A Ca^{+2}-ACTIVATED PROTEINASE IN CHICKEN SKELETAL MUSCLE

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ABSTRACT

A neutral calcium-activated protease of muscle (CAP) has previously been characterised and may play a role in myofibrillar disassembly and turnover.

In this study both CAP and endogenous CAP inhibitor from adult and embryonic chicken skeletal muscle have been partially purified by DEAE-cellulose and Sephadex G-150 chromatography.

CAP from embryonic muscle shows similar properties to the corresponding enzyme from adult tissue with respect to calcium dependence (maximum activity at 1.0 mM Ca^{+2}), pH optimum (7.2) and sensitivity to proteinase inhibitors (inhibited by leupeptin and chymostatin). Both embryonic and adult enzymes were found to have molecular weights of 112000 daltons by gel filtration on Sephadex G-150.

CAP activity was present in cultured skeletal muscle cells and increased with cellular growth and differentiation (five-fold). The presence of an inhibitor of CAP was demonstrated in cell cultures by ion-exchange chromatography, the levels of which decreased with a simultaneous increase in CAP activity.

CAP activity showed an increase in developing muscle from 12-day embryos to 7-week chicks in relation to cellular DNA (3.8-fold), although the extent of this increase did not match the extent of accumulation of myofibrillar proteins.

High levels of CAP inhibitor were found in early embryonic muscle and these decreased markedly during development. CAP inhibitor from embryonic tissue was fractionated into 3 species using DEAE-cellulose in contrast to inhibitor from adult tissue which exhibited only two species.

The results indicate that the levels of CAP greatly increase at a time when myofibrillar content of muscle is rapidly increasing and, in addition, demonstrate that CAP activity may be controlled to a large extent by the levels of an intracellular inhibitor.

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ABBREVIATIONS

A	-	absorbance
AMP	-	adenosine monophosphate
ara-C	-	1-β-D-arabinofuranosylcytosine
C	-	Celsius
CAP	-	Ca^{+2}-activated protease
C_i	-	Curie (3.7×10^{10} disintegrations/second)
cm	-	centimetre
cpm	-	counts per minute
DNA	-	deoxyribonucleic acid
DTT	-	dithiothreitol
EDTA	-	ethylene diamine tetraacetic acid
em	-	emission
ex	-	excitation
g	-	gram
h	-	hour
ℓ	-	litre
λ	-	wavelength (lambda)
m	-	metre
M	-	molar
MEM	-	Eagle's minimal essential medium
mg	-	milligram
min	-	minute
ml	-	millilitre
mm	-	millimetre
mM	-	millimolar
M_r	-	molecular weight

N	-	normal
NAD	-	nicotinamide adenine dinucleotide
SDS	-	sodium dodecyl sulphate
sec	-	second
TCA	-	trichloroacetic acid
TEMED	-	N,N,N',N'-tetramethylethylene diamine
Tris	-	Trishydroxymethyl aminomethane
μ (prefix)	-	micro ($10^{-6}\times$)
U	-	unit
vol	-	volume
v/v	-	volume by volume
w/v	-	weight by volume

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CHAPTER 1

INTRODUCTION

1.1 GENERAL PROTEIN DEGRADATION

It is well-established that tissue proteins undergo a continual process of renewal, referred to as "turnover" and that the renewal rates of different proteins, usually defined as their half-life ($t_{\frac{1}{2}}$), varies over a wide spectrum, even within a given cell type. It can be concluded that levels of tissue proteins are determined by their rates of degradation as well as by their rates of synthesis and that the former as well as the latter may be regulatable. Although considerable information is now available on the mechanism of protein synthesis and some of the factors that might alter the rate of protein synthesis, very little is known about the mechanism for degradation of specific proteins; nor is there a consensus regarding the nature of the cellular compartmentalisation that may be involved (Ballard, 1977; Kay, 1978).

One important function of intracellular protein degradation is probably the removal of abnormal proteins which might arise by mutations, errors in gene expression, denaturation or chemical modification. There is now strong evidence that bacterial and animal cells can selectively hydrolyse abnormal proteins (Goldschmidt, 1970). Protein turnover also serves to increase the organism's ability to adapt readily to changes in its environment. Thus, short half-lives may have evolved

in order that crucial enzymes can fluctuate rapidly with changing physiological conditions (Schimke, 1970). Increased protein degradation also seems to be an important physiological response of most organisms to poor nutritional conditions (Dice et al, 1978). Although little is known about the mechanism of degradation of any specific protein, several correlations have been made relating various physiochemical properties of proteins to their rates of degradation in vivo (Arias et al, 1969; Goldberg and Dice, 1974; Goldberg and St John, 1976; Ballard, 1977). In general, proteins with large subunits, abnormal proteins, glycoproteins, acidic proteins and hydrophobic proteins have a high turnover rate. A general relationship has also been established between catabolism in vivo and the susceptibility of proteins to attack by endopeptidases in vitro. In addition, there are numerous examples where ligands and co-enzymes protect enzymes from degradation.

Most of the well-studied proteolytic enzymes are present in lysosomes (Goldberg and St John, 1976; Segal et al, 1976). Lysosomes of most tissues contain at least three well-defined endopeptidases (cathepsin B_1, cathepsin D and cathepsin L), one exopeptidase (cathepsin C) and several peptidases (e.g. cathepsin A). Because of the large number and high concentrations of these proteolytic enzymes, the lysosome has been widely assumed to be the site of protein degradation. However, strong evidence for this conclusion has only accumulated recently and is still incomplete. It is generally accepted that lysosomes are responsible for the degradation, when it occurs, of proteins normally targeted for secretion from the

cells and for degradation of proteins taken up into autophagic vacuoles. It seems hardly likely that lysosomal action accounts for degradation of all cellular proteins because of conceptual difficulties in accounting for the observed heterogeneity in protein half-lives in terms of such autophagic activity. Recent evidence has pointed to the presence of non-lysosomal proteases in the cell. These proteases have maximum activity in the alkaline pH range (pH 8-9) and are inactive in the acidic pH region that is optimal for most lysosomal proteases (pH 4-5). For example, Katunuma and co-workers (1973) have demonstrated serine proteases specific for pyridoxal or NAD-requiring enzymes in several mammalian tissues including muscle which cleave their substrates by limited proteolysis. These group-specific proteases may play a crucial role in the removal of apoenzymes which lack their cofactors. Their existence has been demonstrated in rat liver, small intestine and skeletal muscle (Katunuma et al, 1975; Kominami et al, 1975; Banno et al, 1975). Skeletal muscle has also been shown to contain a number of neutral peptidases (Bury and Pennington, 1975), a calcium-activated protease that releases proteins from myofibrils (Dayton et al, 1976a, b), an insulin-degrading enzyme (Brush, 1971; Duckworth et al, 1972) and an alkaline protease that is associated with the myofibril (Noguchi et al, 1974; Mayer and Rosen, 1975) (see following section). In addition, a neutral protease has recently been characterised in Ehrlich tumour cells and in liver tissue that is sensitive to inhibitors of serine proteases (Nelson and Traub, 1980). The protease, isolated with the components of the cytoskeleton framework of the tumour cells, demonstrated

a high substrate specificity for a 58000 dalton protein which is the predominant subunit of intermediate-sized filaments in Ehrlich tumour cells.

1.2 MYOFIBRILLAR PROTEIN DEGRADATION

The proteins of muscle tissue represent a large fraction of total body protein, comprising about 50% in an adult. Muscle, therefore, represents a large reserve of protein that can be mobilised during periods of dietary stress. However, because of the highly organised macromolecular nature of the myofibril in striated muscle, the turnover of myofibrillar proteins presents additional complexities that must be accounted for when devising any model for myofibrillar protein turnover. Myofibrils consist of long arrays of repeating sarcomere units, extending unbroken from one end of the muscle cell to the other. Consequently, even a single break in the long series of sarcomeres constituting a myofibril will disable that entire myofibril. Yet, studies have shown that myofibrillar proteins do turn over with half-lives of 5 days for myosin heavy chain and 8 days for actin in the rat (Zak et al, 1978). Structural studies on atrophying muscle where myofibrillar degradation is enhanced have shown that a gradual decrease in myofibril diameter occurs in such muscle but that missing sarcomeres are not observed (Engel and Stonnington, 1974). In addition, Morkin (1970) has reported that newly synthesised myofibrillar proteins are added at the periphery of existing myofibrils which is in accordance with the current concept of myofibril

degradation by a continuous assembly and disassembly of myofilaments (Etlinger et al, 1980). Goldspink has autoradiographic evidence suggesting that in skeletal muscle of young animals, in which myofibrils are rapidly lengthening along the fibre axis, and in hypertrophying cardiac muscle, addition of new filaments also takes place at the ends of myofibrils (Williams and Goldspink, 1971; Griffin et al, 1971).

Very little is known about the proteases that cause degradation of myofibrillar proteins and it was previously assumed that lysosomal cathepsins were responsible for myofibrillar degradation. Indeed, it has been shown that the number of lysosomes in muscle cells increases dramatically during periods of rapid protein degradation such as occurs in muscular dystrophy (Kar and Pearson, 1972). However, several lines of evidence suggest that the lysosomal system alone is not responsible for myofibrillar protein degradation. The presence of myofibrils or thick and thin filaments has not been shown in the lysosomes of atrophying muscle (Lockshin and Beaulaton, 1974a, b) and it seems improbable that lysosomes could engulf whole myofibrils. It therefore appears that the proteins of myofibrils must be degraded to monomers before lysosomal degradation can occur.

The existence of several non-lysosomal proteases in muscle has been demonstrated and the activities of these proteases are consistent with their possible involvement in intracellular protein degradation. Katunuma et al (1975) reported an intracellular serine protease in rat skeletal muscle that is active

in the alkaline pH region. The amount of the protease in various types of muscle differs greatly, the activity being higher in red muscle than in white (Katunuma et al, 1977). The serine protease degrades native myofibrillar proteins, namely myosin, troponins T and I, tropomyosin and actin (Yasogawa et al, 1978). A significant increase in the levels of the serine protease was found in patients with two types of muscular dystrophies (Katunuma et al, 1978). An alkaline protease, "myofibrillar alkaline protease" (MAP), has been reported by Mayer et al (1974), who described some properties and adaptive changes of the enzyme under conditions of muscle protein degradation, although it is not clear whether this alkaline proteolytic activity arises from one distinct enzyme or several enzymes (Pennington, 1977). Dahlmann and Reinauer (1978) purified an alkaline protease from a crude fraction of rat skeletal muscle that is distinct from the former enzymes as it is not inhibited by serine protease inhibitors but is sensitive to N-ethylmaleimide and p-chloromercuribenzoate. They suggest that a thiol group may be involved in its catalytic action. A myofibrillar alkaline protease has also been reported in rat heart, the specific activity of which increases progressively with age and upon prolonged starvation (Griffin and Wildenthal, 1978).

In addition, a Ca^{+2}-activated protease ("CAP") in muscle was first discovered by Busch et al (1972), who demonstrated the ability of this proteolytic factor to remove z-discs from intact myofibrils. This activity was subsequently identified in various muscle types, purified to homogeneity and further

characterised. The substrate specificity of CAP suggests a role for the enzyme in the initial disassembly of the myofibril which might initiate turnover of the myofibrillar proteins (Dayton et al, 1976a, b). Our current knowledge of CAP is reviewed in the following section.

1.3 CALCIUM-ACTIVATED PROTEASE

Discovery of the Protease

A calcium-activated protease was first found by Meyer et al (1964), who described a protein factor from rabbit skeletal muscle capable of activating phosphorylase b kinase from the the same muscle in the presence of Ca^{+2} ions. This protein factor was removed during purification of the kinase and further purified by acid precipitation, ammonium sulphate fractionation and DEAE-cellulose column chromatography. A similar kinase-activating factor was subsequently reported in heart muscle by Drummond et al (1965, 1966, 1968), who suggested that activation of phosphorylase b kinase by Ca^{+2} involves proteolytic cleavage by this factor. More recently, Mellgren et al (1979) isolated three endogenous Ca^{+2}-dependent proteases in rabbit skeletal muscle which degraded rabbit muscle phosphorylase phosphatase to lower molecular weight forms. It is likely that these proteases are related to the activity which activates cyclic AMP-independent protein kinase from rat skeletal muscle (Inoue et al, 1977).

The presence of a Ca^{+2}-activated protease ("CAP") thought to

be related to the activities described in the preceding section and that is capable of removing Z-discs from intact myofibrils was discovered by Busch et al (1972). They examined strips of rabbit psoas muscle by electron microscopy after the muscle had been incubated at pH 7,2 in Ca^{+2}-containing solutions for 9 hours at 37°C and found the removal of Z-discs with little other ultrastructural change of the myofibrils. When purified myofibrils were incubated in the presence of Ca^{+2}, no detectable effect was noticed on the myofibrillar structure. Consequently, it was suggested that Ca^{+2} activates a sarcoplasmic factor (removed upon purification of myofibrils), which then removes Z-discs. To isolate this factor, the sarcoplasmic proteins in the homogenate supernatant were subjected to isoelectric precipitation between pH 4,9 and 6,2. The protein precipitated in this pH range caused the removal of Z-discs when incubated with purified myofibrils in the presence of Ca^{+2} ions.

Although this protein fraction contained Ca^{+2}-activated proteolytic activity, the heterogeneity of the fraction made it impossible to determine whether the Ca^{+2}-activated proteolytic activity was responsible for Z-disc removal. Reddy et al (1975) isolated and partially purified CAP (200-fold) from the post-myofibrillar supernatant fraction of rabbit skeletal muscle. Digestion of isolated myofibrils with partially purified CAP resulted in removal of Z-discs with a loss of α-actinin. Dayton et al (1975, 1976a, b) purified CAP from porcine skeletal muscle (17800-fold) and presented electron microscopic findings that showed the removal of Z-discs from

intact myofibrils by purified CAP.

Distribution of CAP in types of tissue

A Ca^{+2}-dependent protease was detected in human platelets (Philips and Jakábová, 1977) and bovine platelets (Szpacenko et al, 1980), which has similar properties to myofibrillar CAP but would appear to serve a different function. Guroff (1964) isolated a neutral, Ca^{+2}-activated proteinase from the soluble fraction of rat brain, whose function is not clear, but which seems to act on a specific fraction of soluble brain protein. The presence of CAP has also been demonstrated in chicken skeletal muscle (Ishiura et al, 1978), human skeletal muscle (Suzuki et al, 1979), bovine ventricular muscle (Toyo-Oka et al, 1979), canine cardiac muscle (Mellgren, 1980), rat liver cytosol (Nishiura et al, 1978) and porcine skeletal and cardiac muscle (Dayton et al, 1976a, b; Dayton and Schollmeyer, 1980). In addition, calf uterus cytosol contains a Ca^{+2}-activated protease which irreversibly converts the larger molecular states of estrogen receptor into a smaller, salt-stable form (Puca et al, 1977).

Purification and Properties of Ca^{+2}-activated Protease

Purification

CAP was purified from the post-myofibrillar supernatant fraction from rabbit skeletal muscle (Reddy et al, 1975) by a 2-step isoelectric precipitation of the enzyme between pH 6,1 and 4,9, followed by DEAE-cellulose and Sephadex G-200 column

chromatography. The enzyme preparation thus obtained was purified 200-fold over the crude extract but was not a homogeneous preparation. In contrast, Dayton et al (1976a) purified CAP from porcine skeletal muscle 17800-fold. Their purification involved five column chromatographic procedures in succession: (1) 6% agarose; (2) DEAE-cellulose; (3) Sephadex G-200; (4) DEAE-cellulose with a very shallow gradient; (5) Sephadex G-150. Purified Z-disc-removing CAP migrated as a single band during polyacrylamide gel electrophoresis under non-denaturing conditions and comprised 85-90% of the total protein as judged by densitometer scans of the gels.

Other workers used different procedures for the purification of CAP. For example, Ishiura et al (1978) purified CAP 2700-fold over the crude extract from chicken skeletal muscle. Their method involved firstly pH precipitation of the crude extract (pH 4,9), followed by DEAE-cellulose column chromatography, Ultragel AcA 34 chromatography and finally, a second column of DEAE-cellulose. The enzyme thus obtained migrated as a single band on SDS-polyacrylamide gel electrophoresis. CAP was purified from rabbit skeletal muscle (Azanza et al, 1979) by a method involving DEAE-Sephacel chromatography, affinity chromatography on organomercurial-Sepharose and gel filtration on Sephacryl S-200 and Sephadex G-150. SDS-polyacrylamide gel electrophoresis of the enzyme after the final column step revealed the presence of only one band. Toyo-Oka et al (1979) used a novel method for the purification of CAP from bovine ventricular muscle by isoelectric precipitation

of the enzyme at pH 4,7 to remove endogenous inhibitor. Further purification involved affinity chromatography in anti-pain-aminohexyl Sepharose 4B, gel filtration on Sephadex G-200, DEAE-cellulose column chromatography and isoelectric focusing in sucrose density columns (pH 3,5 to 6,0). Their preparation, purified 10000 times over the crude extract, gave a polypeptide band at 80000 daltons on SDS-polyacrylamide gel electrophoresis with a few contaminating bands. The large differences in degrees of purification obtained for CAP from different muscles is likely to be a result of differences in the concentrations of endogenous inhibitors as well as in the concentrations of CAP itself.

Molecular Weight

CAP from porcine skeletal muscle (Dayton et al, 1975, 1976a) migrated as a single band during polyacrylamide gel electrophoresis in the absence of SDS and as two bands in the presence of SDS. The molecular weights of the two bands were 80000 and 30000 daltons, respectively. Elution profiles from gel permeation columns indicated that the undenatured CAP molecule is not larger than 110000 daltons. Suzuki et al (1979) purified CAP from human skeletal muscle, as well as rabbit and monkey skeletal muscle and demonstrated two subunits of 80000 and 30000 daltons, respectively, which is in accordance with Dayton et al (1976a). However, CAP from chicken skeletal muscle (Ishiura et al, 1978) is a monomeric protein consisting of a single polypeptide chain of 80000. By using different purification procedures, Azanza et al (1979) obtained a molecular weight of 73000 for CAP from rabbit skeletal muscle.

Their purification procedure eliminated two contaminants each of about 30000 daltons. CAP from bovine ventricular muscle has a molecular weight of 80000 (Toyo-Oka et al, 1979), whereas Mellgren (1980) obtained a molecular weight of 135000 for partially purified CAP from canine cardiac muscle.

It seems unlikely that the 30000 dalton subunit described by Dayton et al (1976a) is a contaminating protein due to the high degree of purification of their enzyme (17800-fold). However, Ishiura et al (1978) did not employ drastic conditions of purification that could have resulted in the dissociation of the oligomeric enzyme. It is possible that the difference observed by the various authors arises from the sources of the enzyme.

Intracellular localisation of CAP

Reville et al (1976) demonstrated that CAP from porcine skeletal muscle was not located in a membrane-bound subcellular fraction and therefore existed in the sarcoplasm. However, they found that if the muscle was homogenised very mildly, their yields of CAP were decreased, which suggests that more severe homogenisation possibly released CAP from myofibrils or other structures where it was adsorbed. If CAP's physiological role is to degrade Z-discs, the effectiveness of CAP in skeletal muscle cells would be greatly increased if CAP were adsorbed to filaments near the Z-discs or to the Z-disc itself. Recently, the presence of CAP was demonstrated in myofibrils, particularly at the Z-disc, in glycerinated chicken myofibrils,

by an immunofluorescent method (Ishiura et al, 1980). The amount of CAP bound to myofibrils was approximately 4% of the total enzyme present. No immunological difference was found between the myofibril-bound CAP and the soluble enzyme and it is not clear whether there is a functional difference between free and bound CAP. It is possible that a compartmentation of the enzyme would facilitate the control of the proteolytic activity in the muscle cell, namely, that degradation of myofibrillar proteins and activation of soluble enzymes could be achieved independently.

CAP is therefore present in the sarcoplasm of muscle cells with some fraction bound to myofibrils. The significance of the latter is as yet unclear.

Dependence of CAP activity on Ca^{+2} ion concentrations and effect of other divalent cations on CAP activity

Purified CAP from porcine skeletal muscle required 1,0 mM Ca^{+2} for maximum activation while almost no activity was present below 0,1 mM Ca^{+2}. CAP activity was significantly decreased in the presence of 10 mM Ca^{+2} or higher (Dayton et al, 1976b), The effects of other divalent cations on CAP activity were studied. Mn^{+2}, Mg^{+2}, Ba^{+2}, Co^{+2}, Ni^{+2} and Fe^{+2} did not activate CAP when added singly in the absence of Ca^{+2}. In the presence of 1,0 mM Ca^{+2}, the simultaneous presence of 1 mM Mg^{+2}, Mn^{+2} or Ba^{+2} had no effect on CAP activity whereas Co^{+2}, Cu^{+2}, Ni^{+2} and Fe^{+2} inhibited CAP to various degrees when present at 1 mM concentrations.

Ishiura et al (1978) reported a requirement of 1,8 mM Ca^{+2} or 10 mM Sr^{+2} for maximum activation of CAP from chicken skeletal muscle. Other divalent cations did not activate CAP and Zn^{+2} ion (5 mM) completely blocked the activation of CAP by Ca^{+2}.

An optimum concentration of 10 mM Ca^{+2} was reported by Reddy et al (1975) for CAP from rabbit skeletal muscle, whereas Suzuki and Goll (1974) reported maximum activation of CAP at 1 mM Ca^{+2} from the same muscle. They found no proteolytic activity at 0,01 mM Ca^{+2} but appreciable activity at 0,1 mM Ca^{+2}.

CAP from human skeletal muscle crude homogenate required 1 mM Ca^{+2} for optimum activation with Mg^{+2}, Co^{+2} and Mn^{+2} being 52%, 35% and 25% as effective as Ca^{+2} in activating CAP (Kar and Pearson, 1976). Suzuki et al (1979), however, purified CAP from human skeletal muscle and reported a concentration of 5 mM Ca^{+2} for maximum activation of the purified enzyme, whereas Ba^{+2}, Mg^{+2}, Co^{+2} and Ni^{+2} did not activate CAP at concentrations up to 10 mM.

Two Ca^{+2}-dependent proteases from canine cardiac muscle, which appear to have the same molecular weight (135000 daltons) are both activated by Ca^{+2} ions (Mellgren, 1980). Two peaks of protease activity were obtained after chromatography on DEAE-Sepharose CL-6B, Ultrogel AcA 34 and DEAE-Sephadex and were designated peaks 1 and 11, in order of their elution off a DEAE-Sephadex column. Peak 1 protease was active at 40 µM

Ca^{+2}, whereas peak 11 protease had very little activity until the concentration of Ca^{+2} was greater than 0.4 mM. Peak 1 protease was therefore active at free Ca^{+2} concentrations which may be attained in muscle cells during contraction. It is therefore interesting that the Ca^{+2} concentrations usually required to activate CAP are much higher than the intracellular concentrations of free Ca^{+2} in resting, living skeletal muscle (generally 10^{-5} M or less). This factor may regulate or limit indiscriminate destruction of Z-discs *in vivo*. During postmortem storage of muscle, however, sarcoplasmic reticular membranes lose their ability to sequester Ca^{+2} very early and intracellular Ca^{+2} concentrations should increase to 0.1 mM or more. This increase in free, intracellular Ca^{+2} concentration would activate CAP and enable it to initiate the degradation of Z-discs that is observed in postmortem muscle.

On the other hand, the requirement of CAP for Ca^{+2} *in vivo* may be different to the concentration necessary for activation *in vitro* or may be modified *in vivo* in response to certain physiological stimuli such as phosphorylation of a subunit of CAP. Alternatively, intracellular compartmentalisation of Ca^{+2} may occur, such that localised concentrations of Ca^{+2} might reach levels required for activation of CAP. It has been shown that a fraction of CAP activity is adsorbed on to myofibrils in the Z-disc region (Ishiura *et al*, 1980). Possibly Ca^{+2} concentrations in these regions could reach higher levels than the concentration normally found in muscle cells. A protein which mediates control of a large number of enzymes by Ca^{+2} is calmodulin, a small heat- and acid-stable protein that is

not tissue or species specific (reviewed by Klee et al, 1980; Gevers, 1980). It is likely that calmodulin plays a role in regulating intracellular Ca^{+2} concentrations by reversible sequestrations within membrane-bound species or by protein binding.

pH optimum of CAP activity

The pH optimum of CAP activity from porcine skeletal muscle (Dayton et al, 1976b) was near pH 7,5. Significant CAP activity existed between pH 6,5 and 8,0, but the activity decreased rapidly below pH 6,5 or above pH 8,0. A similar pH profile was obtained for CAP activity from both human skeletal muscle (Suzuki et al, 1979) and rabbit skeletal muscle (Azanza et al, 1979).

Optimum pH values of around 7,7, 7,0 and 7,0 to 7,5 were reported for CAP activity from chicken skeletal muscle (Ishiura et al, 1978), canine cardiac muscle (Mellgren, 1980) and bovine ventricular muscle (Toyo-Oka et al, 1979).

The effect of pH on CAP activity is therefore completely different from the effect of pH on lysosomal proteases. Although the reported pH optima for Ca^{+2}-activated proteases differ slightly from species to species, it is significant that all require a pH in the neutral region for optimum activity.

Effect of Inhibitors on CAP activity

CAP requires a reducing agent such as mercapto-ethanol or dithiothreitol to function at maximum efficiency (Dayton et al, 1976b). The proteolytic activity is irreversibly inhibited by iodoacetate under conditions where reaction of iodoacetate with proteins is limited mainly to cysteine side chains. The low molecular weight peptide inhibitors, leupeptin and antipain, which inhibit thiol proteinases, inhibit CAP activity, whereas CAP activity is unaffected by pepstatin, an inhibitor of carboxyl proteinases. Libby and Goldberg (1980) demonstrated inhibition of CAP activity from rat skeletal muscle by chymostatin, a serine protease inhibitor that is also a known inhibitor of the myofibrillar alkaline protease. Sugita et al (1980) recently reported that specific thiol protease inhibitors, d,l-trans-epoxy-succinate derivatives, inhibit CAP irreversibly in vitro and in vivo without any effect on other thiol dependent enzymes. Their results were obtained using dystrophic chicken skeletal muscle. Alpha-1-antitrypsin and soybean trypsin inhibitor (Toyo-Oka et al, 1978; Ishiura et al, 1978) have no effect on CAP activity, whereas CAP activity is inhibited by alkylating agents such as N-ethylmaleimide as well as p-chloromercuribenzoate, 2,2'-dithiopyridine and heavy metals (Azanza et al, 1979). The Ca^{+2}-activated protease is therefore a thiol proteinase which requires a sulphydryl group for activity.

Substrate Specificity

CAP requires exclusively a long polypeptide chain as a

substrate and can cleave tubulin, spectrin and oxidised insulin B chain (Ishiura et al, 1979) but not synthetic substrate such as N-benzoyl-L-arginine ethylester and acetyl-tyrosine (Toyo-Oka et al, 1979). One of the myofibrillar contractile proteins, actin, as well as α-actinin, are not degraded by CAP, whereas the regulatory proteins, tropomyosin, troponins T and I and C-protein, are hydrolysed by CAP (Dayton et al, 1975, 1976b). CAP acts on tropomyosin to break it into a group of fragments having molecular weights in the range 13000-18000 daltons and into a series of smaller pieces. Troponin-T and troponin-I, but not troponin-C, are degraded by CAP producing two kinds of fragments with molecular weights of approximately 10000 and 14000 daltons. Troponin-T, which is hydrolysed more rapidly than troponin-I, is first degraded to a 30000 dalton fragment before being reduced to smaller fractions.

In addition, Pemrick et al (1980) have recently demonstrated that CAP isolated from platelets degrades normal rabbit myosin heavy chain in the unphosphorylated form. They suggest that under phosphorylation conditions, skeletal myosin is resistant to degradation by Ca^{+2}-specific proteases. A Ca^{+2}-activated protease present in human platelets has been shown to degrade native platelet myosin heavy chain producing two major cleavage products of molecular weights 140000 and 100000 daltons (Hathaway, 1980). Under identical assay conditions, smooth muscle myosin is not degraded by the CAP. However, smooth muscle myosin light chain kinase is degraded by the CAP, producing two major peptides as well as several minor peptides.

A recent finding is that CAP can also degrade desmin, a protein of the so-called intermediate filaments that are found in a network around the Z-disc region and are thought to play a role in maintaining the integrated structure of the myofibril (Lazarides, 1980; Schollmeyer and Dayton , 1977). CAP isolated from smooth muscle was similar to the enzyme from skeletal muscle and caused hydrolysis of desmin and disruption of the intermediate filaments in addition to the release of α-actinin from Z-discs (Schollmeyer and Dayton, 1977).

Physiological Regulation of CAP Activity

A significant increase in the levels of Ca^{+2}-activated protease has been demonstrated in skeletal muscle of patients with two types of muscular dystrophies, namely, Duchenne dystrophy and a Becker variant (Kar and Pearson, 1976). Increased CAP levels have also been reported in skeletal muscle of dystrophic hamsters and mice (Neerunjun and Dubowitz, 1979) and a 3,6-fold increase in CAP activity was found in atrophying muscle of vitamin E-deficient rabbits (Dayton et al, 1979).

Ultrastructural studies of vitamin E-deficient atrophying rabbit muscle (Dayton et al, 1979) have shown that the first observable change in myofibrillar structure is disruption of the Z-disc and I-band. Considered together these data suggest that CAP may play a role in the observed Z-disc and thin filament degradation, which in turn may initiate myo-

fibrillar breakdown. Consistent with this hypothesis is that increased levels of Ca^{+2} are found in dystrophic muscle (Oberc and Engel, 1977).

Endogenous inhibitor of Ca^{+2}-activated protease

Waxman and Krebs (1978) reported the occurrence of two protease inhibitors in cell extracts from bovine cardiac muscle. One of the inhibitors was active against trypsin and chymotrypsin and the other inhibited CAP from this tissue. The latter was separated from the enzyme by DEAE-cellulose column chromatography and was a protein of molecular weight 270000 daltons. The inhibitor was present in large excess over the amount of CAP in this tissue and its action was not based on a binding of Ca^{+2} to the inhibitor. Otsuka and Goll (1980) have recently purified the CAP inhibitor from bovine cardiac muscle and characterised its properties. The inhibitor is a protein with a subunit molecular weight of 100000 daltons but has a larger molecular weight in nondenaturing solvents. It seems specific for CAP and does not inhibit by chelating Ca^{+2}. Similar inhibitors of CAP have been reported in rat liver (Nishiura et al, 1978) and rat brain (Nishiura et al, 1979). The partially purified inhibitors were proteins that were heat-stable, acid-stable and both had a molecular weight of 300000 daltons. They were readily inactivated by tryptic digestion. An inhibitor of CAP activity in chicken skeletal muscle with a much lower molecular weight (67000 daltons) was reported by Ishiura et al (1977).

It is possible that a number of cellular proteases have gone

undetected because of the presence of inhibitors such as those described above. The possible presence of specific inhibitors may explain the marked heterogeneity in turnover rates of cellular proteins and why non-lysosomal proteolytic activity has generally been thought to be of lesser importance in overall intracellular protein turnover. However, the role of these inhibitors in regulating cellular proteinase activity is as yet unknown.

Possible Role of CAP in Myofibrillar Protein Breakdown

As previously mentioned, CAP degrades many of the myofibrillar proteins. In addition, increased levels of CAP activity are found in patients and animals with muscular dystrophy and in other muscles exhibiting enhanced proteolysis. Intracellular Ca^{+2} concentrations are raised in muscular dystrophy and ultrastructural studies of atrophying muscle have revealed a marked change in Z-disc structure which is similar to that obtained after CAP degradation of myofibrils *in vitro*.

Suzuki *et al* (1977, 1978a) incubated proteins released from myofibrils by CAP with Z-disc-extracted fibre bundles. Electron microscopy revealed that the materials released from myofibrils by CAP were bound to Z-disc-extracted myofibrils in the Z-disc region. However, the Z-discs removed from myofibrils by CAP were unable to be reconstituted and the appearance of the Z-disc region treated by CAP differed from the Z-disc-extracted fibre bundles. The effect of individual fractions released from myofibrils by CAP on Z-disc reconstitution was determined by Suzuki *et al* (1978b). Released

materials from myofibrils by CAP were resolved on a Sepharose 6B column and the fractions that bound in the Z-disc region consisted primarily of proteins having subunit molecular weights near 100000 and 34000 daltons, which were possibly α-actinin and tropomyosin. These observations suggest that CAP may play a role in the initial steps of myofibrillar disassembly and protein turnover.

Dayton et al (1975) have proposed a role for CAP in myofibrillar protein degradation. A modified schematic representation of their hypothesis is given in Fig. 1.1. Their hypothesis suggests that CAP may initiate myofibrillar turnover by degrading Z-discs and C-protein. This would cause disordering of the three-dimensional array of thick and thin filaments. Degradation of tropomyosin and troponin by CAP could then initiate disassembly of thin filaments to actin monomers and dimers and degradation of the protein of the M-line could initiate disassembly of the thick filaments. The actin and myosin monomers released by this disassembly could then be degraded by lysosomes or, alternatively, could reassemble with new tropomyosin and M-line protein to form new myofibrils. Also represented in Fig. 1.1 is the proposed CAP degradation of desmin, a protein of the intermediate filaments found in a network around the Z-disc region and which is thought to play a role in maintaining the integrated structure of the myofibril (Lazarides, 1980; Schollmeyer and Dayton, 1977).

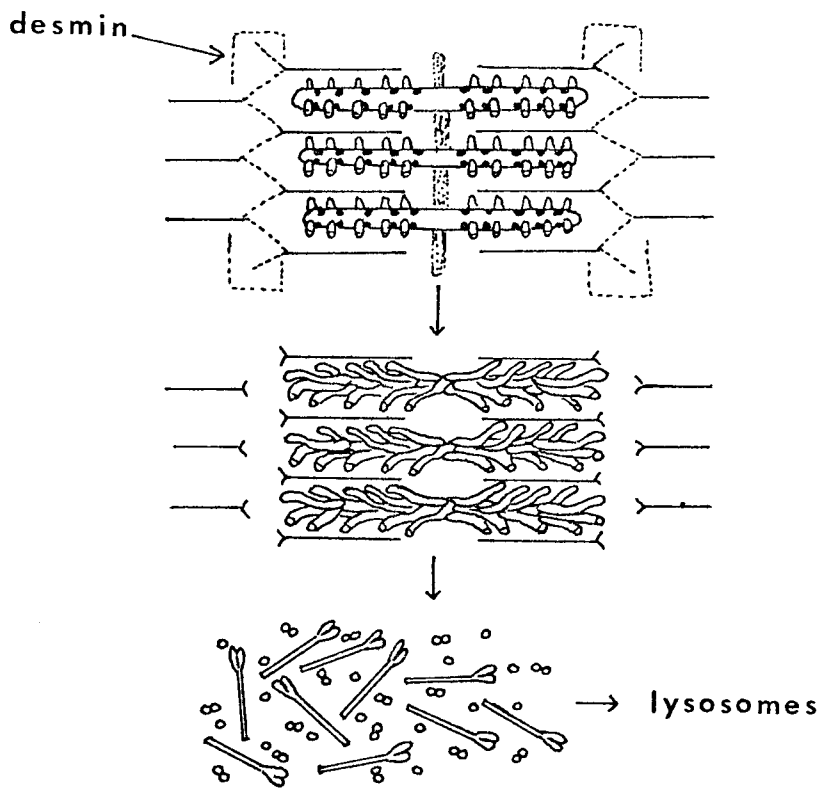


Fig. 1.1 A modified schematic representation of the proposed mechanism of CAP action (Dayton et al, 1975). A single sarcomere of an intact myofibril is shown at the top of the diagram, with thick and thin filaments, Z-discs, M-line and desmin represented. The middle diagram represents the sarcomere after CAP degradation of Z-discs, desmin, tropomyosin, troponin and C-protein. Because the proteins binding actin and myosin monomers into thin and thick filaments have been destroyed by CAP, the thick filaments become disorganised and the thin filaments are freed from the myofibril. After dissociation into monomers and dimers (bottom), actin and myosin molecules are degraded to amino acids by enzymes other than CAP. As indicated, these enzymes may be lysosomal cathepsins.

1.4 OBJECTIVES OF STUDY

No studies have been carried out on CAP activity in muscle that is undergoing a rapid increase in myofibrillar content, such as is found in differentiating muscle. The present investigation was undertaken to characterise CAP activity in differentiating skeletal muscle cultures which undergo marked differentiation between 2 and 6 days' growth in culture. In addition to CAP activity, the presence of any endogenous inhibitor(s) of CAP in muscle cell cultures was also examined. The cultured cell system was chosen since differentiation in cultures has been well-characterised and is a rapid and synchronous event compared to the differentiation occurring in muscle *in vivo* (Yaffe, 1969). Furthermore, muscle cells can be obtained in nearby homogeneous cell populations, whereas the heterogeneity of cell types and the presence of an extracellular fluid space complicates the interpretation of results obtained using normal muscle tissue.

A model system for the characterisation of CAP activity in muscle differentiating *in vivo* was also chosen, namely, developing chicken embryonic breast muscle in which rapid turnover rates of protein occur (Millward, 1980). The presence of CAP inhibitor in this muscle was verified and levels of CAP and CAP inhibitor were measured at various stages of growth and differentiation. Correlations were made between these levels. The protease from embryonic muscle was partially purified and characterised and the enzyme was compared to CAP from adult chicken skeletal muscle.

CHAPTER 2

PURIFICATION AND CHARACTERISATION OF A Ca^{+2}-ACTIVATED PROTEASE AND CAP INHIBITOR

2.1 INTRODUCTION

The presence of a Ca^{+2}-activated neutral protease has been previously reported in cardiac and skeletal muscle from numerous animal species and the levels have been found to vary between species (Dayton et al, 1976a; Ishuira et al, 1978; Toyo-Oka and Masaki, 1979; Dayton and Schollmeyer, 1980; Suzuki et al, 1979a). In addition, increased levels of CAP activity have been found in cases of muscular dystrophy in humans (Kar and Pearson, 1976) and in vitamin E-deficient dystrophic rabbits (Dayton et al, 1979). Although CAP has been characterised in adult cardiac and skeletal muscle, very little is known about the level of CAP activity in muscle that is undergoing a rapid increase in myofibrillar content. I decided to investigate the presence and levels of CAP activity in chicken embryonic skeletal muscle and to compare the properties of the embryonic protease with those of the enzyme from adult tissue.

In attempting to purify the protease, much less activity was found in crude extracts from embryonic muscle than in extracts from adult muscle. After chromatography on DEAE-cellulose, the proteolytic activity in embryonic muscle extracts was greatly increased. When the column fractions were assayed

for inhibitory activity, a region was found which, when added back to fractions containing proteolytic activity, inhibited the calcium-activated proteolytic activity. This prompted the further investigation of this endogenous inhibitor of CAP in embryonic muscle and comparison of this inhibitor species with the inhibitor from adult muscle. The identification and properties of these inhibitor species may shed new light on the existence and distribution of nonlysosomal proteases and their physiological function in initiating and catalysing cellular proteolysis.

2.2 RESULTS

2.2.1 Standardisation of CAP assay

2.2.1.1 Methods of assay

In order to establish a standard method suitable for assay of CAP activity, four methods were tested, each of which involved the use of denatured or modified casein as a substrate. The procedures followed are described in Chapter 4 (4.2). The following substrates and methods of product analysis were used: 1. Denatured casein and A_{280} of TCA supernatant measured; 2. Denatured casein and tyrosine release measured; 3. $[^{125}I]$ - labelled casein and release of TCA-soluble radioactivity measured; 4. Azocasein and measurement of absorbance at 340 nm of yellow TCA-soluble fraction.

Figs. 2.1 to 2.4 show the linearity of the reactions with increasing enzyme concentration, using each of the four

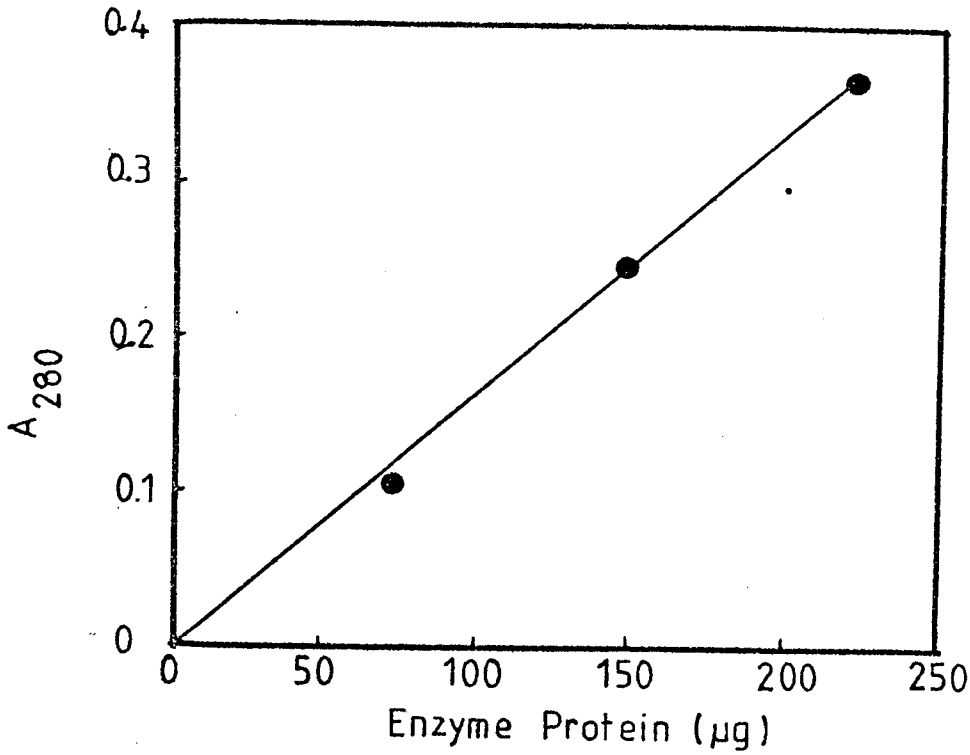


Fig. 2.1 CAP activity using denatured casein as substrate. CAP was prepared as described in 4.6.1 and caseinolytic activity was assayed as described in 4.2.4.2.

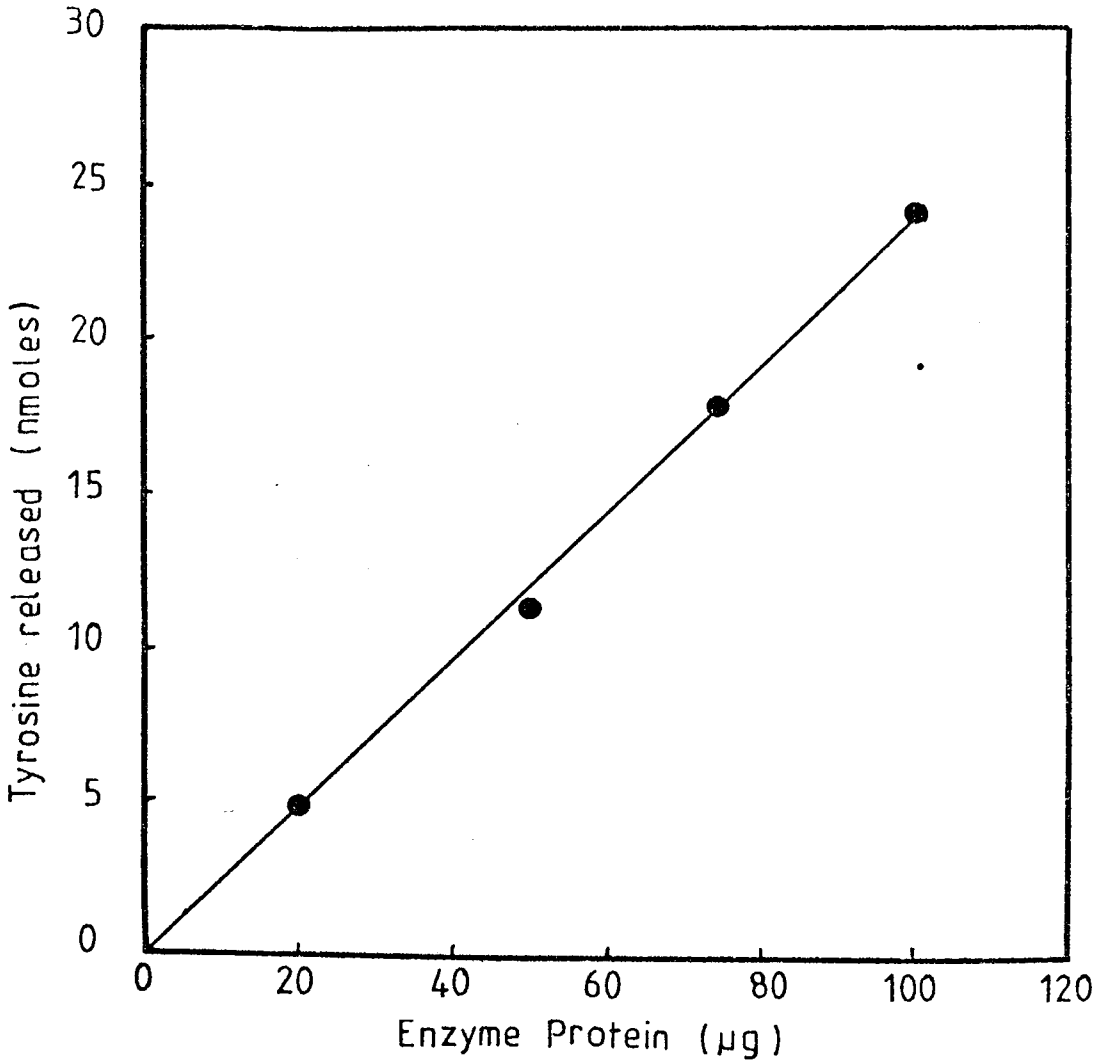


Fig. 2.2 CAP activity measuring tyrosine release. CAP was prepared as described in 4.6.1 and proteolytic activity assayed as described in 4.2.

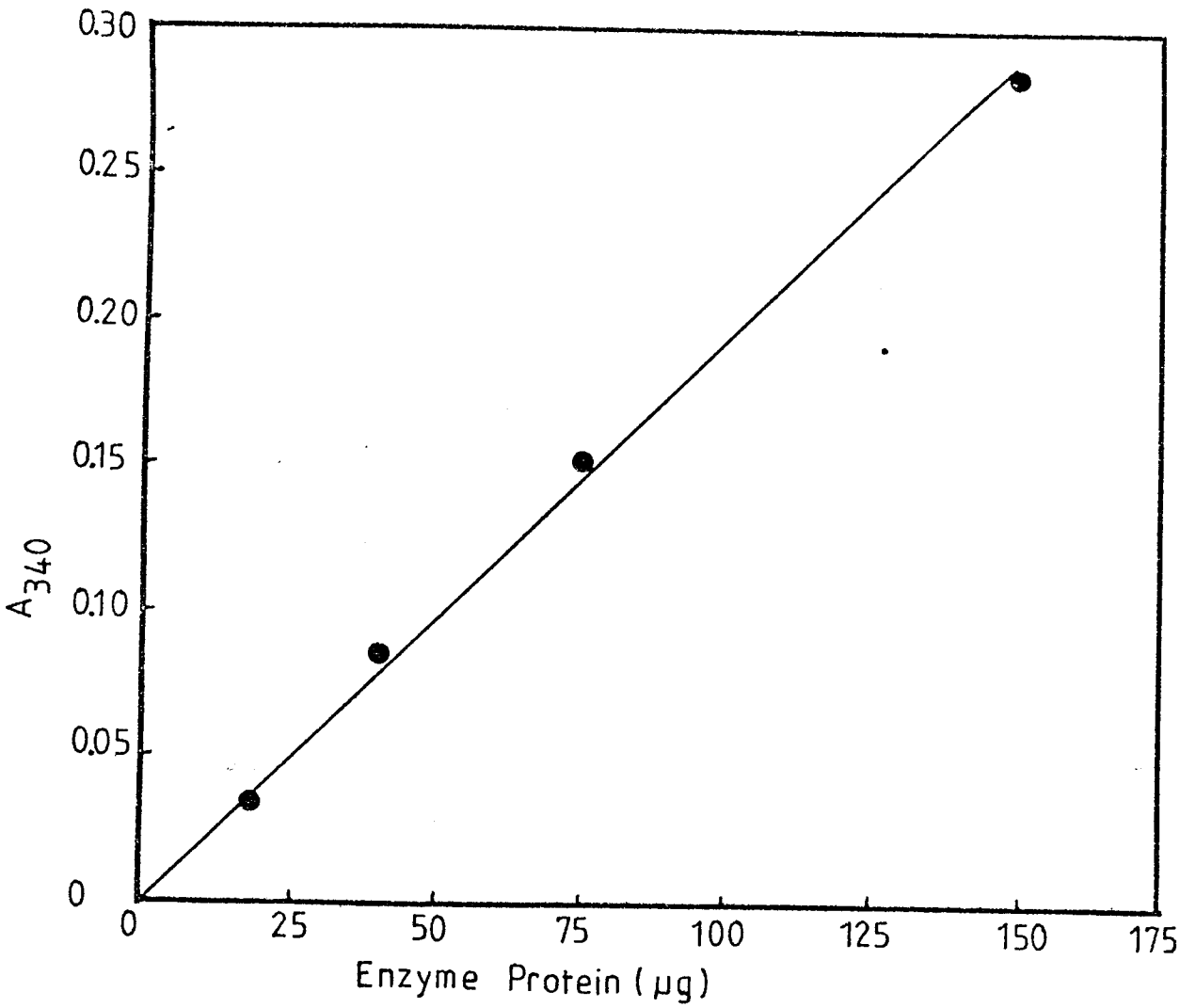


Fig. 2.3 CAP activity using azocasein as substrate. CAP was prepared as described in 4.6.1 and caseinolytic activity assayed as described in 4.2.4.3. The absorbance at 340 nm of the TCA-soluble fraction was measured.

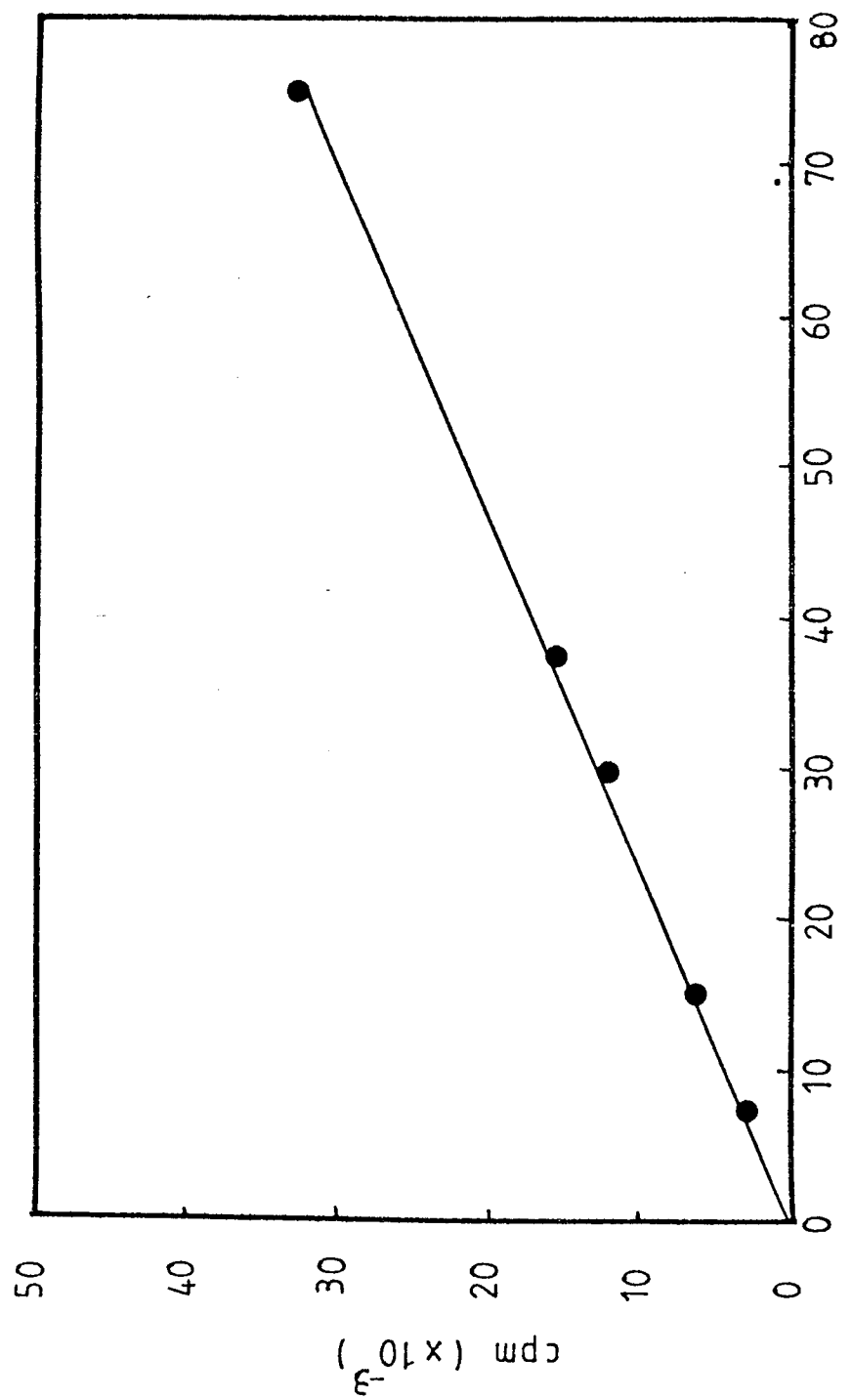


Fig. 2.4 CAP activity using [^{125}I]-casein as substrate. CAP was prepared as described in 4.6.1 and proteolytic activity assayed as described in 4.2.4.1.

substrates described and partially purified CAP prepared as described in 4.6.1. Measurement of radioactivity in the TCA supernatant using the radio-labelled substrate was the most sensitive assay method, followed by the fluorimetric measurement of tyrosine released from unlabelled casein. Absorbance changes of the TCA supernatants were not sensitive enough for the low levels of proteolytic activity detected in many samples assayed in this study. Measurement of tyrosine released in the TCA-soluble fraction was chosen as a standard reproducible method of assay. The standard curve for the fluorimetric determination of tyrosine was linear up to 20 nmoles tyrosine (Fig. 2.5). A unit of CAP activity was defined as the amount of enzyme catalysing the release of one nmole of tyrosine at 30°C in 60 min under the standard assay conditions described in the "Methods" section (4.2.2).

2.2.1.2 Time-dependence of CAP reaction

The time-dependence of CAP activity under the standard reaction conditions is shown in Fig. 2.6. The protease activity was linear for 60 min and then decreased gradually.

2.2.1.3 Dependence of CAP activity on substrate concentration

The effect of increasing casein concentration on CAP activity is demonstrated in Fig. 2.7. Substrate saturation was approached at a casein concentration of approximately 1 mg/ml.

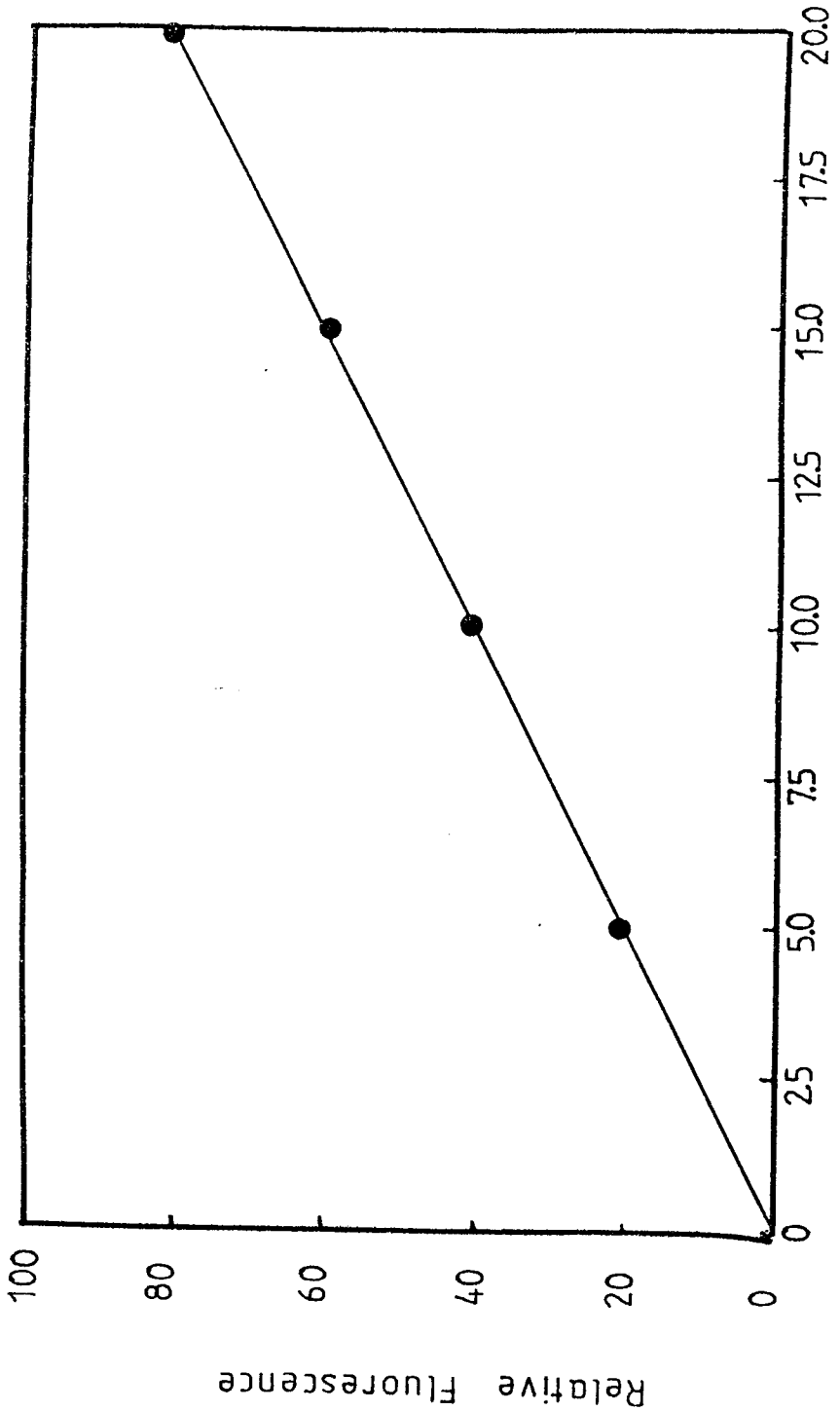


Fig. 2.5 Tyrosine standard curve. Tyrosine was measured fluorimetrically as described in 4.2.3.

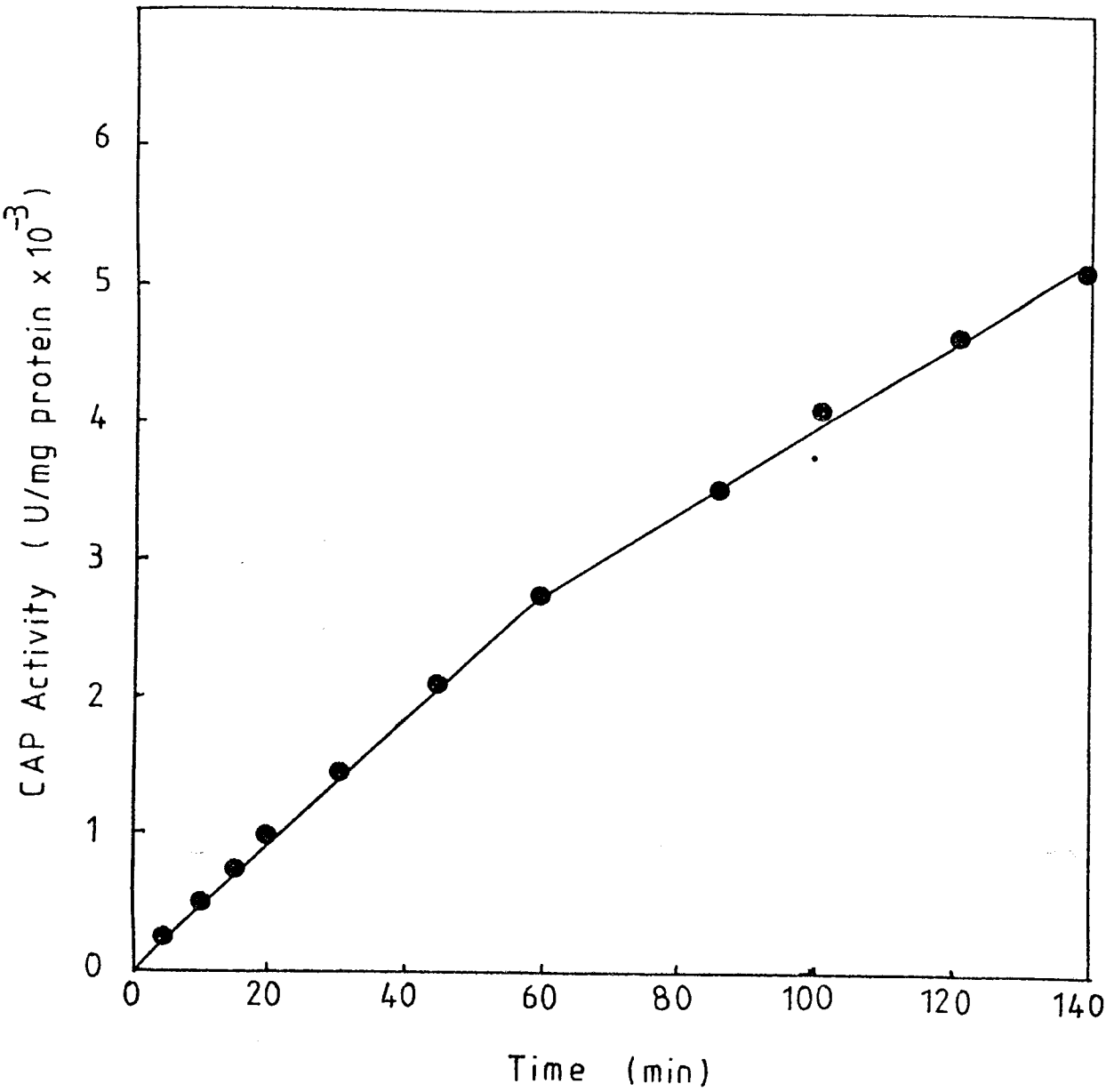


Fig. 2.6 Time dependence of CAP activity. CAP activity (6.5 µg protein) was assayed as described in 4.2.2 and 4.2.3.

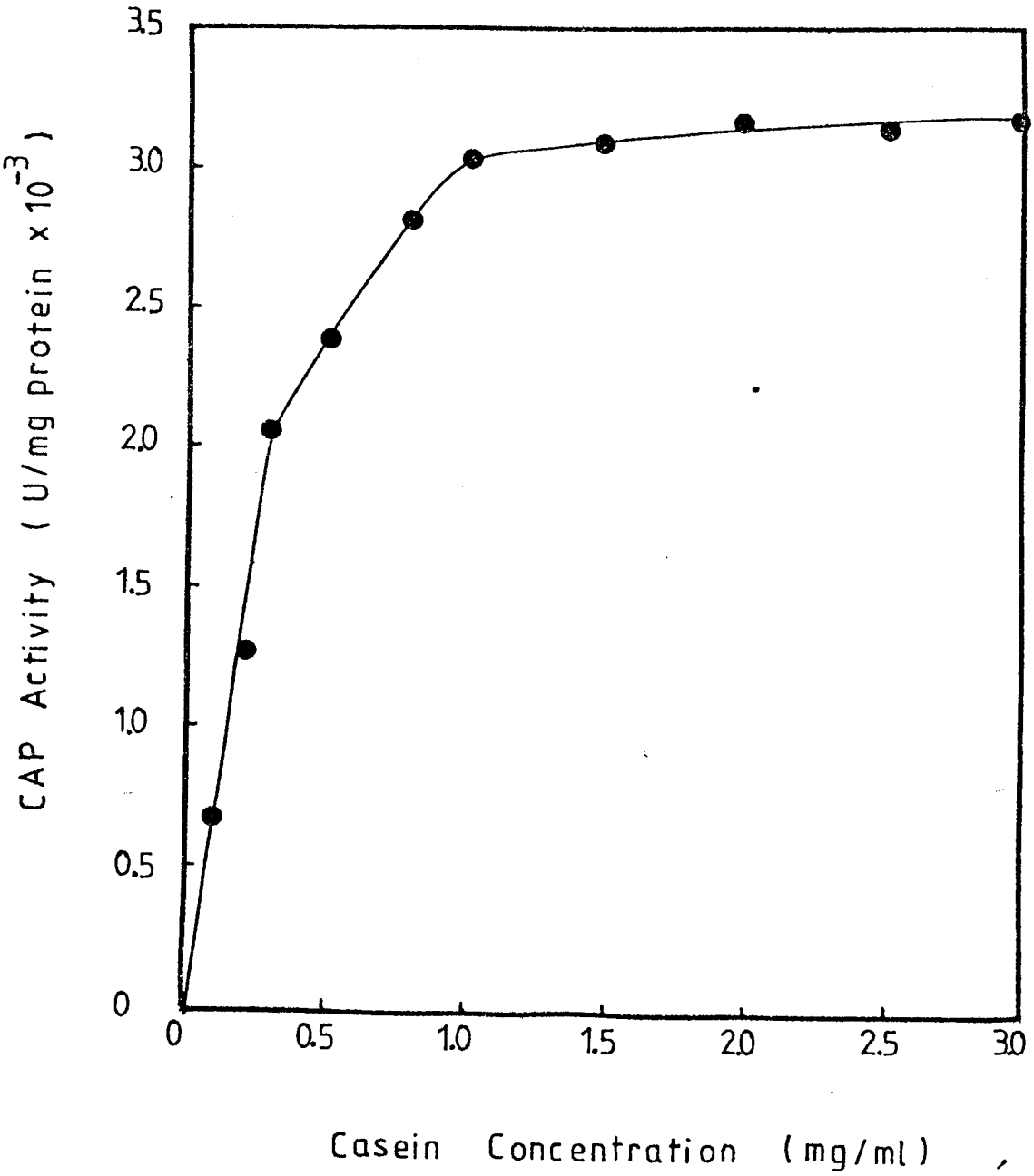


Fig. 2.7 Effect of increasing substrate concentration on CAP activity. CAP activity (6.5 µg protein) was assayed as described in 4.2.2 and 4.2.3 except that the substrate concentration was varied.

2.2.1.4 Effect of enzyme concentration on CAP assay

The effect of increasing the enzyme concentration has been discussed (see 2.2.1.1). The rate of proteolytic activity was directly proportional to enzyme concentration up to a concentration of at least 200 µg/ml in the case of the tyrosine release assay (Fig. 2.2).

2.2.2 Isolation and purification of CAP and CAP inhibitor

2.2.2.1 Sample preparation

Chicken breast muscle (14 g) was trimmed of excess fat and connective tissue, cut into small pieces and homogenised in 2.5 vol of 5 mM Tris, 50 mM NaCl, 4 mM EDTA and 0.1 mM DTT, pH 7.4, as described in 4.6.2. The supernatant was diluted 5 times with ice-cold distilled, deionised water.

2.2.2.2 DEAE-cellulose chromatography

The diluted sample from the above preparation (190 ml) was applied to an equilibrated DEAE-cellulose column and the column washed with 10 vol. of a low ionic strength buffer (see 4.6.2). The enzyme and inhibitor fractions were eluted with a linear NaCl gradient (10-500 mM) and fractions were assayed for CAP activity immediately after elution. The pattern of protein elution was followed using a Bio-Rad colour reagent. After the column was washed with the buffer, no protein was detected just prior to application of the salt

gradient.

In the case of 15-day chick embryonic muscle, protein eluted over a broad range of NaCl concentration gradient (Fig. 2.8). The fractions containing CAP activity eluted in a single peak around 0.3 M NaCl, which followed the bulk of the eluted protein.

Three peaks of inhibitor activity were obtained from embryonic muscle. These were detected by the effect of individual sample fractions on the pooled CAP activity. These peaks eluted distinctly at 0.10 M, 0.17 M and 0.24 M NaCl, respectively and were designated as fractions A, B and C in order of their elution off the column. Peak A eluted almost immediately after application of the NaCl gradient but was considered as initially bound to the column as no inhibitory activity was detectable in the column wash prior to application of the salt gradient.

The specific activity of CAP in embryonic muscle increased from 2.6 U/mg to 848 U/mg after DEAE-cellulose chromatography, an increase of 326-fold (Table 2.1). Total CAP activity was increased 15 times above the activity of the crude homogenate. Since the initial column step removed an inhibitor of CAP as well as protein, the increase in specific activity (326-fold) is therefore an apparent purification and represents both removal of contaminating proteins and an increase in CAP activity through the removal of CAP inhibitor.

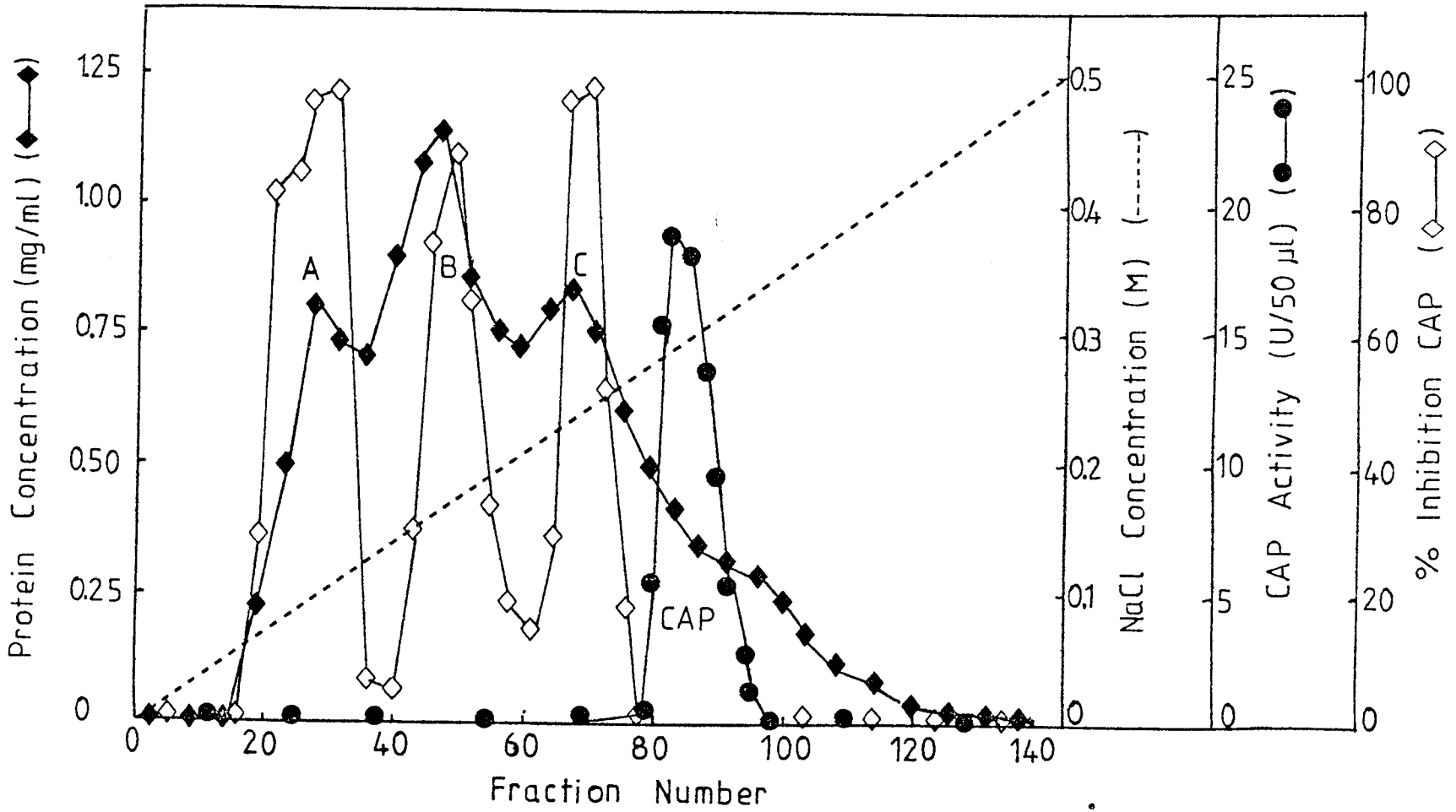


Fig. 2.8 DEAE-cellulose chromatography of CAP and inhibitor from embryonic muscle. CAP and inhibitor from 15-day chick embryos (14 g) were separated on a DEAE-cellulose column (1.6 cm × 25 cm), previously equilibrated in 5 mM Tris, 10 mM NaCl, 0.1 mM EDTA and 0.1 mM DTT, pH 7.4. A sample of 190 ml (194 mg protein) was applied to the column and elution was with a linear NaCl gradient (300 ml) from 10 mM to 500 mM NaCl. The flow rate was 20 ml/h and each fraction contained 2.1 ml. Aliquots of 50 µl were assayed for CAP activity as described in 4.2.2 and 4.2.3. CAP inhibitor was assayed by incubating 50 µl aliquots with 50 µl pooled CAP containing 10 U CAP activity.

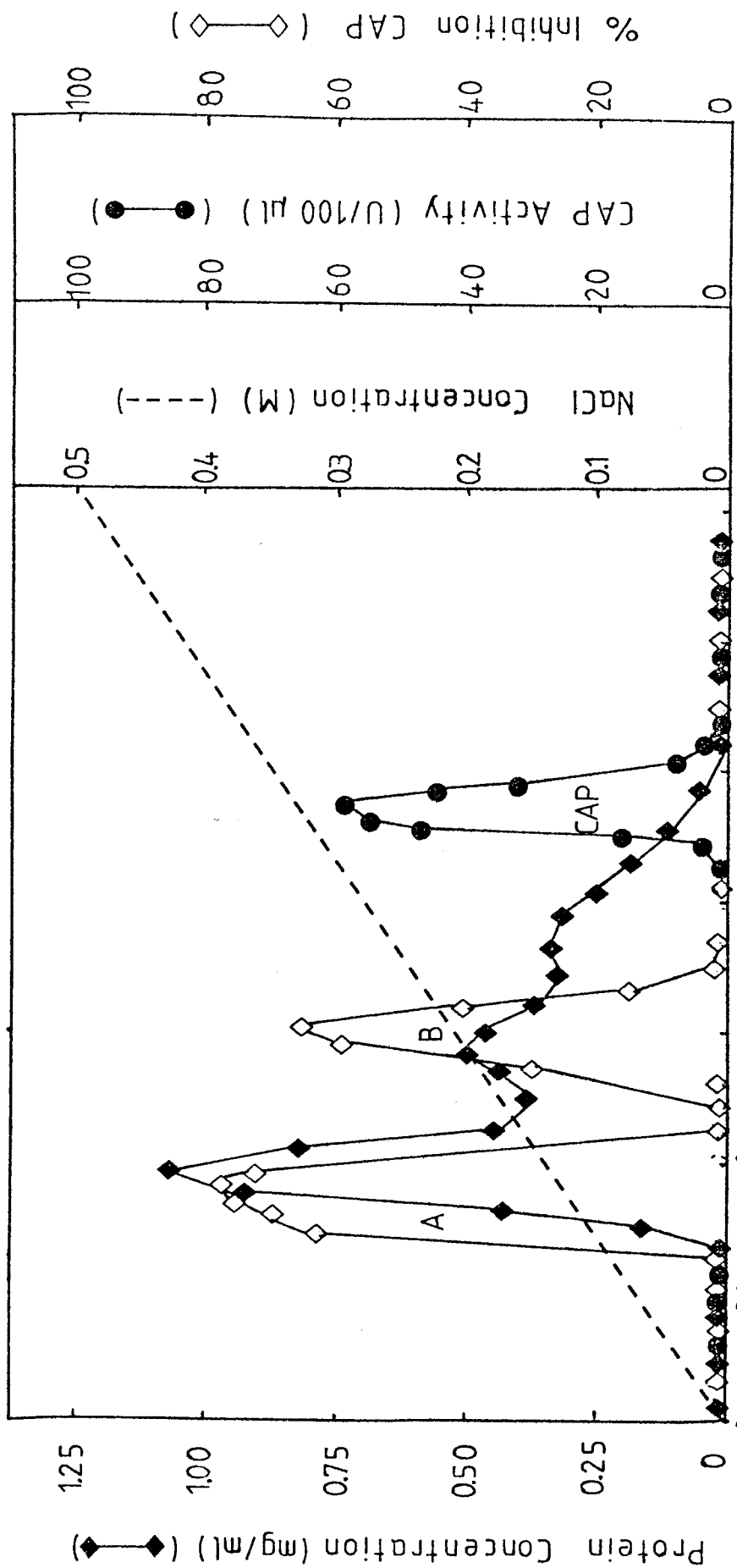


Fig. 2.9 DEAE-cellulose chromatography of CAP and inhibitor from adult chicken breast muscle. CAP and inhibitor from adult skeletal muscle (14 g) were separated on a DEAE-cellulose column (1.6 cm × 25 cm), previously equilibrated in 5 mM Tris, 10 mM NaCl, 0.1 mM EDTA and 0.1 mM DTT, pH 7.4. A sample of 140 ml (404 mg protein) was applied to the column and elution was with a linear NaCl gradient (300 ml) from 10 mM to 500 mM NaCl. The flow rate was 20 ml/h and each fraction contained 2.1 ml. Aliquots of 100 µl were assayed for CAP activity as described in 4.2.2 and 4.2.3. CAP inhibitor was assayed by incubating 200 µl aliquots with 50 µl pooled CAP fractions containing 10 U CAP activity.

TABLE 2.1

PURIFICATION OF CAP FROM 15-DAY CHICK EMBRYO BREAST AND ADULT MUSCLE

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	% activity	Apparent purification (-fold)
15-day embryo:					
Crude extract	194	513	2.6	100	
DEAE-cellulose	9.1	7756	848	1500	326
Sephadex G-150	2.6	7574	2913	1476	1120
Adult:					
Crude extract	404	3834	9.5	100	
DEAE-cellulose	2.8	5376	1920	140	202
Sephadex G-150	0.66	5224	7915	136	833

1 unit of CAP activity corresponds to 1 nmole tyrosine released in 60 min at 30°C

Results obtained for DEAE-cellulose chromatography of CAP and inhibitor from adult muscle (7-week old chickens) are represented in Fig. 2.9. A similar pattern of elution was obtained for CAP, whereas only two inhibitor peaks, A and B, were found, as opposed to three in the embryonic muscle. These peaks eluted at 0.11 M and 0.20 M NaCl, respectively, which were only slightly different to the values of the corresponding peaks from embryonic muscle. It is not known whether these differences are significant. CAP specific activity in adult muscle after DEAE-cellulose chromatography increased from 9.5 U/mg to 1920 U/mg, an increase of 202-fold (Table 2.1). The total activity did not show as marked an increase as CAP from embryonic muscle and increased from 3834 U to 5376 U.

2.2.2.3 Gel Filtration of CAP on Sephadex G-150

Fractions containing embryonic CAP activity after DEAE-cellulose chromatography were pooled, concentrated by vacuum dialysis (approximately 5 times) and a sample of 3 ml applied to a Sephadex G-150 column. CAP activity eluted in a single peak distinct from the main fraction of protein which eluted with the void volume (Fig. 2.10). This purification step resulted in a further 3.4-fold purification of the enzyme from embryonic muscle (Table 2.1).

A molecular weight calibration curve for the Sephadex G-150 column is shown in Fig. 2.11. The molecular weight of partially purified CAP as determined by gel filtration was

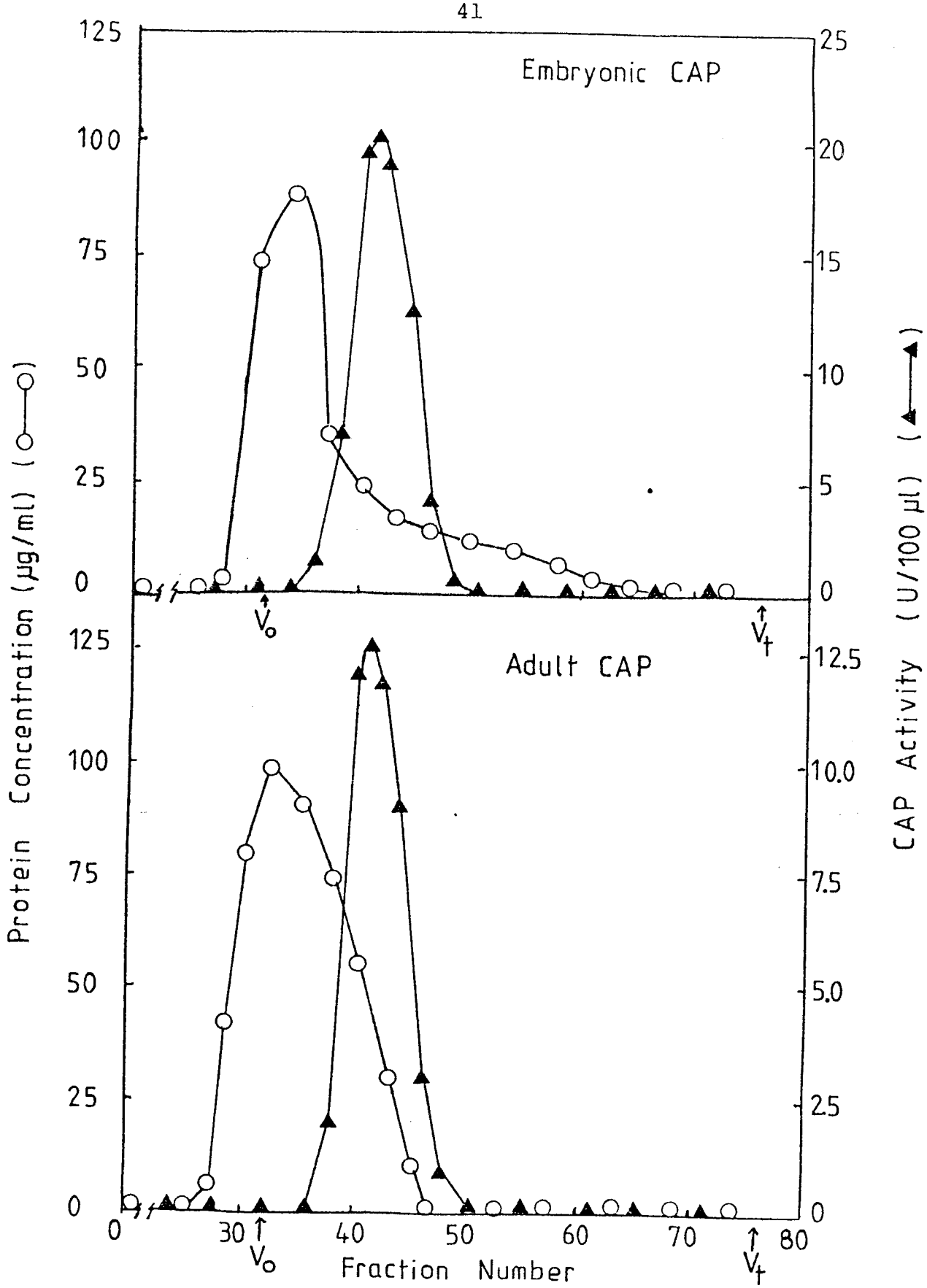


Fig. 2.10 Gel filtration of CAP on Sephadex G-150. CAP, after DEAE-cellulose chromatography, was applied to a Sephadex G-150 column (1.6 cm × 84.5 cm), previously equilibrated in 5 mM Tris, 10 mM NaCl, 0.1 mM EDTA and 0.1 mM DTT, pH 7.4. Samples of 3 ml (9.1 and 2.8 mg protein of 15-day embryonic and adult CAP, respectively) were applied to the column and elution was with the same buffer. The flow rate was 8 ml/h and fractions of 2.25 ml were collected. CAP activity was assayed as described in 4.2.2 and 4.2.3. The void volume of the column was determined using blue dextran 2000.

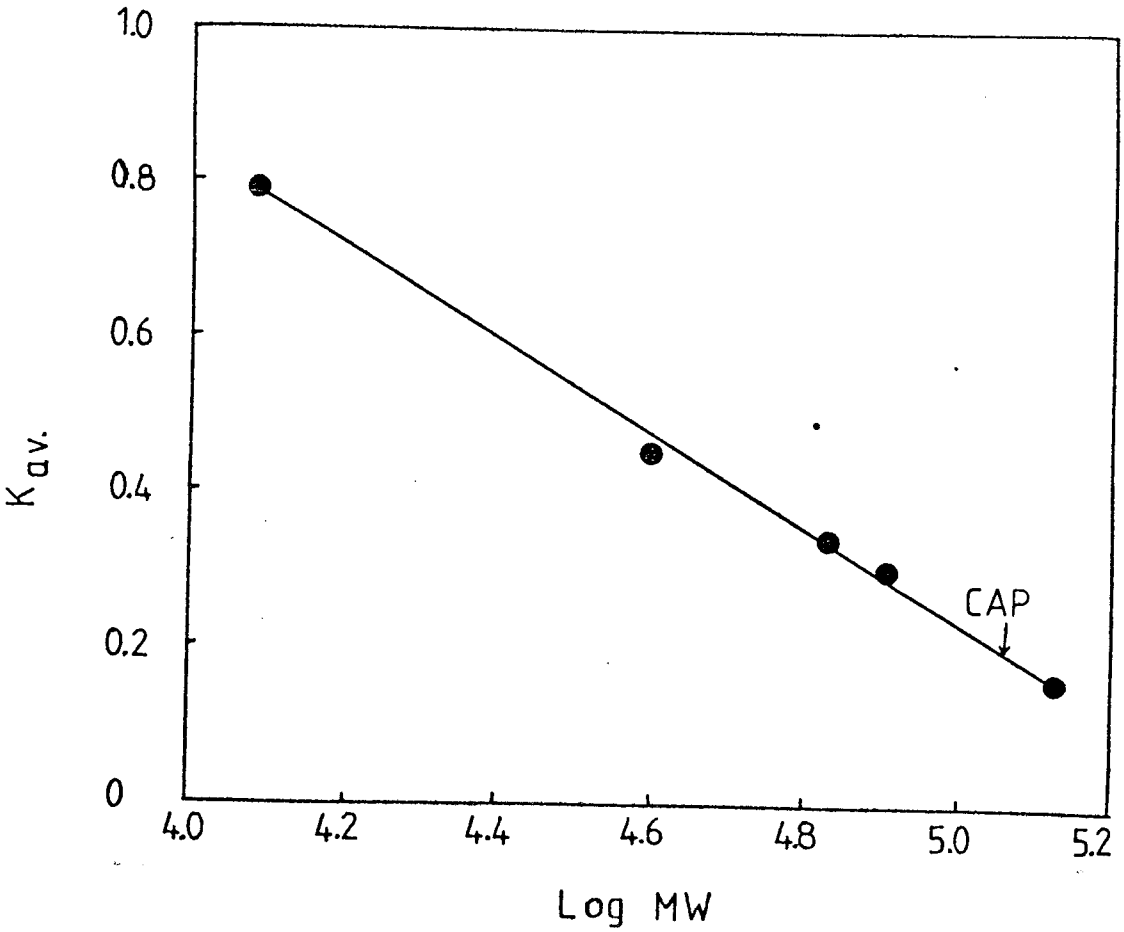


Fig. 2.11 Molecular weight calibration curve for Sephadex G-150 column (1.6 cm × 84.5 cm), equilibrated in 5 mM Tris, 10 mM NaCl, 0.1 mM EDTA and 0.1 mM DTT, pH 7.4. The standards and their molecular weights were: cytochrome c (12000); peroxidase (40000); bovine serum albumin (68000); creatine kinase (81000) and lactate dehydrogenase (138000).

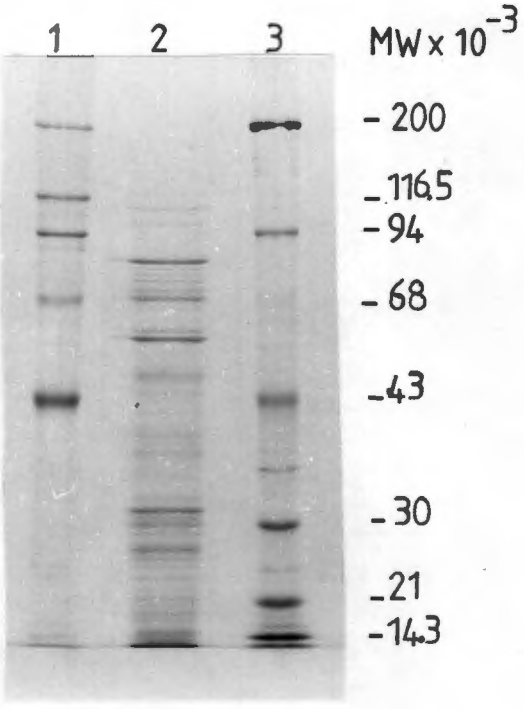


Fig. 2.12 SDS-polyacrylamide gel electrophoresis of CAP from embryonic muscle (15-day) after Sephadex G-150 chromatography. Equal volumes of CAP sample and solubilising buffer were mixed and electrophoresis was carried out as described in 4.8. The direction of migration is from top to bottom. (1) myosin heavy chain (200000), β-galactosidase (116500), phosphorylase b (94000), bovine serum albumin (68000), ovalbumin (43000); (2) 16 µg calcium-activated protease; (3) myosin heavy chain (200000), phosphorylase b (94000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (21000), lysozyme (14300).

112000 daltons. Gel filtration of CAP from adult muscle following DEAE-cellulose purification yielded similar results with a further 4-fold purification (Fig. 2.10). SDS-polyacrylamide gel electrophoresis of the concentrated, partially purified enzyme from embryonic muscle revealed the presence of 6 predominant bands having molecular weights of 83000, 69000, 55000, 33000, 30000 and 26000 daltons (Fig. 2.12).

The overall purification of CAP from embryonic muscle was 1120-fold, whereas CAP from adult muscle was purified 933 times over the crude extract. Ion-exchange chromatography resulted in a larger increase in total activity of embryonic CAP than of adult CAP. The increases were 15 times and 1.4 times respectively, which indicates a difference in levels of CAP inhibitor found in embryonic and adult muscle (see Chapter 3).

2.2.2.4 Fractionation of CAP Inhibitor peaks on Sephadex G-150

Inhibitor peaks A, B and C from embryonic muscle after DEAE-cellulose chromatography (Fig. 2.8) were concentrated by vacuum dialysis (approximately 5 times) and samples of 4 ml applied to a Sephadex G-150 column with the same dimensions as used for fractionation of CAP. The elution profiles of each of the inhibitor fractions is shown in Fig. 2.13 and the details relating to their activities are given in Table 2.2. Inhibitor A eluted with the void volume and therefore had a molecular weight greater than 200000 daltons. Inhibitor B eluted just after the void volume at a molecular weight of approximately 150000 daltons. It is uncertain whether

TABLE 2.2

QUANTITATION OF INHIBITOR PEAKS FROM 15-DAY EMBRYOS AFTER DEAE-CELLULOSE CHROMATOGRAPHY (FIG. 2.13)

	A	B	C
Units inhibitor from DEAE-cellulose chromatography	571200	485500	457800
Units inhibitor loaded on Sephadex G-150 column	130000	112500	155000
Specific activity inhibitor loaded on Sephadex G-150 column (U/mg)	17333	15845	20261
<u>Sephadex G-150 Inhibitor peaks</u>			
Units inhibitor recovered	76730	66070	67800
Protein recovered (mg)	4.70	2.43	1.59
Specific activity inhibitor (U/mg)	16300	27200	42600
Inhibitor recovery (%)	59	59	44
Purification by Sephadex G-150	0.94	1.7	2.1

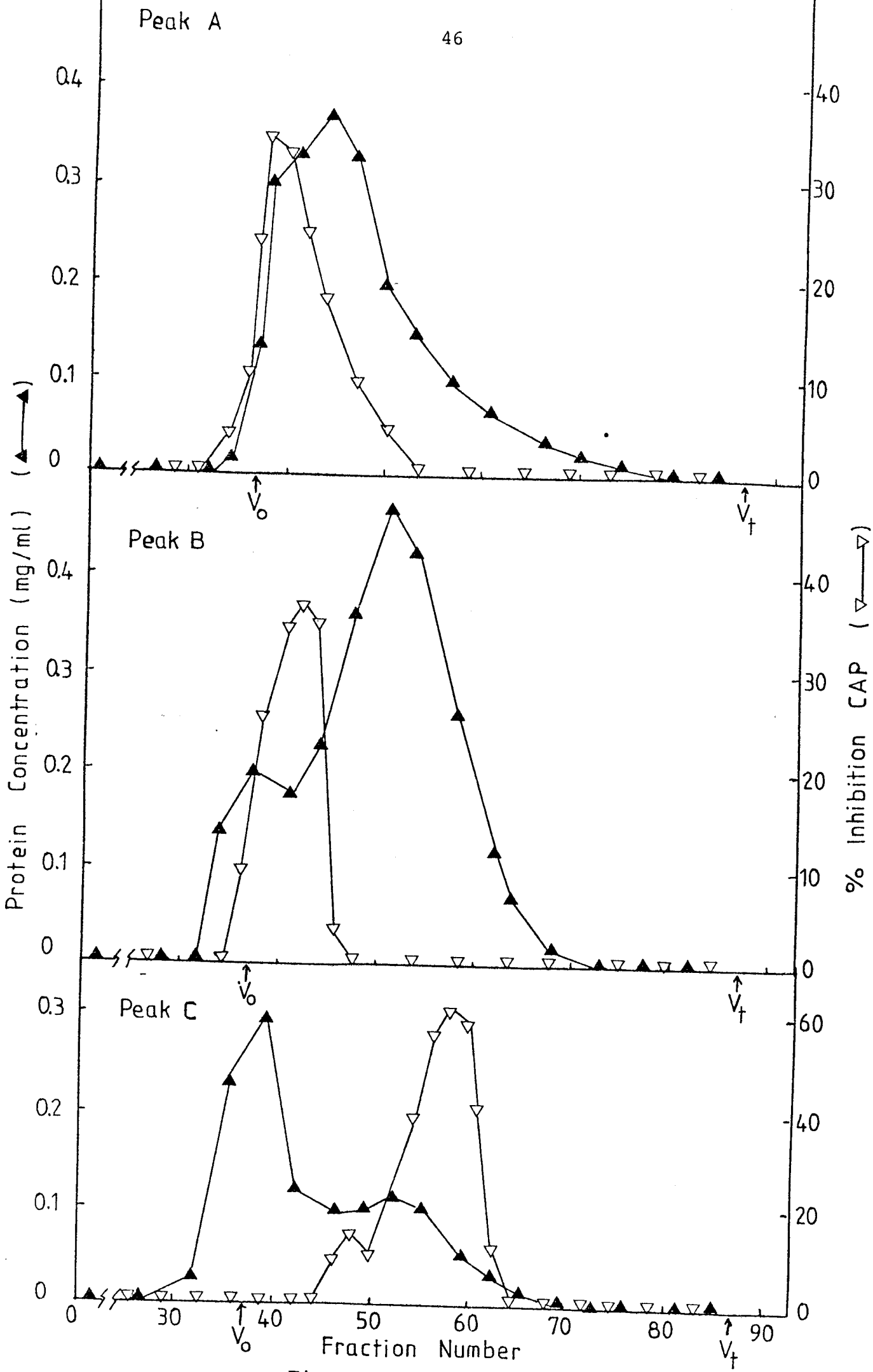


Fig. 2.13 (see overleaf)

inhibitors A and B are in fact different molecular weight species as they eluted at positions very close to each other. Inhibitor peak C, absent in older muscle, was separated from the main protein peak and eluted at a point corresponding to a molecular weight of 50000 daltons. The yields of inhibitor activity in the case of peaks A and B were 59%, while 44% of peak C activity was recovered (inhibitor activity was quantitated in a similar manner as described in 3.2.3). The specific activity of peaks B and C was increased, 1.7-fold and 2.1-fold, respectively, while peak A exhibited a decrease in specific activity after gel filtration (0.94-fold). Although a substantial amount of protein was removed from each inhibitor fraction during gel filtration, greater increases in specific activities were not observed owing to the loss of inhibitor activity which also occurred during purification.

Fig. 2.13. Gel filtration of inhibitor peaks A, B and C on Sephadex G-150. Inhibitor peaks A, B and C after DEAE-cellulose chromatography were applied to a Sephadex G-150 column, previously equilibrated in 5 mM Tris, 10 mM NaCl, 0.1 mM EDTA and 0.1 mM DTT, pH 7.4. Samples of 3 ml (7.5, 7.1 and 7.65 mg protein of peaks A, B and C, respectively) were applied to the column and elution was with the same buffer. The flow rate was 8 ml/h and fractions of 1.9 ml were collected. Inhibitor activity (% inhibition CAP) was assayed by incubating 150 µl aliquots with 17 U CAP activity. The void volume of the column was determined using blue dextran 2000.

The results obtained suggest that inhibitor C is a separate entity to A and B with a molecular weight of 50000 daltons. Gel filtration of peaks A and B from adult muscle also indicated two high molecular weight species, with both peaks eluting with the void volume. It is therefore unclear whether A and B have similar molecular weights and further fractionation is required on columns separating larger molecular weight species.

2.2.3 Properties of partially purified embryonic CAP

Embryonic CAP activity from 15-day old embryos was purified by DEAE-cellulose and Sephadex G-150 chromatography (2.2.2) and the following properties were characterised.

2.2.3.1 Dependence of CAP activity on Ca^{+2} ion concentration

When the caseinolytic activity was measured in a manner similar to standard assay conditions at different $CaCl_2$ concentrations, maximum CAP activity corresponded to a Ca^{+2} concentration of 1 mM (Fig. 2.14). Upon lowering the Ca^{+2} ion concentration below 0.4 mM, CAP activity decreased sharply and there was no detectable activity below 0.2 mM Ca^{+2}. CAP activity decreased slowly with increasing concentrations of Ca^{+2} above 1 mM. CAP from adult muscle exhibited a similar trend with increasing Ca^{+2} concentration, with a maximum activity around 0.8 mM Ca^{+2} (Fig. 2.14).

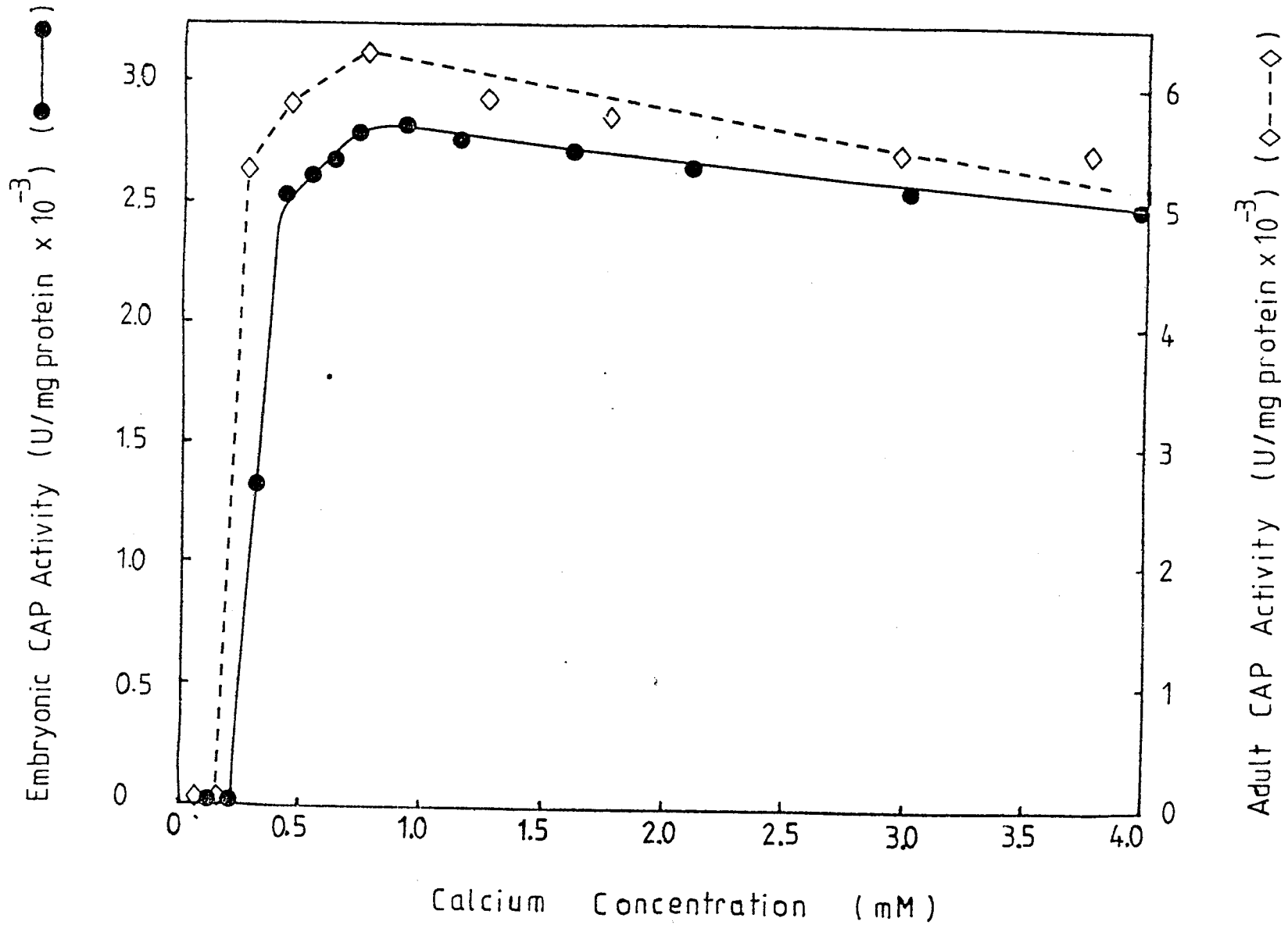


Fig. 2.14 Dependence of CAP activity on Ca^{+2} ion concentration. CAP (6.5 µg protein) was purified from 15-day embryonic muscle by ion-exchange and exclusion chromatography (4.6.2 and 4.6.3). CAP (75 µg protein) was purified from adult breast muscle by isoelectric precipitation (4.6.1). CAP activity of both preparations was assayed as described in 4.2.2 and 4.2.3 using denatured casein as substrate, except that the concentration of $CaCl_2$ was varied.

2.2.3.2 Effect of pH on CAP activity

The effect of pH on CAP activity is shown in Fig. 2.15. Very little CAP activity existed at values less than pH 6 and greater than pH 9. The pH optimum was approximately pH 7.2. CAP from adult muscle exhibited a similar pH profile with a maximum activity around pH 7.4 (Fig. 2.15).

2.2.3.3 Effect of protease inhibitors on CAP activity

The effect of several inhibitors on CAP activity is shown in Table 2.3. Soybean trypsin inhibitor and pepstatin had no effect on CAP activity at concentrations tested. Leupeptin, a thiol protease inhibitor, almost completely inhibited the enzyme, as did iodoacetate, which binds to sulphydryl groups. A marked inhibition of proteolytic activity also occurred in the presence of chymostatin, an inhibitor of chymotrypsin-like serine proteases.

2.2.3.4 Stability of CAP to storage

The stability of CAP under various storage conditions was tested. Both the enzyme isolated by means of isoelectric precipitation and CAP purified by ion-exchange chromatography and gel filtration were stable at $4^{o}C$ for up to two weeks without significant loss of activity. However, storage at $-20^{o}C$ markedly decreased the proteolytic activity and storage in liquid nitrogen resulted in a 50% loss of activity after two weeks.

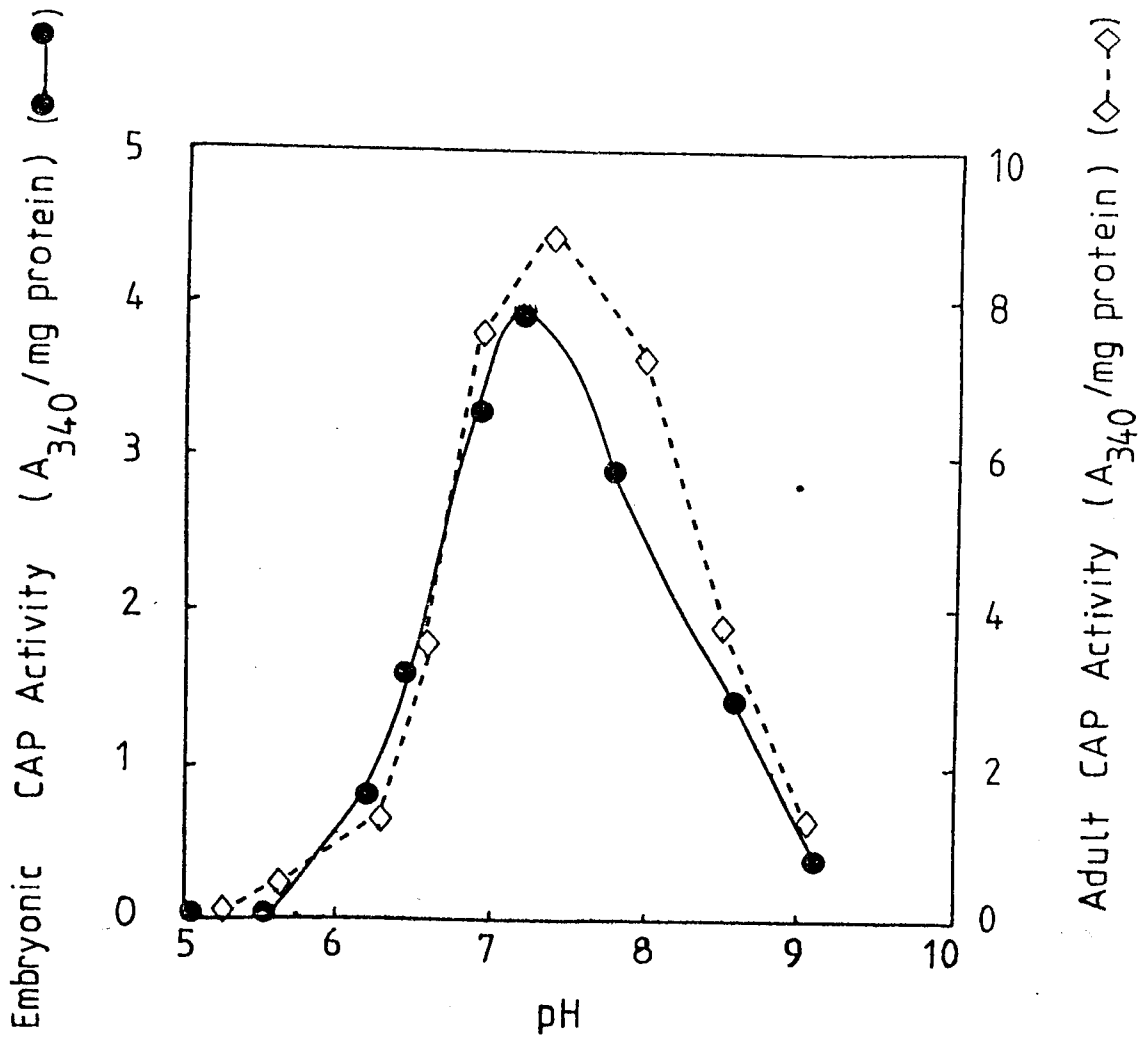


Fig. 2.15 Effect of pH on CAP activity. CAP (6.5 µg protein) was purified from 15-day embryonic muscle by ion-exchange and exclusion chromatography (4.6.2 and 4.6.3). CAP (75 µg protein) was purified from adult breast muscle by isoelectric precipitation (4.6.1). CAP activity of both preparations was assayed as described in 4.2.2 and 4.2.4.3 using azo-casein as substrate and varying the pH.

TABLE 2.3

EFFECT OF INHIBITORS ON CAP ACTIVITY

Inhibitor	Inhibitor concentration (µg/ml)	Caseinolytic activity (%)
*None		100
Leupeptin	40	1
Pepstatin	40	90
Chymostatin	16	20
Soybean Trypsin inhib.	50	98
Iodoacetate	50	17

* CAP activity (6.5 µg protein) was prepared by ion-exchange and exclusion chromatography (4.6.2 and 4.6.3) and assayed as described in 4.2.2 and 4.2.3 using inhibitors where indicated at concentrations stated.

2.3 DISCUSSION

The calcium-activated protease from chicken embryonic skeletal muscle described in this study was purified 1120-fold over the crude extract by two column chromatographic procedures, namely, ion-exchange chromatography and Sephadex G-150 gel filtration. The molecular weight of partially purified embryonic and adult CAP using gel filtration was 112000 daltons, which is similar to the molecular weight for purified CAP from porcine skeletal muscle obtained by Dayton et al (1976a). These workers found two polypeptide bands of 80000 and 30000 daltons, respectively, after SDS-polyacrylamide gel electrophoresis of purified CAP and postulated the existence of two subunits, the larger one being a catalytic subunit and the smaller, a regulatory subunit. Purified CAP from chicken embryonic muscle was not a homogeneous preparation and revealed the presence of 6 predominant bands after SDS-polyacrylamide gel electrophoresis having molecular weights of 83000, 69000, 55000, 33000, 30000 and 26000 daltons. It is likely that the bands observed around 83000 and 30000 daltons are similar to the bands obtained by Dayton et al. Ishiura et al (1978) reported that CAP isolated from adult chicken skeletal muscle was composed of a single polypeptide having a molecular weight of 80000 daltons with the absence of the so-called regulatory subunit found by Dayton et al. It is possible that the preparation of Ishiura and co-workers lacked the light subunit due to dissociation of the enzyme during the purification steps. However, the protease described in this study was not purified to homogeneity and it is therefore not possible

to identify the possible subunits of the enzyme.

Very low activities of CAP were present in crude homogenates of both embryonic and adult chicken skeletal muscle. After chromatography on DEAE-cellulose, the activities of CAP were much higher and analysis of the column fractions revealed the presence of an inhibitory activity towards the protease. This inhibitory activity is probably similar to the supernatant fraction obtained by Drummond et al (1966), which prevented the activation of phosphorylase b kinase. Three peaks of inhibitory activity, A, B and C were identified in embryonic muscle, in order of their elution off a DEAE-cellulose column. It is unclear whether peaks A and B have similar molecular weights as they both eluted with or just after the void volume of the Sephadex G-150 column. The complete absence of inhibitor C in adult muscle lends itself to speculation about the possible role of this inhibitor in the control of CAP activity in embryonic muscle. Assuming a role for CAP in the degradation of myofibrillar proteins, growth of the muscle would be enhanced if the activity of CAP was maintained at a low level during cell differentiation and development. The small inhibitor species could thus play a role in controlling the enzyme activity during muscle cell growth and differentiation.

Ishiura et al (1977) reported the occurrence of an inhibitor of CAP in adult chicken skeletal muscle with a molecular weight of 67000 daltons. This does not correspond with any of the species found in this study and the closest molecular

weight is inhibitor C (M_r = 50000) which, as has been discussed, is not present in adult skeletal muscle. The high molecular weight inhibitor (M_r > 150000) may be similar to the inhibitor from bovine cardiac muscle described by Waxman and Krebs (1978) which has a molecular weight of 270000 daltons. Further purification and chromatography of inhibitors A and B is necessary to determine their possible subunit compositions.

The calcium-activated protease from embryonic chicken muscle exhibited similar properties to the enzyme isolated from adult muscle as well as the well-characterised enzyme from porcine skeletal muscle (Dayton et al, 1976b). Embryonic CAP is active in the pH range between 6.8 and 8.0 with an optimum pH around 7.2. No activity could be detected below pH 5.5, which indicates a non-lysosomal intracellular location of CAP. The pH requirements for CAP activity are therefore consistent with the suggested cytoplasmic role of the enzyme in metabolic turnover of myofibrillar proteins (Dayton et al, 1975).

Embryonic CAP is maximally active at a Ca^{+2} concentration of 1 mM and no activity could be detected below 0.2 mM Ca^{+2}. The intracellular concentration of free Ca^{+2} in living, skeletal muscle is generally 10^{-5} M or less, which is much lower than the concentration of Ca^{+2} required to activate CAP. As has been discussed in Chapter 1, a possible explanation for the discrepancy is the existence of an intracellular compartmentalisation of Ca^{+2} concentration which could reach levels required for activation of CAP. Alternatively, the requirement

of CAP for Ca^{+2} *in vivo* may be different to the concentration necessary for activation *in vitro*.

Treatment of CAP with soybean trypsin inhibitor had no effect on caseinolytic activity, indicating that CAP is not a trypsin-like serine protease. Inhibition of CAP activity by iodoacetate and leupeptin, a potent inhibitor of cellular proteases (Aoyagi *et al*, 1969), suggests that cysteine side chains (thiol groups) are involved either at the active site of the enzyme or in maintaining proper conformation of the molecule. It is interesting to note the effect of chymostatin, an inhibitor of serine proteases, on CAP activity. Libby and Goldberg (1980) are the only workers who have reported a similar inhibition of CAP activity (from rat skeletal muscle) by chymostatin.

From the results presented here, it would appear that CAP of embryonic skeletal muscle is a non-lysosomal, proteolytic enzyme that requires a Ca^{+2} concentration of 1 mM and pH of 7.2 for maximum activation, as well as a sulphyldryl group protector. The activity of CAP in crude extracts of muscle is partially masked by the presence of at least three separable endogenous inhibitors. Such inhibitors may play an important role, together with Ca^{+2} ions, in regulating the activity of the calcium-activated protease by mechanisms as yet unknown, to bring about a controlled degradation of myofibrillar proteins. It should be noted that further investigation of such protease inhibitors is likely to reveal the presence of other cellular, non-lysosomal proteases that

have thus far gone undetected. A greater understanding of the proteolytic enzymes involved in degradation and of endogenous protease inhibitors may help to explain the heterogeneity in the turnover rates of cellular proteins.

CHAPTER 3

CAP AND CAP INHIBITOR IN CULTURED MUSCLE CELLS AND IN CHICKEN SKELETAL MUSCLE DURING DEVELOPMENT

3.1 INTRODUCTION

The previous section described the purification of CAP and its separation from inhibitors in embryonic as well as in adult chicken skeletal muscle. Embryonic CAP was characterised and three species of inhibitory activity were fractionated on DEAE-cellulose. Adult muscle contained only two inhibitor species and the levels were much lower than in embryonic muscle. These results led to an investigation of both CAP and inhibitor in developing muscle, ranging from 12-day old embryos to adult chickens. An increase in CAP activity during muscle cell differentiation and myofibril accumulation would support a possible role for CAP in contractile protein turnover.

CAP activity was measured after purification of the enzyme by isoelectric precipitation, which removed CAP inhibitor. This method did not allow accurate quantitation of inhibitory activity owing to incomplete recoveries and in subsequent experiments, ion-exchange chromatography was utilised to separate and quantitate CAP and inhibitor levels. Low levels of CAP were found in embryonic muscle compared to adult muscle (also see 2.2.2). The results indicated high levels of inhibitor in early embryonic muscle, which decreased very rapidly

until low levels were maintained in adult muscle. CAP levels, expressed in terms of DNA, increased during development but did not match the accumulation of myofibrillar proteins.

The presence of CAP in cultured skeletal muscle cells has not been previously described. Muscle cell primary cultures undergo a marked differentiation *in vitro* and exhibit a marked increased in myofibrillar content between 2 and 6 days in culture (Yaffe, 1969; Konigsberg, 1979). The partial purification and quantitation of CAP and an inhibitor of CAP in muscle cell cultures is reported in this chapter. An increase in the proteolytic activity was observed during cell growth and differentiation.

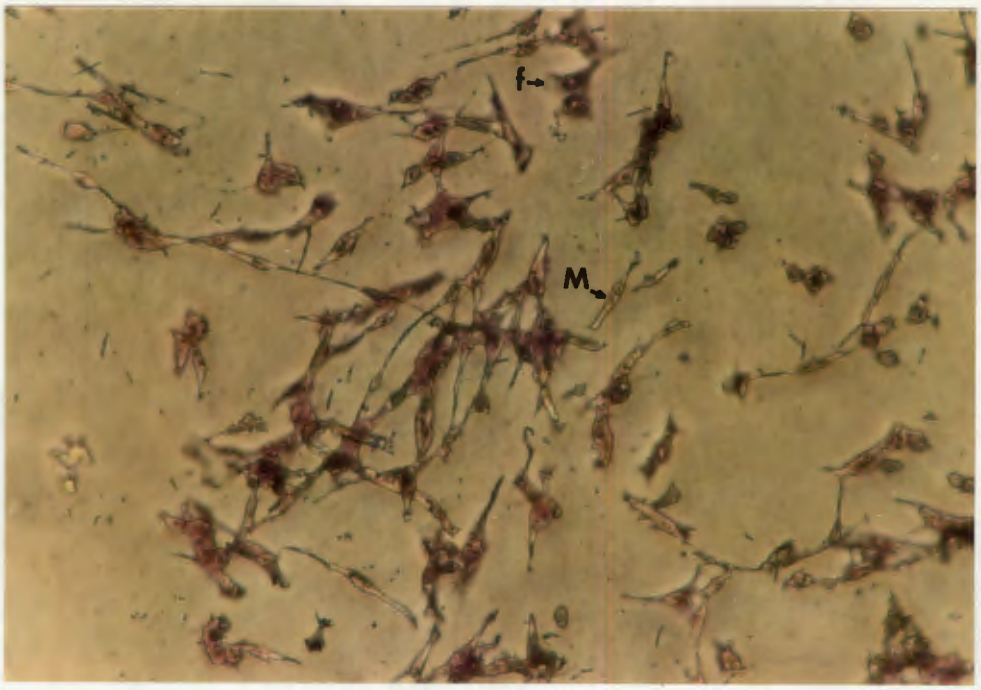
3.2 RESULTS

3.2.1 Characterisation of cultured muscle cells

3.2.1.1 Growth and differentiation of cultured muscle cells

Primary chick skeletal muscle cultures were prepared from thigh muscle of 12-day chicken embryos as described by Van der Westhuyzen (1979)(see also 4.7). Dissected muscle tissue was mechanically dissociated using the method of Tepperman *et al* (1975), which yields cell preparations containing a greater percentage of muscle cells than is obtained by methods using enzyme dissociation of muscle tissue. The morphology of the cells during differentiation in culture is shown in Fig. 3.1. After one day, virtually all the cells present were mono-

A



B

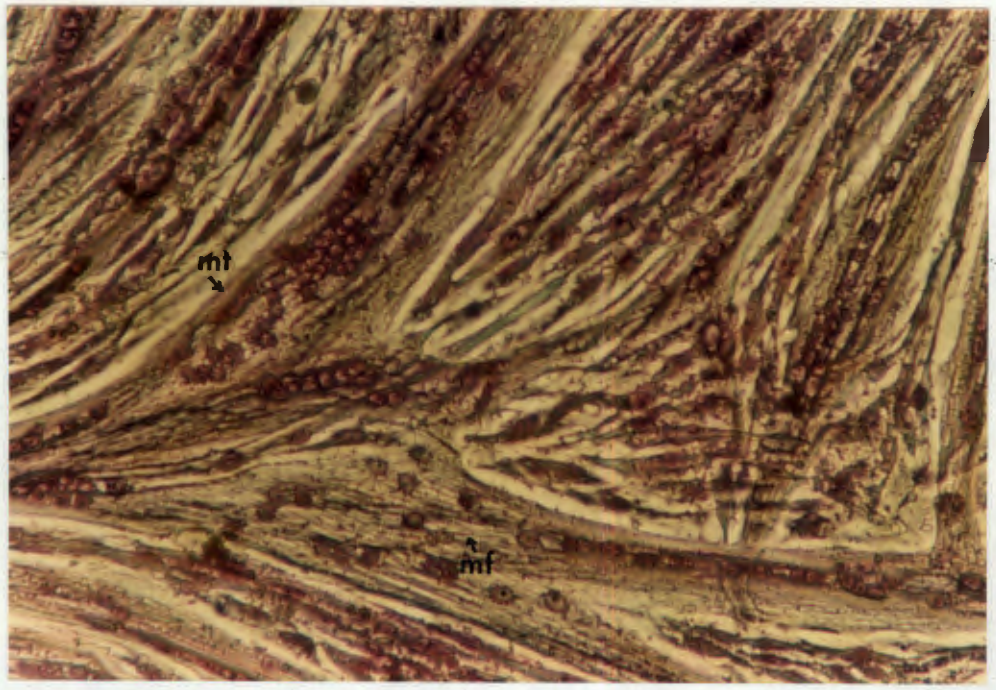


Fig. 3.1 Primary chick skeletal muscle cultures at different stages of growth and differentiation. Cultures were prepared as described in 4.7.3. (A) 1 day cells, (B) 5 day cells.

M = myoblast

f = fibroblast

mt = myotube

mf = striated myofibrils

nucleated and were comprised of dense, bipolar muscle cells and larger non-muscle cells. After approximately two days in culture, mononucleated myoblasts aligned with each other and began to fuse to form large, multinucleated myotubes. The myotubes were observed to contract spontaneously after four days and myofibrillar striations were present in myotubes after four to six days. Treatment of cultures with ara-C after 60 h, when fusion of myogenic cells was virtually complete, resulted in primary cultures in which approximately 80% of the cells were myoblasts. In differentiated cultures, after four to five days in culture, approximately 90% of the nuclei were present in fused myotubes.

The cellular DNA and protein contents per 100 mm dish are shown in Fig. 3.2. An increasing amount of total cellular protein was observed during the culture period, whereas the DNA content increased rapidly until the second day and then remained relatively constant. This is due to the fact that skeletal muscle cells withdraw from the cell cycle and cease to divide after fusion.

As a measure of the degree of differentiation in the muscle cell cultures, the cellular contents of myosin heavy chain and actin polypeptides were quantified by SDS-polyacrylamide gel electrophoresis. As described in the legend to Fig. 3.3, an actomyosin fraction was collected by centrifugation after homogenisation of the cells in a buffer of low ionic strength and subjected to SDS-polyacrylamide gel electrophoresis. The protein present in the myosin and actin bands (Fig. 3.3) was

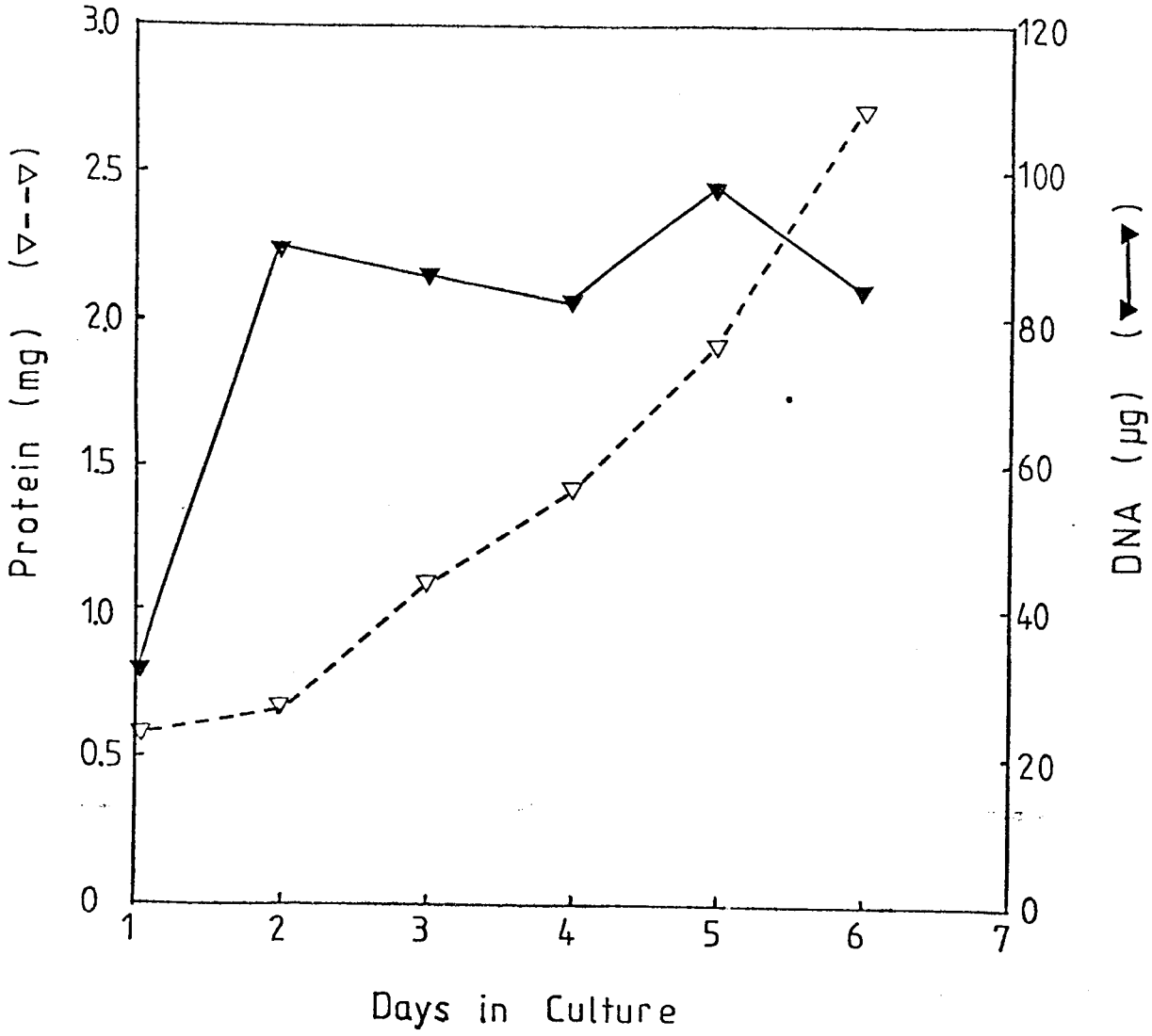


Fig. 3.2 Growth of primary culture of 12-day chick embryo skeletal muscle cells. Cultures were prepared as described in 4.7.3.

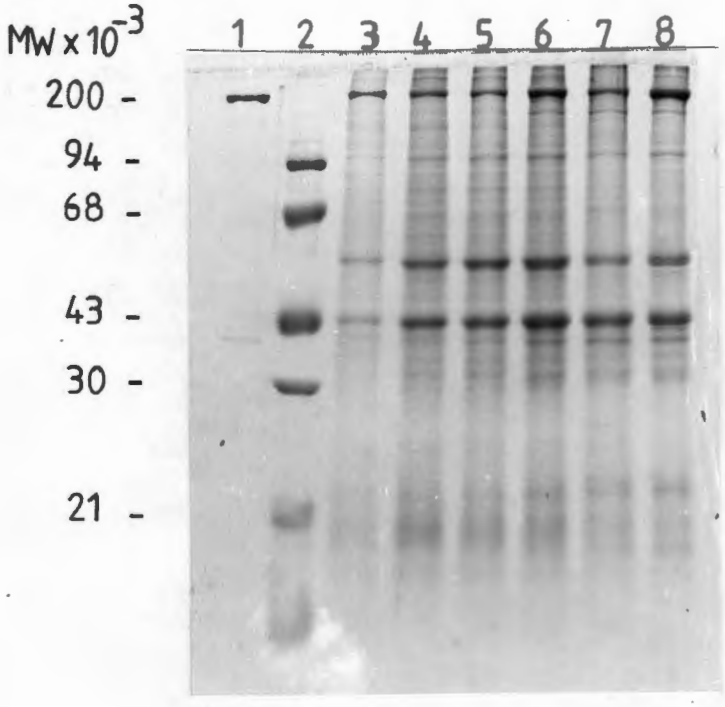


Fig. 3.3 SDS-polyacrylamide gel electrophoresis of pelleted protein from muscle cells after homogenisation at different stages of growth. Cell cultures were harvested as described in 3.2.1.2, centrifuged and 3 volumes of 20 mM sodium bicarbonate, 1 mM EDTA, pH 7.4 added to the pellets. The suspensions were homogenised, centrifuged at low speed and a volume of solubilising buffer added to each pellet. Samples were applied and electrophoresis was carried out as described in 4.8. The direction of migration is from top to bottom. (1) myosin heavy chain (200 000), (2) phosphorylase b (94000), bovine serum albumin (68000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (21000), lysozyme (14300), (3) 1 day cultures, (4) 2 day cultures, (5) 3 day cultures, (6) 4 day cultures, (7) 5 day cultures, (8) 6 day cultures.

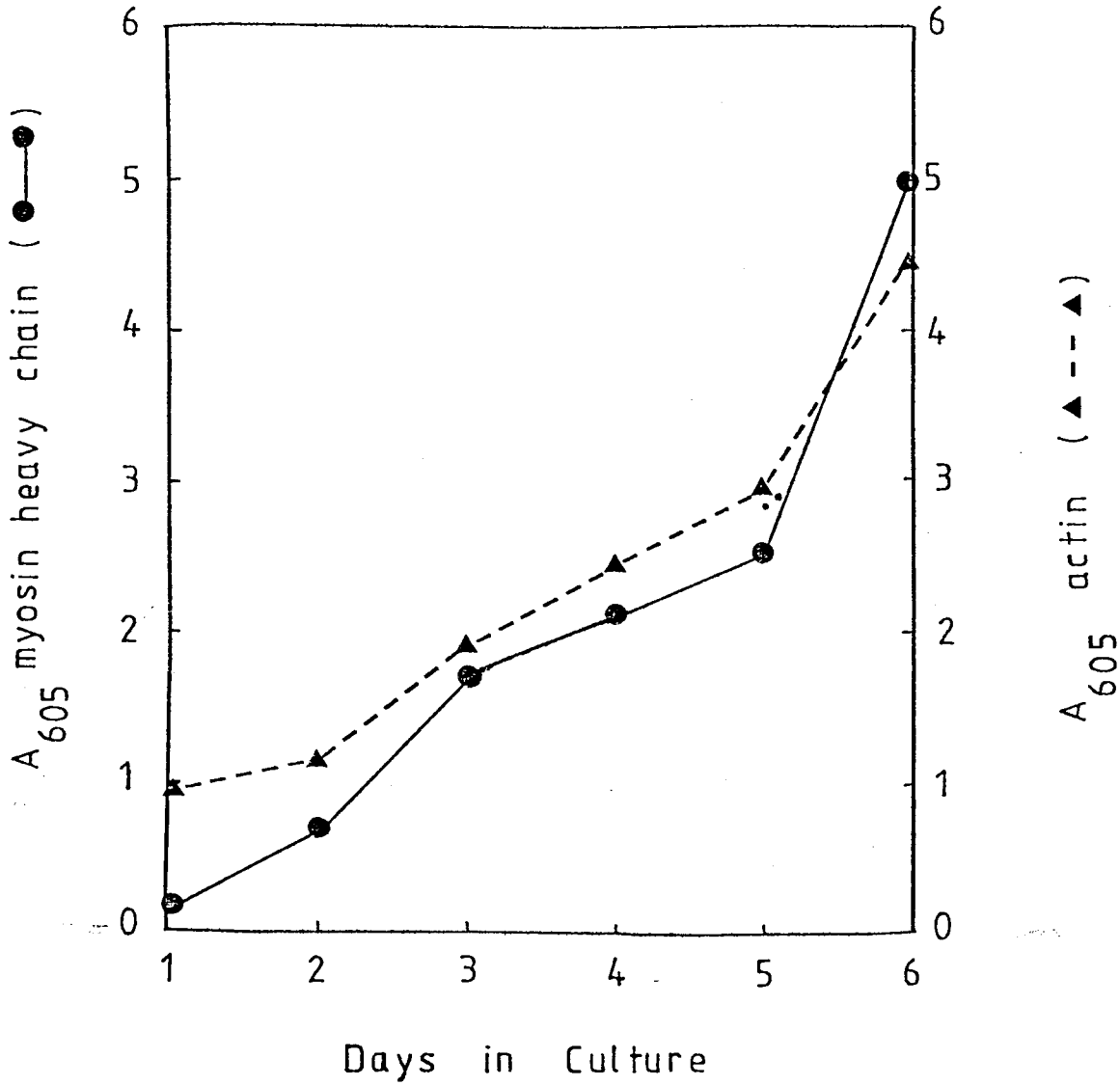


Fig. 3.4 Quantitation of myosin and actin bands of cell cultures after SDS-polyacrylamide gel electrophoresis. The protein present in the bands was quantified as described in 4.9 and expressed as the colour yield quantified per dish for each protein.

quantified as described in 4.9 and the total content of myosin and actin, expressed as the total colour yield per dish for each protein, is shown in Fig. 3.4. A marked increase in myosin heavy chain and actin content was observed from one to six days in culture, with a slight lag during the first two days. These observations were in agreement with the appearance of striated myofibrils in the fused myotubes.

3.2.1.2 CAP activity in cell cultures

CAP activity was measured in muscle cell cultures after isoelectric precipitation of the enzyme at pH 4.9 (4.6.1). The results of two separate experiments are represented in Table 3.1. An increase in CAP activity from 3.5 days to 6 days was observed, both in relation to total cellular DNA and to protein. A five-fold increase in enzyme activity was observed in the first experiment compared with a two-fold increase in the second experiment. CAP levels in fibroblasts derived from the primary muscle cell cultures after 9 days were similar to levels in muscle cells after 3.5 days. CAP activity was therefore present in muscle cell cultures and showed an increase with cellular growth and differentiation. Significant CAP activity was also present in fibroblasts derived from muscle cell cultures.

3.2.1.3 Fractionation of CAP and inhibitor

The established method of CAP isolation by isoelectric precipitation had disadvantages in that (1) the recovery of any

TABLE 3.1

CAP ACTIVITY IN MUSCLE CELL CULTURES

Cell Type	Experiment No.	CAP Activity*.	Days in Culture 3.5	6
Muscle cells	1	U/mg DNA	17	91
		U/mg protein	0.6	2.6
	2	U/mg DNA	22	46
		U/mg protein	0.5	1.0
			9 days in culture	
Fibroblasts	2	U/mg DNA	18	
		U/mg protein	0.5	

* After either 3.5 or 6 days, 20 100 mm dishes were rinsed 3 times with saline G (minus Ca^{+2} and Mg^{+2}), scraped into the same medium and pelleted by centrifugation. The cell pellets were suspended in 3 vol. of a freshly prepared solution of 20 mM sodium bicarbonate, 1 mM EDTA, pH 7.4 and CAP was partially purified as described in 4.6.1. CAP activity was assayed as described in 4.2.2 and 4.2.3.

possible endogenous inhibitor was not described, (2) no information was available concerning the yield of enzyme activity from the procedure and (3) the method was not suitable for small scale preparation as with the small volumes of homogenate obtained from cultured cells. The method of Waxman and Krebs (1978) was therefore adopted to measure CAP activity in cultured cells (see 4.6.2) and the presence of CAP inhibitor in cultured cells was demonstrated by fractionation of CAP and CAP inhibitor on DEAE-cellulose. After 3.5 days or 6 days in culture, 24 100 mm dishes were harvested and homogenised in 5 mM Tris, 50 mM NaCl, 4 mM EDTA and 0.1 mM DTT, pH 7.4 and the diluted high speed supernatant was applied to a DEAE-cellulose column. The elution profiles of CAP and inhibitor from the muscle cell cultures are shown in Fig. 3.5. The protein eluted in a broad band over the whole column and CAP eluted in a single peak at about 0.33 M NaCl in the case of both 3.5 and 6 day cultures, which is similar to the elution of CAP from 15-day embryonic and adult muscles (Chapter 2). The presence of CAP inhibitor was demonstrated in both 3.5 and 6 day cultures. In 3.5 day cells the inhibitor eluted at about 0.13 M NaCl in a broad peak but a broader elution profile, with multiple peaks, was observed for the inhibitor from 6 day cultures. It is possible that the inhibitor peak from 3.5 day cultures consists of more than one inhibitor species and could even contain peaks similar to A and B found in 15-day embryos. Similarly, the two inhibitor peaks fractionated from 6 day cells eluted at similar NaCl concentrations (0.08 and 0.2 M NaCl, respectively) to peaks A and B of 15-day embryos. The inhibitor peak corresponding to peak C in embryonic muscle

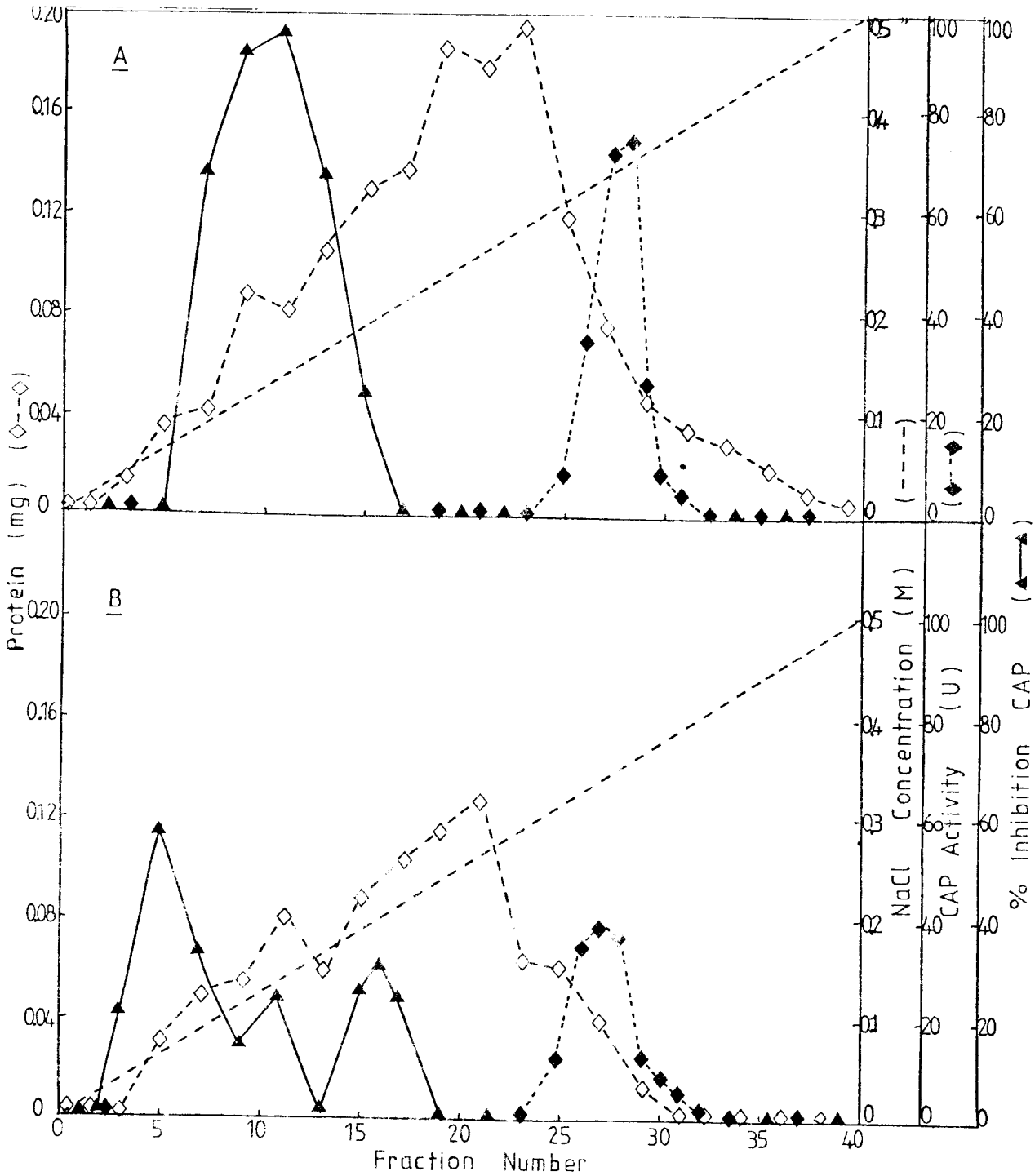


Fig. 3.5 (see overleaf)

Fig. 3.5 DEAE-cellulose chromatography of CAP and inhibitor from muscle cell cultures. CAP and inhibitor were separated on a DEAE-cellulose column (0.6 cm × 14.5 cm), previously equilibrated in 5 mM Tris, 10 mM NaCl, 0.1 mM EDTA and 0.1 mM DTT, pH 7.4. Samples of 15 ml were applied to the columns, which were then washed with ten column volumes of the same buffer. Elution was with a 25 ml linear gradient from 10 mM to 500 mM NaCl. The flow rate was 6 ml/h and each fraction contained 0.63 ml. Protein concentration was determined using colour reagent obtained from BIO-RAD Laboratories. Aliquots of 50 µl were assayed for CAP activity as described in 4.2.2 and 4.2.3. CAP inhibitor was assayed by incubating 40 µl aliquots with 100 µl pooled CAP containing 10 U and 6 U CAP activity in the case of A and B, respectively.
A : 3.5 day cells; B : 6 day cells.

TABLE 3.2

SEPARATION OF CAP AND INHIBITOR FROM MUSCLE CELL CULTURES ON DEAE-CELLULOSE

	3.5 day cells	6 day cells
Cell homogenate		
Protein loaded on column (mg)	4.56	4.0
Specific activity CAP (U/mg)	4	36
Units activity loaded on column	20	144
DEAE-cellulose		
Total CAP activity recovered (U)	400	275
CAP specific activity (U/mg)	714	1146
% CAP activity recovered	2000	200
Apparent purification (-fold)	178	32

CAP activity was assayed as described in 4.2.2 and 4.2.3. Details of column chromatography are given in Fig. 3.5.

was not present in muscle cell cultures. Details of the enzyme purification are given in Table 3.2. The ratio of total CAP activity after DEAE-cellulose chromatography to the activity loaded on the column showed an increase from 3.5 to 6 days, 20-fold and about 2-fold, respectively. The results are consistent with the increase in CAP activity observed after enzyme purification by isoelectric precipitation (3.2.1.2). These observations indicate that CAP inhibitor decreases in cell cultures during differentiation, with a simultaneous increase in CAP activity.

3.2.2 CAP activity in developing muscle

As an alternative approach to the characterisation of CAP during muscle development and owing to the difficulties and time involved in growing sufficient cells in culture to obtain adequate material for CAP purification and measurement, I initially decided to measure CAP activity in breast muscle of developing chickens to obtain a profile of CAP activity with increasing age. Proteolytic activity was measured over a range of ages, from 12-day embryos through to 6-week chickens. CAP activity was measured both in the crude homogenate and in the inhibitor-free fraction obtained after purification of the enzyme by isoelectric precipitation (Fig. 3.6). A marked increase in specific activity of CAP occurred with development, particularly during the period from approximately 4 days to 9 days after hatching. Fig. 3.6 also shows the relationship between total protein and DNA in the developing muscle. A dramatic increase in the ratio was observed during the period

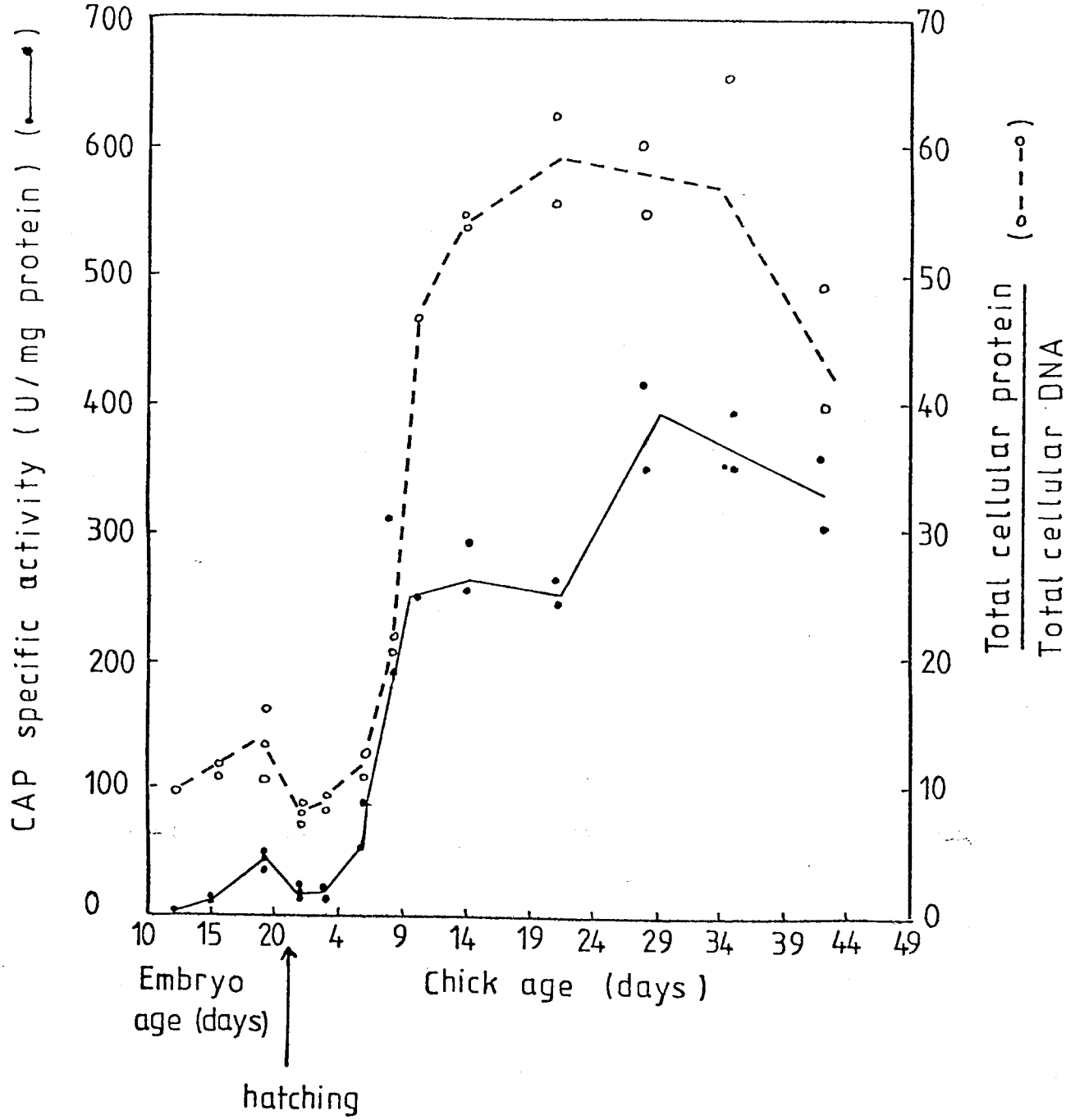


Fig. 3.6 Specific activity of CAP during development. CAP inhibitor-free fraction was obtained by isoelectric precipitation as described in 4.6.1 and CAP activity was assayed as described in 4.2.2 and 4.2.3. Each point represents the result of a different muscle preparation.

of 6 days to 2 weeks after hatching, which indicates a rapid accumulation of proteins during that period with a simultaneous decrease in the rate of DNA synthesis as a result of myoblast fusion.

Fig. 3.7 shows the profile of CAP activity in developing muscle expressed in terms of total cellular DNA and protein. A trend of increasing activity was observed with a peak of activity in 19-day embryos and a marked increase from 4 to 8 days after hatching. After 8 days, CAP activity was decreased until 2 weeks and then a gradual increase in activity was observed in terms of both cellular DNA and protein. Fig. 3.8 shows a similar profile of CAP activity present in the crude homogenate of developing chicken muscle. An overall increase in CAP activity was observed with development both in terms of total cellular protein and DNA, with a more marked increase occurring in the latter. The graph of CAP activity in terms of cellular DNA showed a peak of activity at 19-day embryos but the peak observed in Fig. 3.7 at 8 days after hatching was not present. Overall, both groups demonstrate an increase in CAP activity during muscle development. The presence of CAP inhibitor early in development is indicated by the fact that the levels of activity in the crude homogenates (Fig. 3.8) are lower than those in the fraction partially purified by isoelectric precipitation (Fig. 3.7). The ratio of total CAP activity in the inhibitor-free fraction to CAP activity in the crude homogenate is represented in Fig. 3.9. The significance of the single value for the 12-day embryos is not certain. The general trend observed was a marked decrease

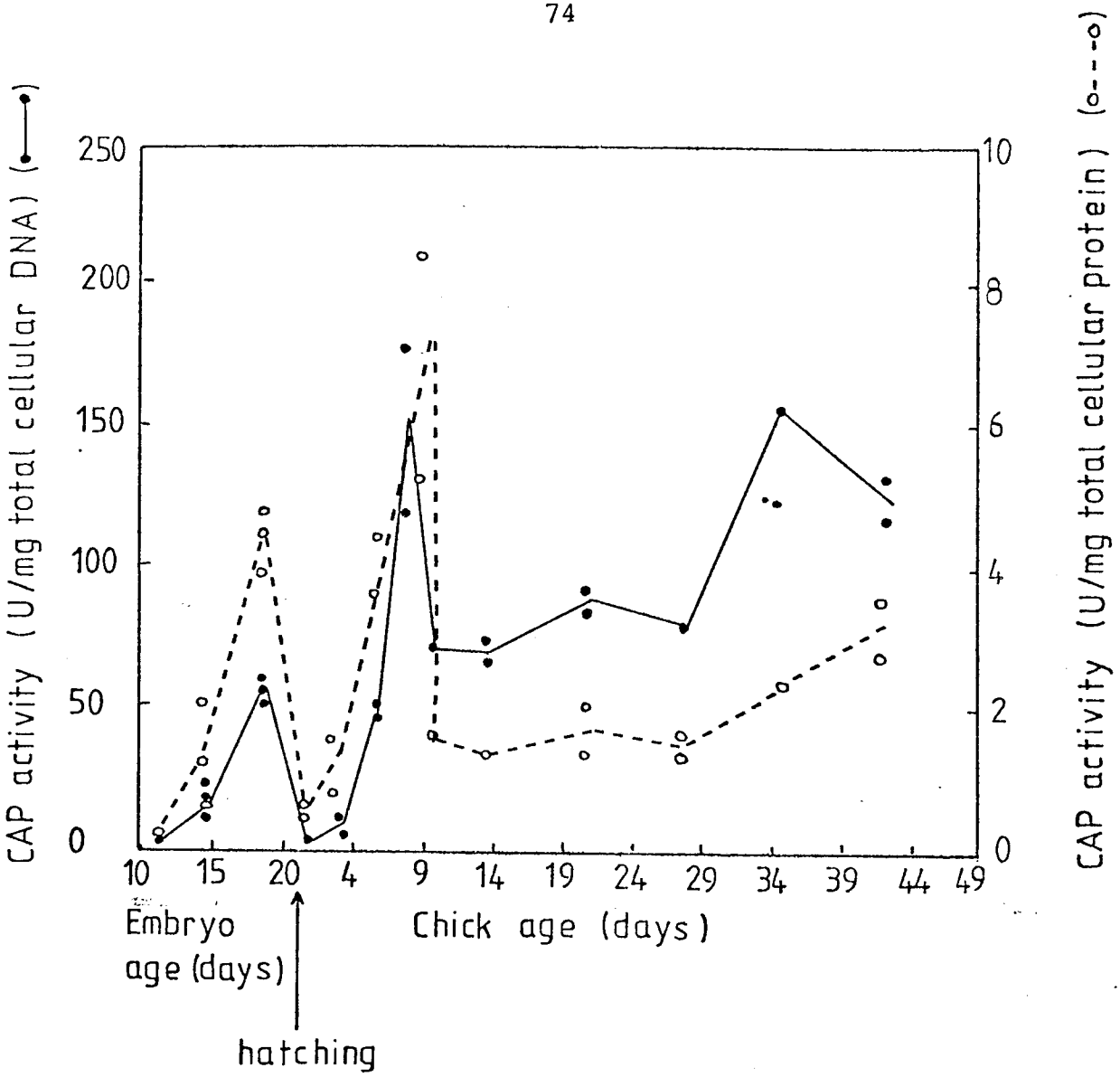


Fig. 3.7 CAP activity in developing chicken muscle. Inhibitor-free CAP fraction was obtained after purification of the enzyme by isoelectric precipitation (4.6.1) and CAP activity was assayed as described in 4.2.2. and 4.2.3. Each point represents the result of a different muscle preparation.

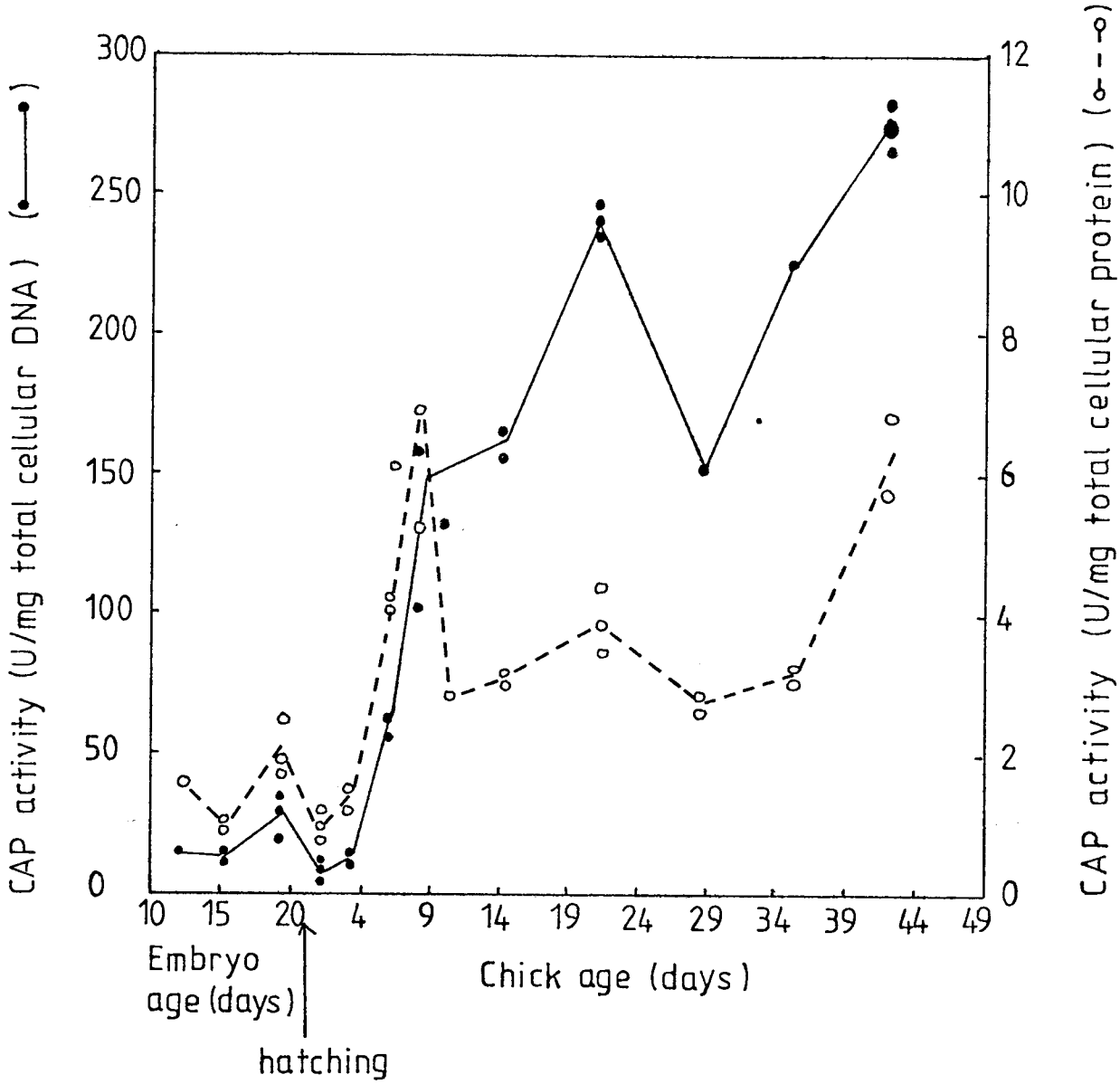


Fig. 3.8 CAP activity in cellular homogenate in developing chicken skeletal muscle. CAP activity expressed in terms of total cellular protein or cellular DNA and assayed as described in 4.2.2 and 4.2.3. Each point represents the result of a different muscle preparation.

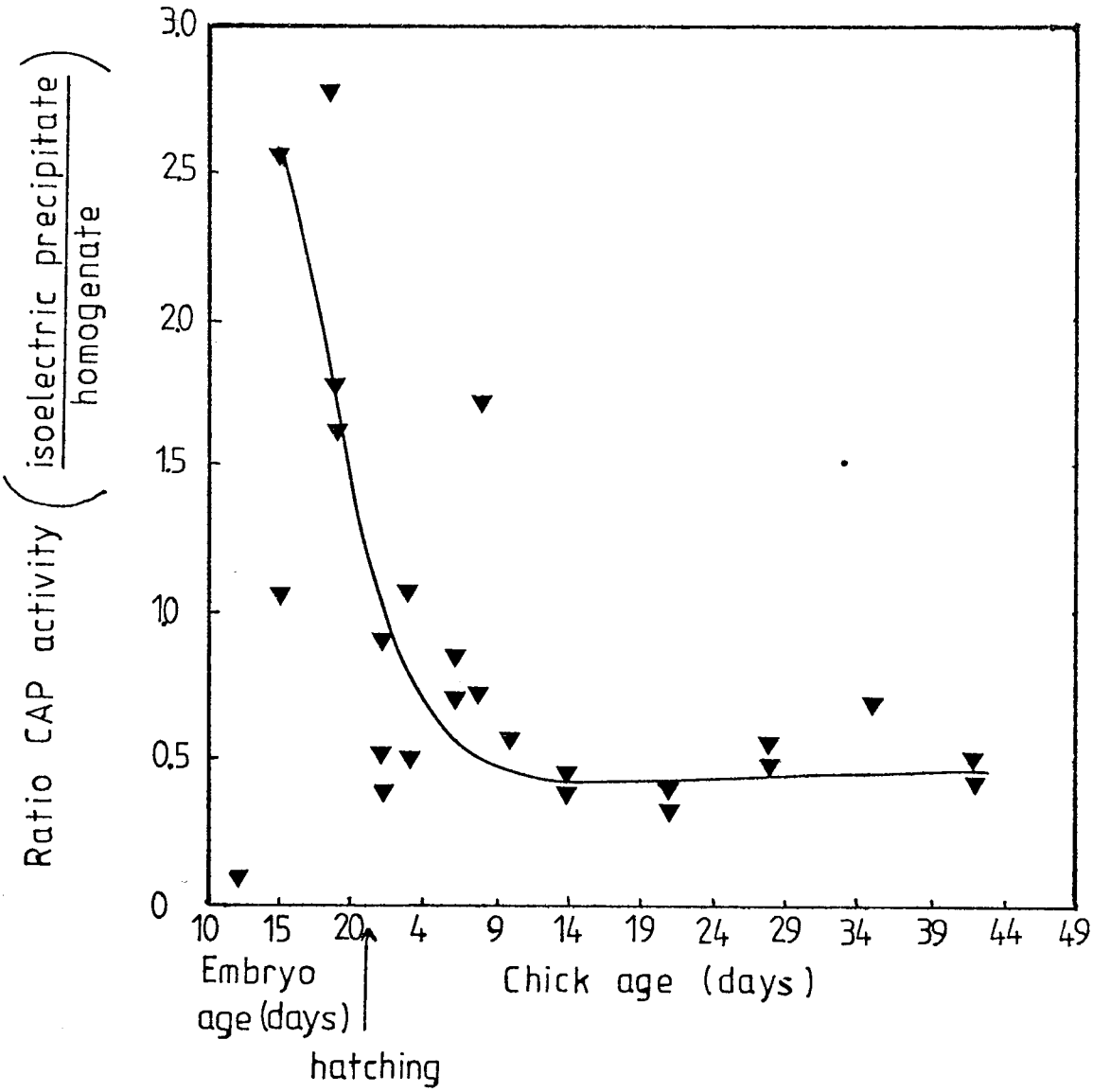


Fig. 3.9 Ratio of CAP activity in inhibitor-free fraction to that in crude cellular homogenate. Enzyme fractions were obtained after purification by isoelectric precipitation and CAP activity was assayed as described in 4.2.2 and 4.2.3.

in the ratio until about 8-9 days after hatching after which a fairly constant ratio was maintained. This ratio is an indication of the amount of inhibitor present in the crude homogenate fraction that is removed during isoelectric precipitation of the enzyme. A high ratio would therefore imply a high concentration of inhibitor present in the homogenate. It should also be noted, however, that the interpretation of the results is complicated by the fact that losses of enzyme activity occurred during the precipitation step, as indicated by the fact that enzyme activities in the precipitated fraction were lower than in the homogenate fraction (ratio <1), in the case of the older activities.

These results indicate both an increase in CAP activity during muscle development and a decrease in CAP inhibitor activity. However, isolation of CAP as described above did not allow the isolation of CAP inhibitor and a direct determination of the levels of inhibitor present in developing muscle as opposed to the CAP activity. It was therefore necessary to separate CAP and inhibitor to allow for the independent measurement of both activities. Ion-exchange chromatography, using DEAE-cellulose as described previously (3.2.1.3) was employed and the levels of both CAP and CAP inhibitor were determined in developing muscle.

3.2.3 Quantitation of CAP and Inhibitor in developing muscle by ion-exchange chromatography

Breast muscle from developing chickens, ranging in age from

12-day embryos to 7-week chickens, was homogenised as described in 4.6.2. The homogenate was centrifuged at high speed and the supernatant was diluted and applied to a DEAE-cellulose column. The column was washed with ten column volumes of a low salt buffer and the enzyme and inhibitor fractions were then eluted with a linear NaCl gradient (10-500 mM). Fractions were assayed for CAP activity and the inhibitor activity in the fractions was subsequently assayed using a given volume of the pooled CAP fractions from the same muscle. In all ages studied, CAP eluted in a single peak at about 0.31-0.33 M NaCl (Figs. 3.10 and 3.11), as shown earlier for adult and 15-day embryonic muscle (Chapter 2). Protein eluted as a broad fraction in all cases. Inhibitor peaks A, B and C were present in embryos up to 17 days of age, while by the age of 19 days, inhibitor C had disappeared. The older muscle had inhibitors A and B present, with very low levels of both in 7-week old chickens. It must be noted that inhibitor values shown in Figs. 3.10 and 3.11 for the different chromatographic separations cannot be compared as they were not assayed with the same CAP activity.

To quantify the levels of inhibitor present in muscle, a standard curve showing enzyme activity in the presence of increasing amounts of inhibitor was established. Such a curve of CAP activity from 15-day embryos with increasing amounts of inhibitor is represented in Fig. 3.12. The graph shows a linear relationship between amount of inhibitor present and inhibition of CAP activity and was used for the quantification of inhibitor present in each age studied. Inhibitor-containing fractions from each DEAE-cellulose chromatographic separation

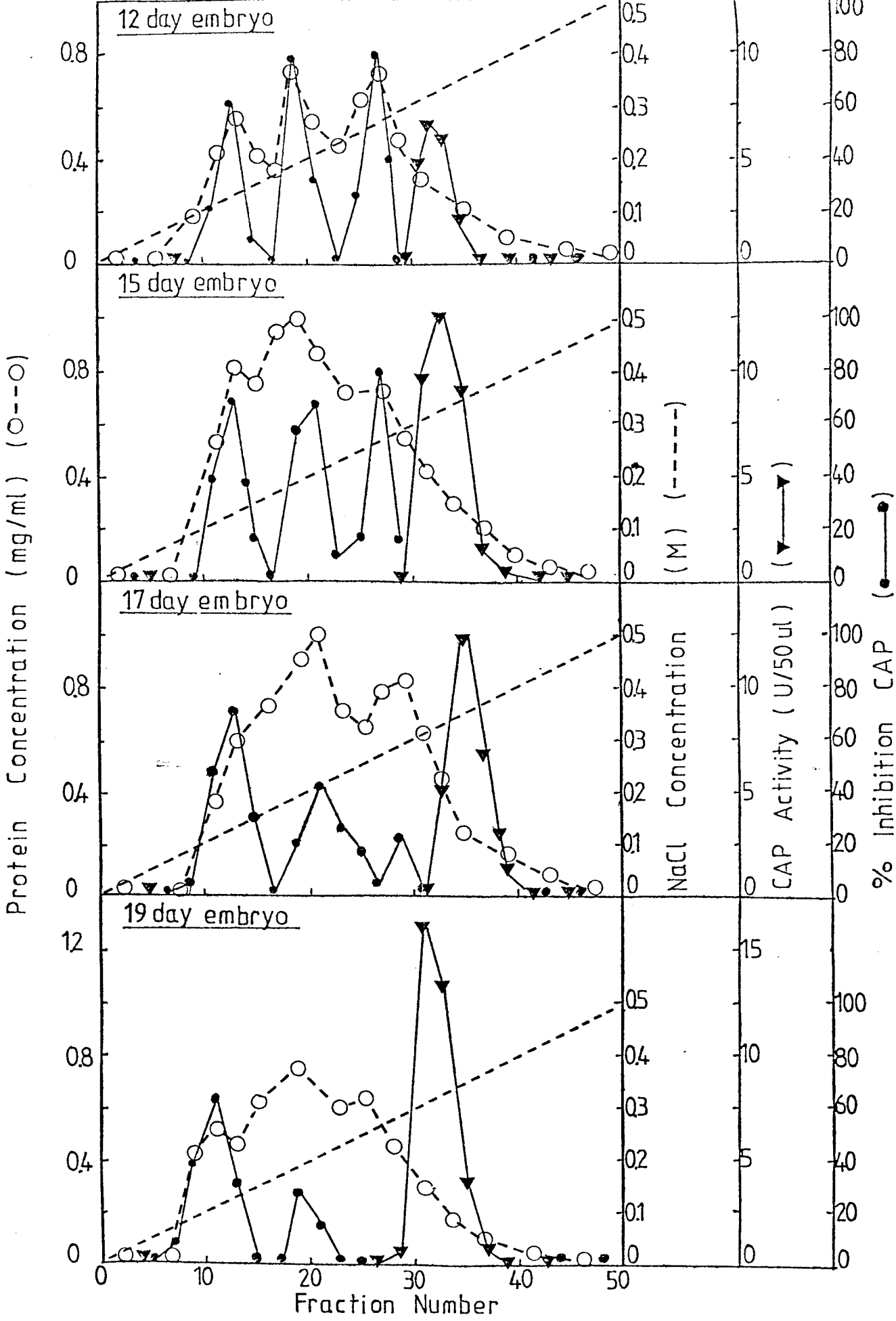


Fig. 3.10 (see overleaf)

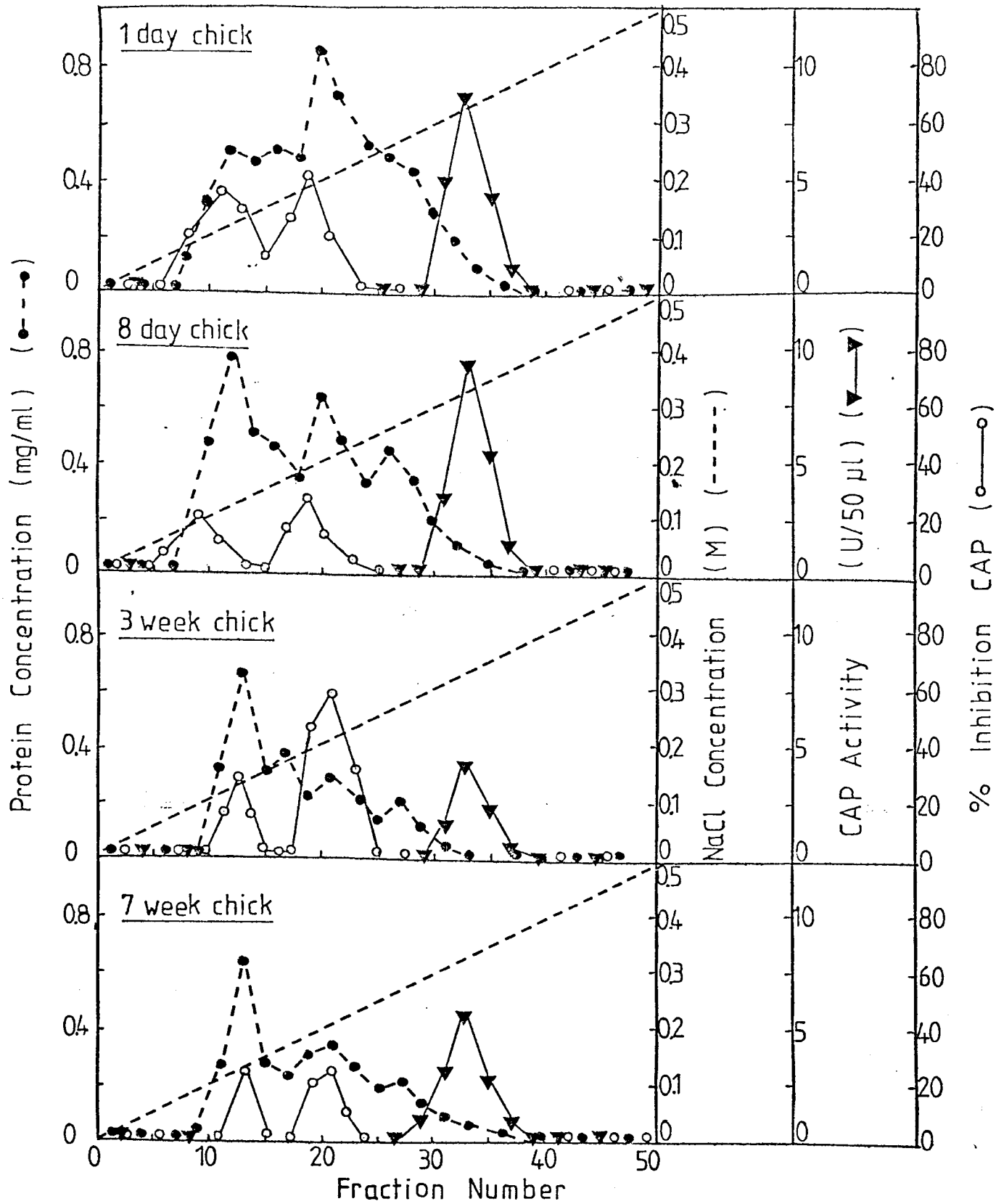


Fig. 3.11 (see overleaf)

Fig. 3.10 DEAE-cellulose chromatography of CAP and CAP inhibitor. Chromatography was carried out as described in 4.6.2, using DEAE-cellulose columns (0.8 cm × 17.5 cm), previously equilibrated in 5 mM Tris, 10 mM NaCl, 0.1 mM EDTA and 0.1 mM DTT, pH 7.4. Samples of cell extract containing 26, 39, 36 and 34 mg protein were applied to columns for 12-day, 15-day, 17-day and 19-day embryos, respectively. Elution was with a linear NaCl gradient in buffer (10 mM - 500 mM NaCl). The flow rate was 10 ml/h and each fraction contained 1.0 ml. Aliquots of 50 µl were assayed for CAP activity as described in 4.2.2 and 4.2.3. CAP inhibitor was assayed by incubating 50 µl aliquots with 50 µl pooled CAP.

Fig. 3.11 DEAE-cellulose chromatography of CAP and CAP inhibitor. Chromatography was carried out as described in 4.6.2, using DEAE-cellulose columns (0.8 cm × 17.5 cm), previously equilibrated in 5 mM Tris, 10 mM NaCl, 0.1 mM EDTA and 0.1 mM DTT, pH 7.4. Samples of cell extract containing 24, 49, 57 and 59 mg protein were applied to columns for 1-day, 8-day, 3-week and 7-week chickens, respectively. Elution was with a linear NaCl gradient in buffer (10 mM - 500 mM NaCl). The flow rate was 10 ml/h and each fraction contained 1.0 ml. Aliquots of 50 µl were assayed for CAP activity as described in 4.2.2 and 4.2.3. CAP inhibitor was assayed by incubating 50 µl aliquots with 100 µl pooled CAP in the case of 1-day and 8-day chickens and 50 µl pooled CAP in the case of 3-week and 7-week chickens.

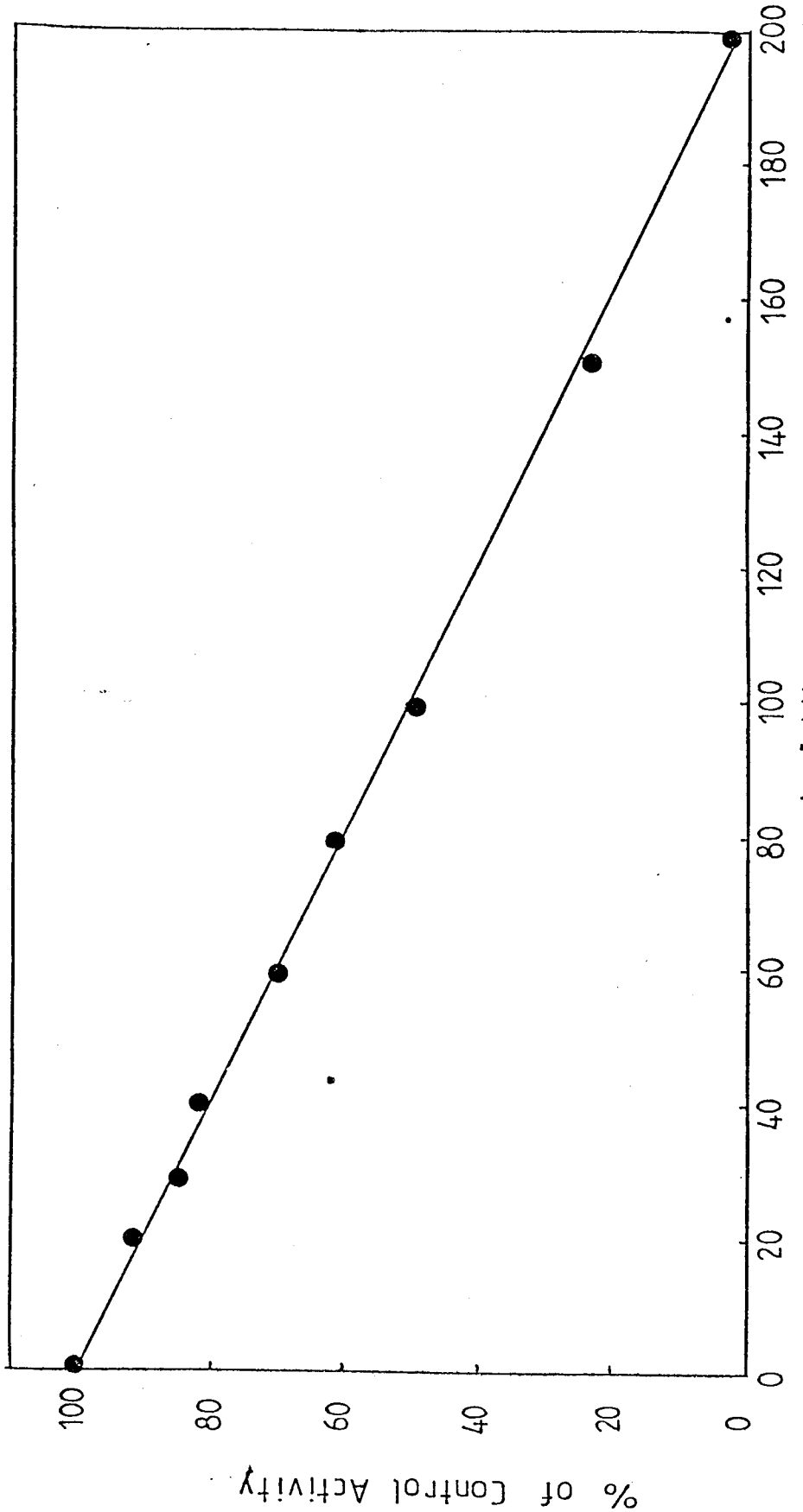


Fig. 3.12 Inhibition of CAP activity (17.4 U) by CAP inhibitor. CAP activity from 15-day embryos was assayed in the presence of varying amounts of inhibitor fractions from the same muscle. CAP and inhibitor activities were prepared by DEAE-cellulose chromatography and assayed as described in 4.2.2 and 4.2.3.

were pooled and assayed with CAP isolated from 15-day embryos. Where two or more inhibitor peaks were present, these were pooled and treated as a single inhibitor. One thousand units of inhibitor activity were defined as the amount which inhibited the activity of a given amount of CAP activity (17.4 U) by 50%.

The inhibitor fractions from all ages tested (Figs. 3.10 and 3.11) were quantified in terms of the titration curve shown in Fig. 3.12 and the results are given in Table 3.3. The initial CAP activity of the crude homogenate showed a large variation with a steady increase from 47 U/g in 12-day embryos to 370 U/g tissue in 7-week chicks. The specific activity of the pooled active CAP peak after DEAE-cellulose chromatography increased dramatically after hatching from 940 U/mg in 1-day chicks to 2022 U/mg in 3-week chicks but then dropped to 1090 U/mg in 7-week chicks. The reason for the drop in activity in 7-week chicks is not known. The ratio of CAP activity after chromatography to crude activity showed a marked decrease from 12 in early embryonic muscle to 1 and 0.8 in 3-week and 7-week chicks, respectively. The amount of CAP in the tissue increased by 3.8-fold with respect to total cellular DNA (65 U/mg to 247 U/mg) (see also Fig. 3.13) but decreased by 6-fold in relation to total tissue protein (12 U/mg to 2 U/mg). This is apparent when considering the protein/DNA ratio of muscle tissue during development (Table 3.3).

The specific activity of the inhibitor after chromatography decreased markedly from 25187 U/mg to 1860 U/mg with a slight increase occurring around 19-day embryos and 1-day chicks.

TABLE 3.3

SUMMARY OF RESULTS OBTAINED FROM THE EXPERIMENTS DESCRIBED IN FIGS. 3.10 AND 3.11. INHIBITOR ACTIVITY REPRESENTS THE POOLED ACTIVITY OF THE POOLED ACTIVE FRACTIONS.

	12-day embryo	15-day embryo	17-day embryo	19-day embryo	1-day chick	8-day chick	3-week chick	7-week chick
Homogenate:								
CAP activity (U/g tissue)	47	120	80	156	99	311	262	370
Protein/DNA ratio	5	7	8	9	14	49	108	120
CAP activity (U/mg cellular protein)	1.0	1.9	1.1	2.0	1.1	1.9	1.6	2.5
DEAE-cellulose fractions:								
Specific activity CAP peak (U/mg)	507	389	378	650	940	1243	2022	1090
Total activity CAP peak (U/g tissue)	567	486	640	584	641	592	272	306
$\frac{\text{Activity peak}}{\text{Activity applied}}$	12	4	8	4	6	2	1	0.8
Specific activity CAP inhibitor (U/mg)	25187	13333	14118	19118	18947	6751	1333	1860
Total U inhibitor/g tissue	115862	65728	76800	46925	82588	22130	3233	1916
$\frac{\text{U inhibitor}}{\text{U enzyme}}$	204	135	120	80	129	37	12	6
U CAP/mg cellular DNA	65	51	66	71	104	177	180	247
U CAP/mg cellular protein	12	8	9	8	7	4	2	2
U inhibitor/mg cellular DNA	13312	6883	7986	5680	13422	6608	2143	1546
U inhibitor/mg cellular protein	2470	1037	1067	609	955	135	20	13

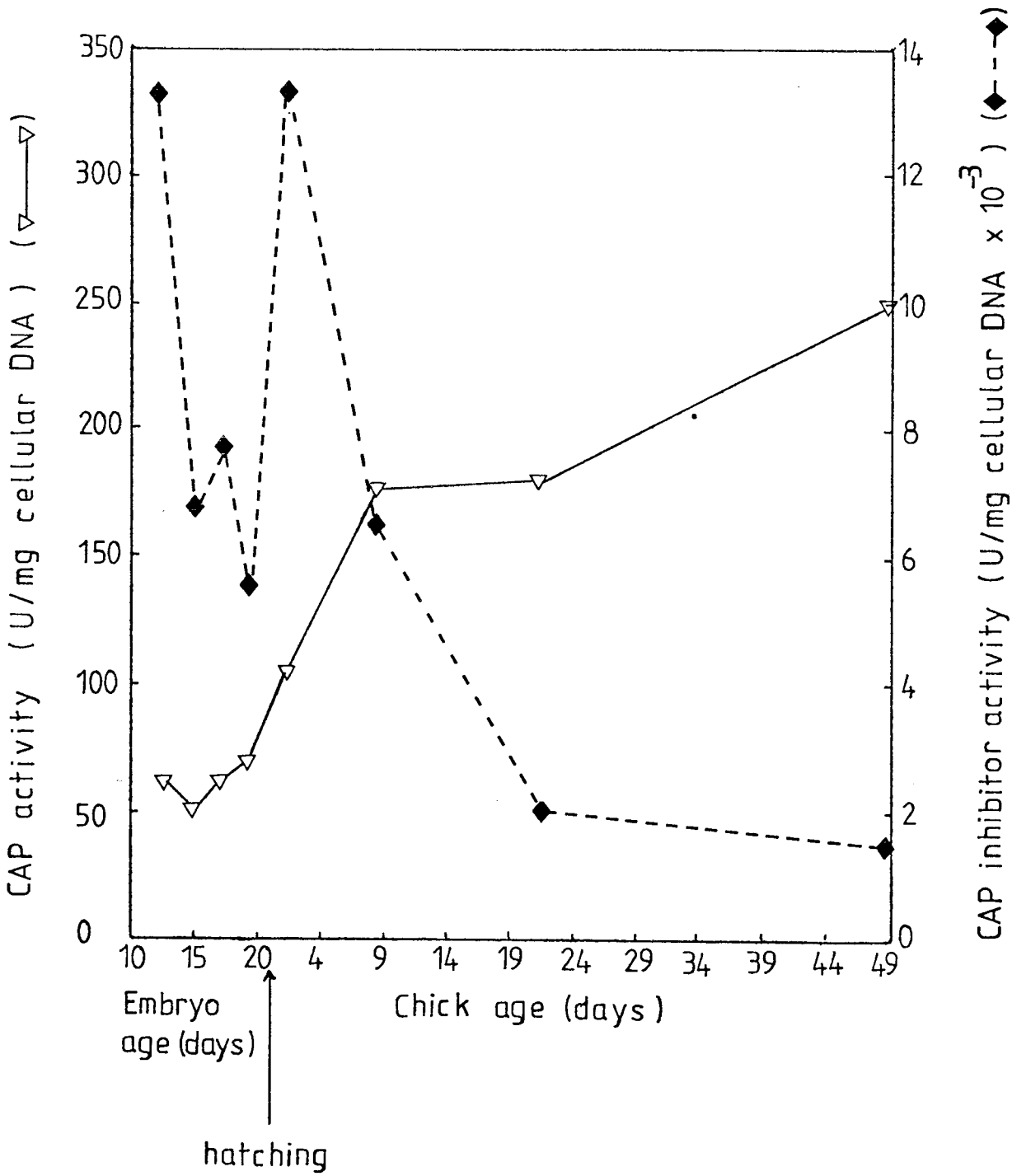


Fig. 3.13 CAP and CAP inhibitor levels in developing muscle after DEAE-cellulose chromatography. Results plotted are those represented in Table 3.3.

This dramatic decrease in inhibitor levels with increasing age is reflected by the drop in the ratio of eluted inhibitor to eluted protease activity of 204 to 6, as well as by a decrease in the ratio of CAP activity after chromatography to crude activity (see above). The amount of inhibitor present decreased both in relation to total cellular DNA (13312 U/mg to 1546 U/mg) (see also Fig. 3.13) and protein (2470 U/mg to 13 U/mg), which represent decreases of 8.6-fold and 190-fold, respectively. Overall, the inhibitor levels decreased more markedly during development than the corresponding increase in CAP levels.

The results obtained reflect an increase in CAP levels expressed in terms of the tissue DNA content in developing muscle with a simultaneous, more rapid decrease in inhibitor levels. The decrease in inhibitor activity would result in an effective increase in CAP activity intracellularly. The increase in the levels of purified CAP, however, did not match the synthesis and accumulation of myofibrillar proteins during muscle development, as reflected by the decrease in activity observed in relation to total cellular protein.

3.3 DISCUSSION

Results of this study demonstrate the presence of both a calcium-activated protease and an inhibitor of the protease in muscle cell cultures derived from 12-day chicken embryos. The enzyme is unlikely to be a contaminating protease from non-muscle cell types such as mast cells, as significant levels

of activity were found in differentiated muscle cell cultures. Park et al (1973) showed that treatment of rats with the mast-cell-degranulating agent 48/80 substantially decreased the activity of the chymostatin-sensitive alkaline protease (MAP) isolated from skeletal muscle. This was confirmed by Libby and Goldberg (1980) and by Woodbury et al (1978a, b), who used immunofluorescent and biochemical techniques to support a mast-cell origin and localisation of MAP. This does not seem to be the same for CAP from skeletal muscle, as has been demonstrated in this study, where pure myoblast cultures were grown. In addition, the presence of CAP inhibitor(s) in muscle cell cultures indicates that the inhibitor is endogenous and not perhaps a contaminating serum protein inhibitor.

Application of crude extracts of muscle cell cultures to DEAE-cellulose columns resulted in the separation of CAP from inhibitory activity. A marked increase in CAP activity occurred with muscle cell growth and differentiation which appeared to be matched by a corresponding decrease in inhibitor activity. The increase in the effective CAP activity (i.e. CAP activity of the total homogenate, containing inhibitor) observed with differentiation and the corresponding increase in myofibrillar protein content, supports the role of CAP in myofibrillar protein turnover postulated by Dayton et al (1975). High levels of inhibitor found during early growth and differentiation could have a protective effect on and control degradation of myofibrillar proteins by CAP.

Measurement of CAP activity in developing muscle by means of

isoelectric precipitation of the enzyme showed an increasing trend of proteolytic activity with age. However, it was uncertain whether any CAP activity was destroyed during the process of lowering the pH to 4.9. Busch et al (1972) described their method of preparation of CAP, which was precipitated when the pH was reduced from pH 6.1 to pH 4.9. No mention was made as to the percentage recovery of the proteolytic activity. Similarly, Ishiura et al (1978) utilised a pH treatment of the crude extract from adult chicken skeletal muscle during their purification of CAP and a loss of CAP activity actually occurred during the pH step. This indicates a possible denaturation of CAP during acid treatment. The results presented here show that a loss of enzyme activity was evident in the case of muscle from older birds since the enzyme activity actually decreased with the isoelectric precipitation step (Fig. 3.9).

Because of the possible loss of CAP activity using the procedure described above and the fact that this method of isolation of CAP yielded no direct information about quantitative levels of inhibitor, I decided to separate and quantify CAP and inhibitor from developing muscle by DEAE-cellulose chromatography. In contrast to the isoelectric precipitation procedure, DEAE-cellulose chromatography did not result in a significant loss of enzyme activity in any of the enzyme preparations and this procedure would therefore appear to be suitable for enzyme purification. The results showed a marked increase in the levels of CAP during development expressed per tissue DNA, especially just after hatching, although

this increase did not match the synthesis and accumulation of myofibrillar proteins. High levels of CAP inhibitor were found in embryonic muscle and these decreased markedly during development. This decrease was more than the corresponding increase in CAP activity and would cause an effective increase in CAP activity intracellularly.

As discussed in the preceding chapter, three peaks of inhibitory activity were present in early embryonic muscle, whereas older muscle contained peaks A and B only, which decreased to very low levels in adult muscle. The possible significance of the third peak of inhibitory activity (peak C) has already been discussed (Chapter 2). The presence of two peaks of inhibitor in adult muscle has not been reported previously. Ishiura et al (1977) reported the presence of only one species of inhibitor from adult chicken skeletal muscle with a molecular weight of around 68000 daltons. In addition, muscle cell cultures appeared to contain inhibitor activities which eluted at similar positions to peaks A and B after DEAE-cellulose chromatography, with the absence of an inhibitor species similar to peak C.

The existence of intermediate filaments has recently been demonstrated in smooth and skeletal muscle (Granger and Lazarides, 1979; Lazarides, 1980), which contain the proteins, desmin and vimentin, which have been found to co-exist at the periphery of the Z-disc. These proteins are thought to play a role in maintaining the integration of the myofibrillar ultrastructure by transversely linking adjacent myofibrils

at their Z-discs. Schollmeyer and Dayton (1977) have shown that smooth muscle CAP caused hydrolysis of desmin and disruption of the intermediate filaments in addition to the release of α-actinin from Z-discs. These observations, together with the results obtained in this study, further support the role of CAP in myofibrillar protein turnover as proposed by Dayton et al (1975), whereby CAP may initiate myofibrillar disassembly by degradation of the elements that hold the thin and thick filaments together. The proposed role of CAP is also supported by the presence of increased levels of the protease in dystrophic human muscle (Kar and Pearson, 1976) and skeletal muscle of dystrophic hamsters and mice (Neerunjun and Dubowitz, 1979), in which increased muscle breakdown occurs.

In addition to initiating myofibrillar protein degradation, it is possible that CAP may play a role in the insertion or internalisation of new contractile proteins into the myofibril. Morkin's electron microscopic autoradiographic studies of postnatal skeletal muscle suggested that newly synthesised myofilaments are added to the periphery of pre-existing myofibrils (Morkin, 1970). Goldspink also suggests that addition of new filaments takes place at the ends of myofibrils and that myofibrils may grow in diameter and then split longitudinally once a specific myofibrillar diameter has been reached (Williams and Goldspink, 1971; Griffin et al, 1971). It is suggested that CAP may play a role in intial proteolytic steps necessary for myofibril splitting and myofilament release.

However, Libby and Goldberg (1980) have recently found that, in contrast to the rat muscle, overall protein breakdown in cultured myotubes derived from chick embryos showed little or no inhibition by leupeptin and chymostatin and thus appears not to be associated with lysosomes. By the same argument, they suggested that CAP, which is also inhibited by leupeptin and chymostatin, may also not play a major role in protein breakdown under those conditions and that the critical proteases involved in overall protein breakdown in muscles have not yet been identified.

In conclusion, the evidence reported by other investigators over the last few years strongly suggests that CAP does play a fundamental role in the initial stages of myofibrillar protein turnover. Furthermore, the activity of the enzyme may be controlled both by the levels of the CAP enzyme itself and also by the concentrations of various endogenous inhibitors of the enzyme.

CHAPTER 4

MATERIALS AND METHODS

4.1 MATERIALS

All operations were carried out at 4°C unless otherwise stated.

Glass double-distilled water was used for all solutions.

Centrifugation was performed with a Beckman Model J21-C centrifuge and appropriate rotors.

Homogenisation, where described, was done with an Ultra-Turrax homogeniser.

Chickens and chick embryos were purchased from Golden Grove Poultry Farm, Cape Town.

Horse serum was prepared from freshly-collected blood obtained from the State Vaccine Institute, Cape Town.

Eagle's Minimum Essential Medium was obtained from GIBCO and Urografin from Schering (Germany).

Penicillin (Novopen) was purchased from Novo Industries (S.A.) and streptomycin sulphate from Glasco-Allenburys (S.A.)

Sephadex G-150 was obtained from Pharmacia (Uppsala, Sweden). When not in use, columns of Sephadex G-150 were stored in the presence of sodium azide to prevent microbial growth. DEAE-cellulose was purchased from Whatman (Kent, England).

Leupeptin and chymostatin were purchased from the Peptide Institute, Osaka, Japan. Pepstatin, trypsin, creatine kinase, soy bean trypsin inhibitor, 1-β-D-arabinofuranosyl-cytosine and calf thymus DNA (Type 1) were obtained from Sigma (U.S.A.). Dithiothreitol, cytochrome c, peroxidase, lactate dehydrogenase and bovine serum albumin (fraction V) were purchased from Miles (Cape Town). Double-distilled glycerol, Tris base, EDTA and 1,2-dichloroethane were purchased from Merck, Germany.

Standards for gel electrophoresis and concentrated dye reagent for protein determination were obtained from BIO-RAD Laboratories, California.

All chemicals not further described were of Analar grade or equivalent thereof, supplied by various companies.

4.2 CAP ASSAY

4.2.1 Preparation of denatured casein

Denatured casein was prepared as follows: 2.5 g casein was added to 10 ml of 500 mM Tris-HCl, pH 7.4 and the mixture pre-incubated at 37°C for 20 min, after which 0.75 ml of 2N NaOH was added. After heating in a boiling waterbath for 20 min, the solution was cooled and the volume made up to 50 ml with 500 mM Tris-HCl, pH 7.4. The solution was then dialysed overnight against 10 vol. of the same buffer and the pH adjusted to 7.4.

4.2.2 Conditions of assay

The method used routinely for the assay of CAP activity was the measurement of tyrosine released from denatured casein as a substrate. The assay incubation mixture contained the following: 100 µl 20 mM $CaCl_2$, 50 µl denatured casein in 500 mM Tris-HCl, pH 7.4, 50 µl 1mM DTT and 0-300 µl enzyme fraction in a total volume of 0.5 ml. A corresponding control was set up for each sample with 100 µl 10 mM EDTA present instead of $CaCl_2$. The mixture was incubated at 30°C for 60 min and the reaction terminated by the addition of an equal volume of 20% TCA. The mixture was left at 4°C for a minimum of 60 min and then centrifuged at 800 g for 10 min. The tyrosine content of the supernatant was determined as described below (4.2.3).

4.2.3 Measurement of tyrosine release

Tyrosine content was measured fluorimetrically using the method of Waalkes and Udenfriend (1957). 1.0 ml of 0.1% 1-nitroso-2-naphthol in 96% ethanol was added to 2.0 ml of TCA supernatant containing released tyrosine. The solution was mixed and 1.0 ml of 0.05% sodium nitrite in nitric acid (diluted 5 times) was added. After mixing, the tubes were stoppered, incubated at 55°C for 30 min and then cooled. The unreacted substrate was extracted with 9 ml 1,2-dichloro-ethane. The tubes were shaken well and centrifuged briefly at 200 g to separate the aqueous and organic layers. The fluorescence of the upper, aqueous layer was measured on a

Perkin-Elmer fluorescence spectrophotometer ($\lambda_{ex.}$ = 460 nm; $\lambda_{em.}$ = 570 nm). Tyrosine (0-20 nmoles) was used as a standard. Each sample was assayed in duplicate, unless otherwise indicated. Enzyme activity was determined from the difference between the activities in the presence and absence of Ca^{+2} (CAP activity = activity$_{Ca+2}$ - activity$_{+EDTA}$). An enzyme unit is defined as the activity corresponding to the release of 1 nmole tyrosine in 60 min at 30°C.

4.2.4 Other methods tested

4.2.4.1 [^{125}I]-labelled casein as substrate

[^{125}I]-labelled casein was prepared according to a modified method of Fleisher et al (1973). All solutions used were prepared in 0.05 M sodium phosphate buffer, pH 7.5. To 5 µl of [^{125}I] (0.5 mC$_i$) was added 1.0 ml of casein (2.5%). 250 µl chloramine T (4%) was then added and the mixture allowed to stand for 5 min at 4°C. After the addition of 250 µl 5% sodium metabisulphite, the mixture was slowly applied to a Sephadex G-75 column (0.9x 30 cm), previously equilibrated with the same buffer and the sample washed with 3 sample volumes of 10% potassium iodide solution in water. The sample was eluted with the same buffer at a flow rate of 6 ml/h and 1.0 ml fractions were collected. Aliquots were counted in a Packard PGD Auto-Gamma counter. [^{125}I]-labelled casein eluted with the void volume and the unreacted [^{125}I] eluted at a later stage.

CAP activity was assayed as described in 4.2.2 except that 10 µl of [^{125}I]-labelled casein and 40 µl of denatured casein were used as a substrate instead of 50 µl denatured casein only. Aliquots of 50 µl were removed from the incubation mixture at zero time to obtain total radioactivity at zero time. Background radioactivity was measured by substituting the enzyme solution by the buffer only and the cpm thus obtained subtracted from the sample cpm.

4.2.4.2 Measurement of A_{280} of TCA-soluble fraction

Conditions for assay of CAP activity were as described in 4.2.2 except that the absorbance at 280 nm of the TCA-soluble fraction was measured instead of tyrosine released.

4.2.4.3 Azocasein as substrate

A solution of azocasein (5%) was prepared in 500 mM Tris-HCl, pH 7.4 and CAP activity was assayed as described in 4.2.2. except that azocasein was used as a substrate instead of denatured casein. The reaction was terminated by the addition of an equal volume of 5% TCA instead of 20% TCA and the absorbance of the yellow TCA-soluble fraction was measured at 340 nm.

4.3 CAP INHIBITOR ASSAY

An aliquot of inhibitor sample was added to a given activity of CAP (10-20 units) and the mixture incubated at 0°C for 15 min. To initiate the proteolytic activity of CAP, the

aliquots were then added to the remaining components of the standard CAP assay incubation mixture. Control tubes contained aliquots of buffer instead of inhibitor. When assaying column fractions for inhibitor activity, inhibition was expressed as a percentage of control activity.

In order to quantify the amount of inhibitor in a given fraction as carried out in the profiles of inhibitor activity in developing muscle, a standard curve was set up using purified CAP and inhibitor fractions from 15-day embryos. Varying volumes of inhibitor were incubated with a given amount of enzyme and an inhibition curve was constructed. Activity of other inhibitor samples was expressed relative to the curve obtained as follows: 1000 U of inhibitor is defined as that amount of inhibitor required to inhibit a given amount of CAP activity by 50% (a given constant amount for each series of experiments) as indicated in "Results".

4.4 PROTEIN DETERMINATION

Protein was measured spectrophotometrically by the method of Lowry *et al* (1951), using crystalline bovine serum albumin as a standard. Interfering compounds were removed by precipitating the protein and then dissolving the dry TCA pellet in 0.5 N NaOH. In some cases, where indicated, the protein concentration of column fractions was measured according to Bradford (1976), using colour reagent obtained from BIO-RAD Laboratories.

4.5 DNA DETERMINATION

DNA was measured spectrophotometrically by the reaction with diphenylamine according to the procedure described by Abraham et al (1972), using calf thymus DNA (Type 1, Sigma) as a standard with the omission of the amyl acetate extraction step. In the case of samples containing protein, the DNA was first hydrolysed by heating at 70°C in 1 N perchloric acid for 20 min and the supernatant was analysed after centrifugation at 800 g for 10 min.

4.6 ISOLATION AND PURIFICATION OF CAP

4.6.1 Purification of CAP by isoelectric precipitation

CAP was separated from an endogenous CAP inhibitor by precipitation of the enzyme at pH 4.9 according to a modified method of Busch et al (1972). Chicken breast muscle was trimmed of excess fat and connective tissue, cut into small pieces and homogenised in 3 vol. of a freshly prepared solution of 20 mM sodium bicarbonate, 1 mM EDTA, pH 7.4. The homogenate was centrifuged at 10000 g for 15 min and the supernatant filtered through glass wool to remove fat materials. The pH of the crude extract was adjusted to 4.9 with 1 N acetic acid under gentle stirring conditions and after 10 min the resulting precipitate was sedimented by low-speed centrifugation. The sediment was then dissolved in a buffer consisting of 20 mM Tris-HCl, 0.1 M NaCl, 5 mM EDTA and 0.1 mM DTT, pH 7.4. The pH was adjusted to 7.4 by the addition of 1 N NaOH with gentle

stirring. The resulting turbid solution containing CAP activity was centrifuged at 50000 g for 40 min to remove insoluble materials.

4.6.2 DEAE-cellulose chromatography

Chicken breast muscle (usually 10-15 g) was trimmed of excess fat and connective tissue, cut into small pieces and homogenised in 2.5 vol. of a buffer consisting of 5 mM Tris-HCl, 50 mM NaCl, 4 mM EDTA and 0.1 mM DTT, pH 7.4 (Waxman and Krebs, 1978). The homogenate was centrifuged at 50000g for 40 min and the supernatant diluted five times with ice-cold distilled deionised water. The diluted supernatant was applied to a DEAE-cellulose column (DE-52; 1.6 cm × 25 cm), previously equilibrated in 5 mM Tris-HCl, 10 mM NaCl, 0.1 mM EDTA and 0.1 mM DTT, pH 7.4. The column was washed with 10 vol. of this buffer following application of the sample. A linear gradient was run, 150 ml on each side, of 10 mM to 500 mM NaCl and fractions of 2.0 ml were collected at a flow rate of 25 ml/h. Aliquots of 100 µl were assayed for CAP activity.

Columns of different dimensions were used for CAP preparations from muscle cells and smaller samples of embryonic tissue. These are described in the relevant sections.

4.6.3 Sephadex G-150 gel filtration

Pooled CAP and CAP inhibitor fractions from DEAE-cellulose chromatography were concentrated by vacuum dialysis

(approximately 5 times). Samples of 3 ml were applied to a Sephadex G-150 column (1.6 cm × 84 cm), previously equilibrated in 5 mM Tris-HCl, 10 mM NaCl, 0.1 mM EDTA and 0.1 mM DTT, pH 7.4 and were eluted with the same buffer. The flow rate was 8 ml/h and fractions of 2.0 ml were collected.

4.7 CELL CULTURES

4.7.1 Preparation of chick embryo extract

Embryo extract was prepared from 11-day chick embryos under sterile conditions. Embryos were removed from their shells, rinsed three times with saline G (0.137 M NaCl, 5 mM KCl, 1 mM KH_2PO_4, 0.1 mM $CaCl_2$, 0.7 mM $MgCl_2$, 5 mM glucose and 5.0 mg/l phenol red, pH 7.4) and forced through a 50 ml sterile syringe. An equal volume of saline G was added and the mixture centrifuged at 400 g for 30 min. The supernatant was removed and filtered through coarse nylon. The filtrate was divided into aliquots and frozen until required. Before use, the extract was thawed and centrifuged at 400 g to remove insoluble materials.

4.7.2 Gelatin coating of tissue culture dishes

1 g of gelatin was allowed to swell in 100 ml of cold, distilled water. The pellets were centrifuged and the supernatant discarded. The washing procedure was repeated 8 times and the final suspension (1%) placed in a sterile flask and heated for 20 min in a boiling waterbath. 100 mm tissue

culture dishes were coated by the addition of 5 ml 1% gelatin and left to stand for 6 h. The gelatin was then removed and the dishes allowed to dry overnight.

4.7.3 Primary chick embryonic skeletal muscle cultures

Primary chick skeletal muscle cultures were prepared from thigh muscle of 12-day chick embryos as described by Van der Westhuyzen (1979). Dissected muscle tissue was mechanically dissociated using the method of Tepperman et al (1975). A muscle suspension in complete medium (Eagle's minimum essential medium, 10% horse serum, 3% chick embryo extract, supplemented with 100 units/ml each of penicillin and streptomycin), at 0.2 - 0.3 g of tissue/ml, was agitated vigorously in a conical tube in a vortex mixer with 60 × 1-sec bursts. The resulting suspension was diluted with an equal volume of complete medium and filtered consecutively through a coarse and a fine nylon filter to obtain a single-cell suspension. The cell suspension was enriched for muscle cells by a buoyant density centrifugation procedure developed by T. Easton (T. Easton, unpublished results). The cell suspension (4 ml) was layered on 2 ml complete medium containing 16% urografin and centrifuged at 400 g for 20 min. The fibroblasts remained at the interphase between the two layers and the pellet containing predominantly myoblasts was collected. Cultures were seeded at 1.75×10^6 cells/100 mm gelatin-coated petri dish and incubated at 37°C in 5% CO_2 in air. The medium was changed after 24 h and subsequently every two days. To prevent overgrowth by non-muscle cells, cultures were treated with 10^{-5} M

1-β-D-arabinofuranosylcytosine (ara-C) after 60 h in culture, when fusion of myogenic cells was virtually complete, and thereafter, every 24 h. These conditions of preparation resulted in primary cultures in which approximately 80% of the cells were myoblasts after 60 h in culture. In differentiated cultures, after four to five days in culture, approximately 90% of the nuclei were present in fused myoblasts.

4.7.4 Primary chick embryonic fibroblasts

In order to obtain cultured fibroblasts, cells were prepared as described above except that the procedure for the enrichment of myoblasts using urografin was not carried out. The cells were plated directly on gelatin-coated dishes. After 3 days the cells were harvested by trypsinisation (0.05% trypsin, 0.002% EDTA in phosphate-buffered saline) and myotubes removed by filtering the cell suspension through a fine nylon filter. The single-cell suspension was then seeded at 2.0×10^6 cells/100 mm petri dish and the process repeated after 3 days. After a further 3 days in culture, fibroblasts were harvested and assayed for CAP activity.

4.7.5 Staining of cultures

Cultures were washed 3 times with saline G and the sides of the dishes wiped dry. The dishes were then flooded with methanol for 1 min and then rinsed several times with distilled water. They were then stained for 10 min with 0.25% May-Grunwald stain in methanol and then for a further 10 min

with Giemsa stain diluted 1 in 10 with distilled water. Cultures were scored on the basis of the total number of multinucleated cells on each 100 mm dish, using a Nikon microscope. A multinucleated cell was defined as a cell containing two or more nuclei.

4.8 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-polyacrylamide gel electrophoresis was performed on 15-8% acrylamide slab gels according to a modified method of Laemmli (1970). The two gel solutions were poured into a gradient maker so as to give a 15% to 8% linear acrylamide gradient. The two solutions contained the following:

8% acrylamide	15% acrylamide
0.1% SDS	0.1% SDS
5% by volume glycerol	10% by volume glycerol
0.37 M Tris-HCl, pH 8.8	0.28 M Tris-HCl, pH 8.8

The gels were polymerised chemically by the addition of ammonium persulphate and N,N,N',N'-tetramethylethylenediamine (TEMED) to a final concentration of 0.03% (w/v) and 0.025% (v/v), respectively. The stacking gel contained 5% acrylamide, 0.1% SDS and 0.1 M Tris-HCl, pH 8.8 and was polymerised by the addition of 0.05% v/v TEMED and 0.1% w/v ammonium persulphate. The electrode buffer consisted of 0.025 M Tris-HCl, 0.19 M glycine and 0.1% SDS, pH 8.8.

Protein samples were dissolved in a sample buffer consisting of 2% SDS, 10% v/v glycerol, 5% v/v 2-mercaptoethanol and

0.05 M Tris-HCl, pH 6.8 and boiled for 2 min. Electrophoresis was carried out for 3½ h at a constant current of 25 mA. Gels were fixed and stained overnight in 200 ml of 0.025% Coomassie blue, 25% isopropyl alcohol and 10% acetic acid. Gels were destained with shaking in 10% acetic acid.

4.9 QUANTITATION OF PROTEIN BANDS AFTER SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Quantitation of polypeptide bands was carried out as described by Fenner *et al* (1975). The stained polypeptide bands obtained after SDS-polyacrylamide gel electrophoresis were cut out and added to vials containing varying volumes of 25% pyridine, depending on the colour intensity of the bands. The vials were stoppered and shaken for 24 hours to extract the Coomassie blue stain. After 24 hours the absorbance of the extracted dye in 25% pyridine was measured at 605 nm and the protein content expressed in terms of total absorbance of that sample.

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