibody affinity may be considered as the summation of attractive and repulsive non-covalent intermolecular forces resulting from the interaction of the antibody binding site and the homologous antigenic determinant. These forces which contribute to the stabilization of the immune complex are similar to those involved in the stabilization of the specific configuration of proteins and other macromolecules (Pillot and Peltier 1973; Stewart, 1977). The weak precipitate formed by antibodies of the second bleeding antiserum is likely to be due to the interference of non-precipitating antibodies which are considered by Bach (1976) to be low affinity antibodies.

b) Immunoabsorption

i) Advantages of the immunoabsorption method

Immunoabsorption is a quantitative method ideal for detecting antibodies which do not precipitate readily with antigen of low valency. It is also useful for measuring the valency of the immobilised antigen and the antibody titre for high affinity antibodies. Another possible use may be in the detection of heterogeneity of the distribution of the antibody populations specific for each determinant.

Thus in a system where the concentration of an antibody population specific for one determinant is greater than the concentration of an antibody population specific for the other determinant, it would be possible to isolate those populations of antibodies specific for one determinant that are present in the highest concentrations, i.e. at stoichiometric amounts as indicated by the arrows in Figs. 15, 16.

The amount of antibodies adsorbed by the immobilised antigen, is equal to the protein content of the gel after immunoabsorption and washing off the unbound antibodies minus the protein content of the gel before immunoabsorption. Thus the validity of the protein estimation of the immobilised immune complex depends on the washing step, (removal of unbound antibodies from the gel after immunoabsorption).

ii) Special problems associated with the immunoabsorption technique

Immobilisation of the enzyme may interfere with the antigen-antibody reaction. (Ladzunski et al. (1975) and Louvard et al. (1976) studied the fundamental problems of protein surface alteration and protein surface accessibility associated with the attachment of *E. coli* alkaline phosphatase to the matrix. They were able to determine the number of antigenic determinants that were buried or exposed in different conformational states of the matrix bound protein; up to six antibodies were found to bind to the alkaline phosphate dimer (mol. wt. 83,000). The immobilised CK was prepared in such a way that the enzyme maintained its full native enzymic activity; the low degree of substitution of the gel was shown to favour single point attachment (Bickerstaff and Price, 1976, 1978). Smith et al. (1978) using another immunological system, showed that 10% of their antibodies could absorb non specifically to the matrix.

However, control experiments in the present work using the Ig fraction of a non-immunised sheep showed that there was no binding to the immobilised CK.

The advantage of using elution by citric acid (0.1M; pH 2.2) is that it appears not to dissociate the CK dimer. Some CK appears in the neutralised antibodies eluate (Fig. 17) but is only 7% of the enzyme originally present; this may correspond to the elution of enzyme that is non-covalently bound to the matrix which is solubilised by the acid eluent.

iii) Mechanism of immunoabsorption

Although in the course of immunoprecipitation antibodies can bind via both F(ab) regions, in immunoabsorption they can only normally bind via one F(ab) region; thus when the immunoabsorbent column is saturated with its specific antibodies (i.e. when no more antibodies bind to the antigen and when specific antibodies are detected in the unretarded Ig fraction), it is possible to calculate the antigenic valency. A simplified form of the binding equation is summarised as follows:



This is a reversible equilibrium equation; thus it is possible to apply the mass action Law which states:

$$\frac{[\text{Ab}] [\text{Ag}]}{[\text{Ab} - \text{Ag}]} = K_{\text{Diss}}$$

where $[\text{Ab}]$ = concentration of free antibodies

$[\text{Ag}]$ = concentration of an immobilised antigenic determinant unattached to antibodies

$[Ab - Ag]$ = concentration of the immobilised immunocomplex at that antigenic determinant.

On mixing an excess of antibodies with the immobilised antigen, the antigen will only become saturated with antibodies when

$$[Ab] > K_{Diss}$$

On washing, the antibodies only remain bound when they have a very much lower K_{Diss} than the original $[Ab]$ added. Some specific antibodies must dissociate on washing, but if K_{Diss} is sufficiently low, the free $[Ab]$ in the wash is so low that a negligible proportion of the bound antibodies will be removed. Thus in immunoabsorption only high affinity antibodies remain attached to the matrix bound antigen while low affinity antibodies may be expected to dissociate on washing.

iv) Interpretation of the results

Table 12 shows that there is a higher valency for CK binding to antibodies of the first bleeding than to those of the second. This may explain why a weaker immunoprecipitate was found with the second bleeding than with the first bleeding. However, both bleedings have about the same antibody titres; therefore there must be a larger number of certain kinds of antibody molecules in the second bleeding than in the first bleeding. The antibodies of the second bleeding are mostly directed against one determinant. Only a few antibodies appear to be directed against another determinant; this may indicate their absence or that their low affinity did not allow them to remain bound; Fig. 15 indicates that for the

Table 12. Comparative immunological activity of antibodies from the first and second bleeding.

The antibody titre was measured either by immunoprecipitation or by immunoabsorption.

The valency (i.e. $\frac{\text{antibody}}{\text{antigen}}$ molar ratio) was measured either at saturation of the immobilised CK column by the Ig fraction from the first and second bleeding (see Figs. 15-16) or from the antibody excess region of the precipitin curve (see Figs. 12 and 13).

For the antiserum of the second bleeding, precipitation was difficult to achieve; therefore it was impossible to measure the titre or valence in this case.

Bleeding	Antibody titre, mg/ml antiserum measured	
	by immunoprecipitation	immunoabsorption
First	0.38	0.35
Second	-	0.32
Bleeding	Valence of CK measured	
	by immunoprecipitation	immunoabsorption
First	4	3.5
Second	-	2.9

first bleeding there is a fairly even distribution of the antibody populations directed against each determinant.

Table 12 also shows that the valency of CK measured by immunoprecipitation is somewhat higher than that found by immunoabsorption. However, for the immunoprecipitation experiments the conditions are such that even low affinity antibodies bind their homologous antigenic determinants in the high antibody excess region as seen in Fig. 13.

The capacity of the antibodies of the second bleeding to elute at $\text{pH} \leq 3$ more readily than antibodies of the first bleeding may further be ascribed to their lower affinity. However, Table 4B shows that half the antibodies of the first bleeding which are not eluted by low pH are recovered from the immunoabsorbent by urea (8 M), the other half remains on the gel; this is unusual as urea (8 M) is able to dissociate immunocomplexes completely. Again this could be explained by disulfide bond formation of the free thiols of IgG and CK during elution by urea (8 M) at neutral pH. Half of the CK dimer immunocomplex is dissociated by urea 8M, and the other half remains covalently bound to the matrix (immobilised monomer covalently linked to antibodies via disulfide bond); in this case elution by urea (8 M) containing DTT (1mM) would be desirable.

To conclude, there are more high affinity and less low affinity antibodies in the first bleeding than in the second bleeding.

5) Antibody Binding Capacity of the Immobilised Monomer

It was found by immunoabsorption that each monomer molecule bound 1.2

antibody molecules of the second bleeding. This is the expected value since the valence of the dimer determined under the same conditions was 2.5 (Table 2). Hence the CK monomer is recognised by all anti-native CK antibodies showing that the conformation of the monomer is the same in the dimer as in the isolated subunit as suggested by previous biochemical studies (Bickerstaff and Price, 1978). Furthermore, this indicates that few steric hindrance effects occur on immobilisation of the antigen at the matrix.

6) Inhibition of CK by its Antibodies

Earlier experiments (Samuels, 1961) showed that chicken antibodies inhibited rabbit muscle CK. The rate of this inhibition reaction was surprisingly slow extending over a few minutes; the complex formation leading to inhibition must be due to the summation of a number of antibody-antigen reactions, some of which may be quite slow. In the present work the inhibition of CK by sheep antibodies was found to reach a maximum value within a period of at most 5 minutes after incubation of CK with the antibodies. Measurements of residual activity by the phosphate assay were found to be more reproducible than those by the pH stat assay; presumably the reaction mixture containing cysteine in the pH stat assay reduces the disulphide bonds of antibodies (present in high concentration in the incubation mixture); this process may also yield cystine which has proven to be a potent inhibitor of CK (Jacobs et al., 1978).

i) Mechanism of inhibition

The inhibition of CK by its antibodies may be explained in three ways; it could simply be due to the precipitation and removal of CK from the suspension.

There does not appear to be any precipitation (p. 35); moreover, precipitation is a slow phenomenon (see the Methods section, 1) and could not take place within 5 min; all the solutions were also resuspended before measuring the enzymic activity. However, under these conditions immunocomplexes are undoubtedly present.

Inhibition might also be caused by the steric blocking effect of antibody at the active site; this occurs especially with large molecular weight substrates (Cinader, 1965; Branster and Cinader, 1967).

Another explanation for inhibition is that there is a conformational change in the enzyme on binding to an antigenic determinant near the catalytic site (Celada, 1972); in this instance the degree of inhibition depends on the quality of the antibodies as discussed on p.52. This mechanism is the more plausible for explaining CK inhibition.

ii) Interpretation of the results

The inhibition of soluble CK appears to be complete with a high concentration of antibodies from the second bleeding but not with those of the first bleeding.

Fig. 23 shows that low amounts of antibodies from the first bleeding inhibit more than those of the second bleeding and the graph of enzymic activity on addition of increasing amounts of antibodies of the first bleeding is not linear; this suggests some sort of competition by non inhibiting antibodies. However, this effect is not found with the second bleeding where the corresponding graph is linear.

Complete inhibition is only observed with amounts of antibodies much

greater than those needed to saturate the column (especially for the second bleeding). The inhibiting antibodies of the second bleeding must be present in lower concentration in the original antiserum than in that of the first bleeding and probably represent only a small proportion of the total anti CK antibody population.

The results of adding antibodies to the immobilised enzyme are quite different. Table 13 shows that there is less inhibition and that antibodies of the second bleeding give less inhibition of the immunoabsorbent than those of the first bleeding. In addition, Table 8 shows that the unretarded antibodies of the first bleeding retain only a limited ability to inhibit CK whereas the unretarded antibodies of the second bleeding appear to contain most of the inhibiting antibodies that were present before immunoabsorption; this suggests that, since in immunoabsorption only high affinity antibodies are likely to be detected, the first bleeding contains inhibiting antibodies of relatively high affinity which bind to the immunoabsorbent whereas the second bleeding contains inhibiting antibodies of lower affinity which are not detected by the immunoabsorption technique.

The immobilised enzyme shows only partial inhibition by antibodies of the second bleeding, even at high antibody concentration (seen in Fig. 23); a comparable amount of inhibition of soluble CK is found with an excess of F(ab) fragments of the same bleeding. In all these cases antibodies can only bind via one F(ab) region while in solution intact antibodies may bind via both or all of the F(ab) regions to the soluble CK; this cross-linking may be expected to result in an increased avidity of binding especially for IgM. This could

Table 13 Inhibition of creatine kinase by the Ig fraction from the first and second bleeding in solution and in the immunoabsorbent.

The data for the percentage of enzymic activity are drawn from Fig. 23 and Table 7.

Stoichiometric antibody/antigen ratios mean that the minimum amount of antibodies was added that saturated the antigen according to the immunoabsorption results.

		Bleeding			
		First		Second	
		Stoichiometric antibody/antigen ratios	excess antibody	Stoichiometric antibody/antigen ratios	excess antibody
% specific activity of	soluble CK	50	20	80	0
	immobilised CK	60	-	85	50

explain the different patterns of inhibition in solution from that of the immuno-
absorbent.

Merler et al. (1968) found a value of 10 for the valency of IgM molecules. They can bind in solution via all the ten F(ab) regions; this enhancement of binding makes them complete more effectively than IgG molecules for the same antigenic sites in solutions whereas on an immunoabsorbent both IgM and IgG bind via only one F(ab) region. Evidence suggesting that the low affinity antibodies may have a considerable IgM component are presented in Figs. 11 and 17. The molar ratio of IgM/IgG is respectively higher than 0.28 in the immune precipitate (Fig. 11) and less than 0.05 in the antibodies eluted from the immunoabsorbent column (Fig. 17).

7) Substrate induced Conformational Changes: isolation of site specific antibodies

It has been suggested that antibodies bound to CK induce a conformational change which inactivates the enzyme; conversely if CK is incubated with the substrate analogue, it may be expected to undergo a conformational change which prevents binding of the inhibiting antibodies. Table 14A shows that CK combined with creatine, NO_3^- and MgADP, under conditions in which it forms the TSA complex, binds only 1.6 antibody molecules of the first bleeding. The other antibodies appear to be present in the unretarded Ig fraction.

It is reasonable to assume from these results that under these conditions the enzyme undergoes a conformational change which has modified the shape of a neighbouring antigenic determinant resulting in a decrease of affinity of binding

Table 14. Comparative antigenic reactivity of CK in the presence and absence of substrates.

Part of these data are drawn from Tables 4 and 5 and Figs. 21 and 22.

A) With antibodies of the first bleeding

B) With antibodies of the second bleeding

A) First bleeding

	Strength of precipitate in gel media	Valency of CK measured by immunoabsorption
CK	medium	3.4
CK + MgADP + creatine	strong	3.6
CK +(MgADP + creatine + NO ₃ ⁻)	weak	1.6

B) Second Bleeding

	Strength of precipitate in gel media	Valency of CK measured by immunoabsorption
CK	weak	2.1
CK + MgADP + creatine	-	-
CK +(MgADP + creatine + NO ₃ ⁻)	weak	1.9

of specific antibodies. This effect was only observed with antibodies from the first bleeding and not with those of the second bleeding as shown in Table 14B. However, the unbound antibodies recovered from the TSA immunoabsorbent using the Ig fraction of both bleedings inhibited the enzyme in solution (Table 15) to the same extent as before immunoabsorption.

The results also indicate that the inhibiting antibodies which appear to (normally bind at about two antigenic determinants of the CK molecule) did not precipitate the native enzyme in gel media. Thus the lack of precipitate probably arises from the difficulty of forming a 3-dimensional lattice which cannot form if there are only two antigenic determinants on an antigen. Reichlein (1975) reported in his review that bivalent antigens form soluble immune complexes; Pillot and Peltier (1973) also reported that a minimum of three antigenic determinants is required for lattice formation and precipitation. However, Table 14A shows that CK combined to creatine-MgADP -NO₃⁻ did precipitate although a valence of 1.6 was found by immunoabsorption. This effect is again explained by the increased avidity of binding of antibodies in solution. The enhancement of their functional affinity causes them (the antibodies which do not bind the TSA immunoabsorbent) to bind to the TSA complex though with lower affinity than to native CK. Thus by precipitation all antibodies (high or low affinity) are detected whereas by immunoabsorption only high affinity antibodies are detected.

For the second bleeding Table 15 shows that approximately the same number of antibodies bind to the TSA immunoabsorbent as to that without ligand; similarly both the corresponding unretarded antibody fractions were found to be inhibiting. This may be explained by the low affinity of the antibodies of the

Table 15. Comparative antigenic reactivity of CK with unretarded Ig resulting from immunoabsorption of antibodies (from first and second bleeding) by the immobilised CK combined with (MgADP-creatine-NO₃⁻)

Unretarded Ig fraction	Precipitate	% inhibition of CK in solution
First	No	100
Second	No	90

Data are drawn from Tables 6 and 8.

second bleeding.

Table 14A shows that CK bound to MgADP and creatine precipitated better than the free enzyme with antibodies of the first bleeding. The DE complex recognised most of the anti CK antibodies moreover no antibodies were eluted from the DE complex immunoabsorbent by low pH.

On formation of this complex, CK presumably undergoes a conformational change which modifies the environment of an antigenic determinant near the catalytic site resulting in an increase of affinity of binding of those antibodies which have not bound to the TSA immunoabsorbent. Table 4B shows that only half of the protein content of the gel is recovered from the DE complex immunoabsorbent by urea (8 M); a similar observation was made for the antibodies which remain bound to the CK immunoabsorbent after low pH elution, and was discussed on p. 56.

8) Comparative antigenicity of CK and its Derivatives

Table 16 summarises these results. Proteinase K cleaved CK cross reacts completely with the native enzymes using the first bleeding. The enzyme alkylated at the essential thiol residues shows an unusual cross reaction pattern with the native enzyme; the immunoprecipitation gel, carried out using the first bleeding, is seen in Fig. 25; the pattern seen is understandable in terms of a higher avidity of the alkylated enzyme for the antibodies. Table 16 also indicates that the alkylated enzyme may bind rather more antibodies than the unmodified enzyme; the antibodies bound to the alkylated enzyme on the column were not eluted by low pH; all these observations suggest that the alkylation has modified the shape of an adjacent antigenic determinant which in turn has

Table 16. Comparative antigenicity of CK and its derivatives with Ig of the first bleeding in the presence and absence of substrates.

PCK refers to proteinase K cleaved CK

Iac CK refers to the enzyme modified at the single "essential" thiol per subunit.

The valence was measured by immunoabsorption at saturation of the column with Ig molecule of the first bleeding.

These data are drawn from Figs. 21 and 25 and Table 10.

Derivative	Ig First Bleeding		
	Precipitate strength in gels	Cross reaction with the native CK	Valency measured by immunoabsorption
CK	medium	+	3.5
Proteinase K cleaved K	medium	+	-
Iac CK	stronger	±	3.8
PCK +(MgADP + creatine)	medium	+	-
Iac CK +(MgADP + creatine)	stronger	±	-
PCK + (MgADP + creatine + NO ₃ ⁻)	weaker	+	-
Iac CK + (MgADP + creatine + NO ₃ ⁻)	strong	±	-

increased the affinity of binding of homologous antibodies. A similar observation was reported by Samuels (1961) for adenylate deaminase; he found that the enzyme inhibited by phosphate ions bound more antibodies.

All the above effects were found with the first bleeding; using the second bleeding the alkylated CK and the native enzyme were found to bind the same number of antibody molecules (shown in Table 17). However, a preliminary result showed that CK with its essential thiol group modified to form a mixed disulphide group apparently precipitated better with antibodies of the second bleeding than the native CK.

Preliminary experiments have shown that the proteinase K cleaved CK, which has less than 10% of the enzyme activity of the native enzyme, exhibits similar immunological effects with antibodies of the first bleeding to those of the native CK in the presence of either Mg ADP + creatine or Mg ADP + creatine and NO_3^- , as seen in Table 16. It is not known whether the cleaved CK binds to the complex of Mg ADP, creatine and NO_3^- ; some experiments given in Fig. 21c have also shown that, in the presence of Mg ADP and creatine, the alkylated CK and the native enzyme still exhibit the unusual cross reaction pattern seen in Fig. 25.

9) Immunological Behaviour of the Antibodies from Both Bleedings

In the present work evidence has been presented to show that anti CK antibodies of the same sheep bled at an interval of $3\frac{1}{2}$ days have shown significant differences in their immunological activity. This must be an expression of different idiotypes (as defined by Oudin, 1974) in the two antibody populations.

Table 17. Comparative antigenicity of CK and its derivatives with Ig of the second bleeding.

Iac CK refers to the enzyme modified at the single essential thiol of each subunit.

CK-S cysteine refers to the enzyme with its essential thiol group modified to form a mixed disulphide group with cystine (resulting from air oxidation of cysteine).

Data are drawn from Figs. 8, 9, 10 and 20 and from Table 10.

	Precipitate strength	Valency measured by immunoabsorption
CK	weak	2.4
Proteinase K cleaved CK	-	-
Iac CK	weak	2.5
CK-S cysteine	medium	-

The second bleeding taken $3\frac{1}{2}$ days after the first bleeding was expected to contain nearly all the antibodies detected in the first one. The rate of catabolism of sheep IgG is not known but it must be very rapid compared to human myeloma IgG for which the half lives of the subclasses vary between 7 and 21 days (Capra and Kunkel, 1970; Morell et al., 1970). The rate of IgG biosynthesis is expected to be rapid according to the theoretical work of Brambell et al. (1964).

The observation that some of the antibodies of the second bleeding have a lower affinity than those of the first bleeding may be compared with the studies of Cinader (1965) and Macario and Macario (1975); their results using a variety of antigens indicate considerable variability in the affinity of antibodies during the course of immunisation. They commonly find that, during one particular response, there may be an increase in affinity followed by a decrease during the latter part of the response.

10) Hypothetical Models

The results of the immunological activity of the two bleedings may be interpreted in terms of a model system where CK has four antigenic determinants with two on each subunit; this is illustrated in Scheme 2. This shows that antibodies of the first bleeding against determinant (I) inhibit CK activity; conversely CK in the tightly bound TSA complex prevents antibodies against determinant I from binding. Antibodies from the second bleeding against determinant I do not bind strongly and thus these effects cannot be detected. It is concluded that determinant (I) is located near the catalytic site.

Antigenic effects	Bleeding			
	First		Second	
	I	II	I	II
Determinants				
Binding of antibodies to immobilised CK	Yes	Yes	almost none	Yes
Elution by low pH	Yes	No	-	Yes
Affinity	Medium	Strong	Low	Medium
Displacement by TSA	Yes	No	-	No
Inhibition of CK activity	Yes	No	Yes	No
Enhancement of antibody binding with $\text{ICH}_2\text{CONH}_2$ - treated enzyme	Yes	-	No	-

Scheme 2: Proposed scheme of the different antigenicity of creatine kinase detected by antibodies from the first and second bleeding.

Each determinant has been arbitrarily designated I and II.

A further model suggesting a relationship between antigenicity and enzyme activity of CK in various conformational states is presented in Scheme 3.

11) Possible use of antigenicity of CK to detect muscular diseases

Since CK is a weak immunogen, the techniques of clonal selection and cell fusion (hybridoma, Herzenberg et al., 1978) open up the possibility of making gram quantities of antibodies. These antibodies might be useful in clinical chemistry for detecting muscular diseases. All the methods of assay that are suitable for clinical use so far are reported to be of poor diagnostic value (Morin, 1977, 1978, the Lancet Editorial article, 1978). The reason for this is that workers (Gerhardt et al., 1977; Wretou and Pflleiderer, 1978) have been interested in the inhibition of enzyme activity rather than measuring antigenicity; the problems arise because many potent CK inhibitors are found in the plasma (Jacobs et al., 1978; Gruber, 1978). With regard to MB; determination of the low concentration present in the serum at the early phase of heart disease will serve as a sensitive and valuable index of heart damage.

Recently (Steirteghem et al., 1978; Zweig et al., 1978) introduced a radioimmunoassay for estimating CK isoenzymes in human serum using ^{125}I -CK.

As the effect of the labelling reagent they used is unknown (e.g. dissociation of the enzyme, alteration of antigenic structure, ...) I propose a radioimmunoassay using ^{14}C iodoacetamide treated enzyme since the antigenicity of CK is either unchanged or increased. Another advantage of ^{14}C over ^{125}I is the much longer half-life of the labelled MB CK reagent, consequently the longer shelf-life in clinical laboratories.

Conformation of CK	Enzyme activity	Binding of antibodies
alkylated	inactive	more
native	resting	medium
working *	active	less

Scheme 3: Relationship between antigenicity and conformational change at the catalytic centre.

* This refers to the data for the enzyme bound as the TSA complex; it is likely that this has the same conformation as that of the working enzyme.

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