To the Graduate Council:

I am submitting herewith a thesis written by Margaret Diane Corey entitled "Tissue and Species Specificity of Antibodies Produced Against 'Native' and SDS-Denatured Smooth Muscle Actin and Myosin." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Carl J. Wust, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

TISSUE AND SPECIES SPECIFICITY OF ANTIBODIES PRODUCED AGAINST "NATIVE" AND SDS-DENATURED SMOOTH MUSCLE ACTIN AND MYOSIN

A Thesis

Presented for the

Master of Science

Degree

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Margaret Diane Corey

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To the Lord, the source of all my creative endeavors.

ABSTRACT

Antisera were produced against "native" and SDS denatured forms of actin and myosin purified from chicken gizzard to investigate the following questions:

1. Is SDS denaturation prior to immunization necessary to elicit antisera against a protein previously classified as "nonantigenic" in the native state (actin)?
2. Will SDS denaturation of actin and myosin prior to immunization alter the tissue and species specificity of the corresponding antisera?

The detection of precipitating antibodies and the tissue and species specificity of the antisera were analyzed by Ouchterlony double immunodiffusion and indirect immunofluorescent staining of fixed frozen sections. Chicken gizzard, chicken cardiac, and chicken skeletal tissues were used to test the tissue specificity of the antisera while pigeon gizzard, turtle gut, guinea pig gut, and toad gut were used to test the species specificity.

Since two out of three rabbits injected with "native" actin produced precipitating antibodies, it was shown that SDS denaturation prior to immunization is not necessary for antibody production.

Differences in the tissue and species specificity of antisera produced against "native" and SDS denatured forms of the two proteins were not observed. All anti-actins were found to be equally tissue and species nonspecific. In addition, all anti-myosins were found to be tissue specific for smooth muscle but species nonspecific.

Since SDS denaturation unfolds globular proteins into more extended polypeptide chains, it can be concluded from these studies that some antigenic determinants governing species cross-reactivity must be formed by linear amino acid sequences (sequential determinants) rather than conformationally induced juxtapositions of non-sequential amino acids (conformational determinants).

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LIST OF ABBREVIATIONS

αA	alpha actinin
A	actin
Ac	filamentous actin
aDAc	anti-denatured actin
aDMy	anti-denatured myosin
aNAc	anti-native actin
aNMy	anti-native myosin
CC	chicken cardiac
CS	chicken skeletal
CG	chicken gizzard
DAc	denatured actin
DMy	denatured myosin
DTT	dithiothreitol
gAc	globular actin
GPG	guinea pig gut
L_1	light chain (20,000 daltons)
L_2	light chain (16,000 daltons)
MHC	myosin heavy chains
My	myosin
NMy	"native" myosin
NAc	"native" actin
PBS	phosphate buffered saline
PG	pigeon gizzard
Pr	preimmune serum
TG	turtle gut
ToG	toad gut

I. INTRODUCTION

The importance of protein conformation in determining its ability to stimulate antibody formation and interact with the antibody was discovered early in the development of immunochemistry when Landsteiner (1945) found that changes in protein conformation due to chemical modification led to changes in antigenicity. Since that time, specific chemical modification of proteins and the corresponding changes in immunochemical properties have been studied in an effort to understand the relationship between conformation and antigenicity (reviewed by Crumpton, 1974).

The following review and research deals with the effect of sodium dodecyl sulphate denaturation on two protein immunogens. Specifically, answers to the following questions were sought:

1. Is SDS denaturation prior to immunization necessary to elicit antisera against a protein previously classified as "nonantigenic" in the native state (actin)?
2. Will SDS denaturation of proteins prior to immunization alter the tissue and species specificity of the corresponding antisera (actin and myosin)?

Properties of sodium dodecyl sulphate relevant to this study, structural characteristics of the actin and myosin, and past immunological data are reviewed below, followed by the research protocol used to investigate these questions.

The interrelationship between sodium dodecyl sulphate and proteins has been studied for the past three decades. SDS is a nega-

tively charged detergent. When combined with proteins in the presence of heat and sulfhydryl reducing agent, SDS is capable of dissociating oligomeric proteins into their polypeptide chains. Shapiro et al, (1967), were the first to discover that the electrophoretic mobilities of SDS denatured proteins are inversely proportional to the log of their molecular weights. When the SDS monomer concentrations are above .02% (8×10^{-4} M), 1.4 gm of SDS are bound per gram of protein. This corresponds to approximately one SDS monomer per two amino acid residues. The protein SDS bond is noncovalent; however, once formed, the SDS cannot be appreciably removed by dialysis, and the presence of a reducing agent is no longer required to keep the sulfhydral bonds from reforming. The conformation of the dissociated polypeptide chains is controversial; several models ranging from rod-like, ellipsoid to random coil have been proposed to explain the hydrodynamic properties of the SDS protein complexes (reviewed by Payne, 1976).

The significance of SDS in the field of immunology can be illustrated by reviewing early studies of the "contractile" protein actin. Actin is a globular protein found not only in muscle cells, but in all eucaryotic cells (Pollard and Weihing, 1974). Actively motile cells such as amoebas and platelets contain as much as 20% of their protein as actin. In the past few years, this protein has been found to participate in a variety of cell functions including motility, endocytosis, exocytosis, cytokinesis, membrane ruffling, and maintenance of cell shape (Buckley and Porter, 1967; Goldman and Knipe, 1972;

Spooner et al, 1971; Stossel, 1971; Lazarides, 1976; Fugiwara and Pollard, 1976; Orr et al, 1972).

The cytoplasmic actins resemble muscle actins in many ways. Monomeric actins have an apparent molecular weight of about 42,000, bind one mole of ATP per mole of protein, and polymerize in .1 M KCL to form filamentous actin (F-actin), a double stranded helix with a half pitch of 35 nm. The F-actin filaments are capable of binding heavy meromyosin, activating the Mg^{++}ATPase of myosin, and inhibiting DNase I activity (reviewed by Pollard and Weihing, 1974; Korn, 1978). Considering these similarities, it is not surprising that most actins have very similar amino acid compositions and partially conserved peptide maps (Korn, 1978; Vanderchove and Weber, 1978).

The highly conservative structure of actin was thought to be the reason why early attempts to produce antibodies in rabbits to "native" chicken skeletal actin failed (Wilson and Finck, 1971). Later, Lazarides and Weber found that injections of denatured mouse fibroblast actin [separated by SDS polyacrylamide gel electrophoresis (PAGE)] into rabbits elicited antisera that reacted with the homologous antigen as well as chicken skeletal actin in Ouchterlony plates. Thus, these antisera were considered to be species and tissue non-specific; they reacted with cytoplasmic and muscle actins from two different vertebrate species.

In addition, Lazarides produced antibodies in rabbits against denatured actins (separated by SDS PAGE) from chicken gizzard and calf

thymus. These antibodies were equally tissue and species nonspecific as those previously produced. The antisera gave indistinguishable staining patterns on cultured rat embryo fibroblasts and lung cells and guinea pig vascular smooth muscle cells (Lazarides, 1975).

Since that time anti-actins have been produced against NaOH denatured, glutaraldehyde fixed, alum adsorbed, aged, and ammonium sulphate precipitated actin (Hirabayashi and Hayashi, 1972; Herman and Pollard, 1979; Jockusch, et al, 1978; Trenchev and Holborow, 1976; Groeschel-Stewart, 1977). Anti-actins have also been found in patients with chronic active hepatitis (Gabbiani et al, 1973; Lidman et al, 1976). All the antisera were observed to have tissue and species nonspecificity and were all produced against altered or denatured forms of actin. This might support the postulation that denaturation exposes "hidden" antigenic determinants, thus increasing the antigenicity of actin and the species and tissue cross-reactivity of the corresponding antisera (Trenchev and Holborow, 1976). However, Groeschel-Stewart (1977) was successful in producing antiserum against "native" chicken gizzard actin in only one out of seven rabbits that was equally tissue and species nonspecific as that produced against denatured actin. A note added at the end of her paper stated that the injection of less than 1 mg of "native" actin led to more success in antibody production, but these results have not been further amplified.

Thus, small amounts of SDS denatured or "native" actin have been injected into rabbits to determine if denaturation is a requirement

for antigenicity. In addition, since the production of antisera against "native" actin has been rare in the past, the tissue and species specificity of the antisera was characterized and compared with that produced against SDS denatured actin. The effect of SDS denaturation on the specificity of the antisera produced against "contractile" protein, myosin, was also investigated.

Myosin, like actin, is found in muscle as well as nonmuscle cells. The myosin molecule is composed of two identical heavy chains (200,000 daltons) which are folded into a rod-like helical conformation approximately 1400 A^0 in length. The heavy chains combine with 2-3 light chains (16,000 - 25,000 daltons) to form globular heads (approximately 100 A^0 in diameter) that contain the ATPase and actin binding sites (reviewed by Stephens, 1977, Lowey and Steiner, 1972). Despite similarities in size, shape, and susceptibility to certain proteolytic enzymes, myosins from different tissues have different amino acid compositions and immunological properties (Groeschel-Stewart, 1976).

Studies concerning the tissue and species specificity of anti-myosins are diverse. For example, Groeschel-Stewart et al (1976) found that fluorescinated anti-gizzard myosin would stain cultured smooth muscle of human, mouse, guinea pig, and rabbit origin in addition to rat, rabbit, and human frozen sections. However, the anti-gizzard myosin did not cross-react with skeletal or cardiac tissue. Thus, it was concluded that anti-smooth muscle myosin was tissue specific for smooth muscle but species nonspecific. In agreement with

Groeschel-Stewart, Bagby and Pepe (1978) found anti-gizzard myosin to be tissue specific for smooth muscle. However, they found minimal cross-reactivity with other species tissues such as guinea pig ileum and taeni-coli, which led them to conclude that anti-gizzard myosin was not only tissue specific but species specific also. To complicate matters, Fairfax and Groeschel-Stewart (1977) discovered an autoantibody in a patient with bronchiectasis that would cross-react with smooth muscle, nonmuscle, and skeletal muscle myosins in immunofluorescent staining and adsorption tests. Therefore, this anti-myosin was tissue and species nonspecific.

Although various factors such as injection schedules and immunization periods could affect the specificity of these anti-myosins, it could be postulated that changes in the conformation of the protein has led to changes in the specificity of the corresponding antisera. Thus, this assumption has been investigated by comparing the tissue and species specificity of antisera against native and SDS denatured myosin from chicken gizzard.

In summary, Ouchterlony double immunodiffusion and indirect immunofluorescent staining were used to detect the production and to characterize the specificity of antisera produced against SDS denatured and "native" forms of actin and myosin. Chicken gizzard, chicken cardiac, and chicken skeletal tissues were used to test the tissue specificity of the antisera while pigeon gizzard, turtle gut, toad gut, and guinea pig gut were used to test species specificity.

II. MATERIALS AND METHODS

A. Preparation of Immunizing Agents

Unless stated otherwise, all steps were carried out at 4^{o} C, and protein determinations were made using the Biorad Protein Assay with bovine serum albumin as standard. Purification procedures were monitored by SDS PAGE according to Weber and Osborn (1969).

1. Myosins

A crude extract was obtained from chicken gizzards according to Wachsberger and Pepe (1974). Myosin was concentrated by ammonium sulphate fractionation and purified by DEAE chromatography (Richards et al, 1976) and high speed centrifugation. The detailed procedure follows:

One kilogram of fresh chicken gizzards was obtained from Knoxville Poultry and Egg Company Incorporated. The gizzards were stripped free of fat and connective tissue yielding approximately 500 gm of trimmed tissue. The tissue was then washed three times with 4:1 v/w deionized water and ground twice in a chilled meat grinder equipped with 1 mm holes. The ground tissue was washed two additional times with deionized water (4:1 v/w); each wash was followed by centrifugation at 4000 xg for ten minutes. The pellet was suspended in extraction buffer to give a total of 3 x the volume of the ground tissue and to give a final concentration of .025 M KCL, 1019 M PO_4, 1 mM ATP, .5 mM Dithiothreitol, hereinafter referred to as DTT, (pH 7.5). The tissue suspension, in extraction buffer, was

homogenized in a chilled Waring blender for 20 seconds, and stirred gently overnight.

Twelve hours later the ATP concentration was increased to 2 mM, and the suspension was immediately centrifuged for 30 minutes at 4000 xg. The supernatant solution, which contained the crude actomyosin, was filtered through 4 layers of cheesecloth to remove suspended fat. The suspension was then warmed to room temperature and $CaCl_2$ was added to a final concentration of 15 mM. A white precipitate formed, and the suspension was centrifuged at 10,000 xg for 10 minutes. The pellet was suspended in .4 M KCL, .05 M PO_4, .5 mM DTT, (pH 7.5), and stirred in the cold for 1 hour. The protein concentration was adjusted to 10 mg/ml, and the solution was made 10 mM in both ATP and $MgCl_2$ prior to fractionation with ammonium sulphate. The 55-60% ammonium sulphate fraction was dissolved in 40 mM sodium pyrophosphate (pH 7.5) and dialysed against 400 volumes of the same buffer overnight.

The following morning the solution was centrifuged at 20,000 xg for 1 hour. Approximately 400 mg of crude actomyosin (10 mg/ml) was washed onto a DEAE Sephadex A-50 column (2.5 x 90 cm) equilibrated with 40 mM sodium pyrophosphate (pH 7.5). A linear gradient consisting of 1 L of 40 mM sodium pyrophosphate and 1 L of 1 M KCL in 40 mM sodium pyrophosphate (both at pH 7.5) was used to elute the myosin with a flow rate of 20-25 ml/hr. The myosin was eluted within 24 hours and was precipitated overnight by dialysis against several volumes of .05 M KCL, .006 M Phosphate buffer (pH 7.0).

The suspension was centrifuged at 20,000 xg, and the pellet was suspended in .6 M KCL, .05 M DTT and made 10 mM in both ATP and $MgCl_2$. The solution was centrifuged for 3 hours at 38,000 xg to remove any aggregated myosin and actomyosin. The pure myosin was stored in 50% .4 M KCL, .05 M PO_4 (pH 7.4), 50% glycerol at -20° C.

Twenty mg of the purified myosin were divided into two aliquots and prepared for immunization. One aliquot was dialysed against .8 M NaCl, .1 M PO_4 (pH 7.4), hereinafter referred to as "native" myosin (NMy). The second aliquot was dialysed against .8 M NaCl, .1 M PO_4, .1 M DTT, 1% SDS for 24 hours and then heated in a boiling water bath for five minutes. The solution was then redialysed against .8 M NaCl, .1 M PO_4, .2% SDS, hereinafter referred to as denatured myosin (DMy). To each aliquot 50% glycerol v/v was added, and they were stored at -20° C.

2. Actins

An acetone powder was prepared from chicken gizzards according to Carsten and Mommaerts, (1963). Actin was extracted and purified from the powder by repeated polymerization and depolymerization (Spudich and Watt, 1971). The detailed procedure follows:

Approximately 500 gm of fresh chicken gizzards were ground twice in a meat grinder equipped with 1 mm holes. The mince was first extracted with 3:1 v/w .1 M KCL and second with 3:1 v/w .05 M $NaHCO_3$. Each extraction lasted 15 minutes, and all extractions and washes were followed by filtration through cheesecloth. The mince was washed once with 10:1 v/w .001 M EDTA and twice with 10:1 v/w H_2O.

The residue was then washed twice with 2:1 v/w of acetone (0^{o}) and once with 1:1 v/w of acetone for a few seconds while blending. The fibrous mass was air-dried overnight and stored at -20^{o} C.

Ten gm of the acetone powder were extracted at 0^{o} C for 30 minutes with 200 mls of 2 mM Tris-Cl, .2 mM ATP, .5 mM β mercaptoethanol, .2 mM $CaCl_2$ (pH 8.0), hereinafter referred to as Buffer A. The suspension was suction filtered, and the pulp was washed with an additional 100 ml of Buffer A. The filtrates, containing the crude actin, were combined and clarified by centrifugation at 10,000 xg for 1 hour. KCL and $MgCl_2$ were added to a final concentration of 50 mM and 2 mM respectively. The actin was allowed to polymerize for two hours. The KCL was then increased to .6 M and the solution was stirred for 1.5 hours. Centrifugation for 3 hours at 80,000 xg followed to pellet the F-actin. The pellet was dissolved in 20 ml of Buffer A, and the F-actin was depolymerized to G-actin as the sample was dialysed against several changes of Buffer A over a period of 4 days. The G-actin was clarified by centrifugation at 38,000 xg for three hours. The purified actin was then divided into two aliquots. One aliquot was stored in Buffer A to keep the actin in the globular form. Another aliquot was polymerized and stored in the filamentous form by dialysis against .8 M KCL, .1 M PO_4 (pH 7.4). To each aliquot 50% glycerol v/v was added, and they were stored at -20^{o} C.

Later, 20 mg of the purified globular actin were divided into two aliquots and prepared for immunization. One aliquot was dialysed against Phosphate Buffered Saline , (PBS pH 7.4), hereinafter re-

ferred to as "native" actin (NAc). A second aliquot was dialysed overnight against PBS containing .1 M DTT and 1% SDS. The sample was heated in boiling H_2O bath for 3 minutes and redialysed against PBS containing .2% SDS, hereinafter referred to as denatured actin (DAc). To each aliquot 50% glycerol v/v was added, and they were stored at -20^{o} C.

B. Immunization

Twelve male New Zealand white rabbits (weighing 5-7 kg each) were divided into four groups of 3 and used to obtain antisera against NMy, DMy, NAc and DAc. Approximately 30 ml of preimmune serum were obtained from each rabbit to serve as controls in the experiments. Short term immunization schedules were employed and precipitating antibodies were detected by double immunodiffusion. The detailed immunization schedules follow:

1. Anti-myosins

a. Day 1: Approximately 1 mg of myosin emulsified with an equal volume of Freund's Complete Adjuvant (Difco Laboratories) was injected intramuscularly into the hindquarters.

b. Day 10: Approximately 1 mg of myosin emulsified with an equal volume of Freund's Incomplete Adjuvant was injected subcutaneously at multiple sites along the back.

c. Day 20: 100 ug of myosin was injected intravenously into the left marginal ear vein.

d. Day 27: 50 ml of blood were obtained from the right

marginal ear vein.

e. Day 30: 100 ug of myosin were injected intravenously into the left marginal ear vein.

f. Day 35: 50 ml of blood were obtained from the right marginal ear vein.

g. Day 36: The rabbits were anesthetized with nembutal (27 mg/kg) and totally exsanguinated by cardiac puncture.

2. Anti-actins

a. Day 1: Approximately 500 ug of actin emulsified in an equal volume of Freund's Incomplete Adjuvant were injected intramuscularly into the back legs.

b. Day 10: Approximately 500 ug of actin emulsified in an equal volume of Freund's Incomplete Adjuvant were injected subcutaneously at multiple sites along the back.

c. Day 20: 100 ug of actin were injected intravenously into the left marginal ear vein.

d. Day 26: 50 ml of blood were obtained from the right marginal ear vein.

e. Day 40: 500 ug of actin emulsified in an equal volume of Freund's Incomplete Adjuvant were injected intradermally at multiple sites along the back.

f. Day 50: 50 ml of blood were obtained from the right marginal ear vein.

g. Day 58: The rabbits were anesthetized with nembutal (27 mg/kg) and totally exsanguinated by cardiac puncture.

C. Preparation of Serum

The serum was freed of lipids and erythrocytes and the IgG fraction was separated according to Garvey et al (1977). The detailed procedures follow:

To reduce the amount of lipids in the serum the rabbits were fasted twelve hours prior to bleeding. The blood was allowed to clot at 23° C for 2 hours and further incubated overnight at 4° C. The serum was freed of erythrocytes by centrifugation at 10,000 xg for 15 minutes. Any remaining lipids were removed by centrifugation at 15,000 xg for 30 minutes at 0° C followed by filtration through glass wool. The serum was stored at -20° C.

The IgG was fractionated from the serum at 4° C by the slow addition of ammonium sulphate (pH 7.4) to a final concentration of 37%. After stirring for 3 hours, the suspension was centrifuged at 1400 xg for 30 minutes. The white precipitate was dissolved in PBS to restore the volume of the solution to that of the original serum. The ammonium sulphate fractionation was repeated two additional times and the final precipitate was dissolved in a minimum amount of PBS. The salts were removed by dialyzing against several changes of PBS for three days. The solution was then clarified by centrifugation at 1400 xg for 30 minutes. The protein concentration was adjusted to 10 mg/ml and the IgG was stored in small volumes at -20° C.

D. Evaluation of Antisera

All twelve IgG fractions were screened for purity, tissue and species specificity using Ouchterlony double immunodiffusion analysis. The test antigens for purity were actin, ∝actinin, myosin, tropomyosin, and crude actomyosin, all of which were derived from chicken gizzard. Test antigens for tissue and species specificity included crude extracts from chicken gizzard, chicken heart, chicken pectoral muscle, turtle gut, toad gut, guinea pig gut, and pigeon gizzard.

One IgG fraction from each of the four groups was tested for additional analysis of tissue and species specificity by indirect immunofluorescent staining of fixed frozen sections. The fixed test antigens were the same types as those used for crude extracts.

The preimmune IgG from each rabbit served as controls for non-specific precipitation and staining.

1. Preparation of Test Antigens

Antigens purified from chicken gizzard: Myosin and actin were prepared as specified previously. Tropomyosin and ∝actinin were provided by Dr. Roland Bagby (1980).

Crude extracts from smooth muscle: Crude extracts containing actin and myosin as well as many other proteins were obtained from the following smooth muscle tissues: chicken gizzard (CG), pigeon gizzard (PG), guinea pig gut (GPG), toad gut (ToG), and turtle

gut (TG). The following extraction methods were modified from Sobieszek (1975).

Each tissue was stripped of fat and connective tissue, minced and washed twice with 4:1 v/w deionized H_2O. All washes were followed by centrifugation at 4000 xg for 10 minutes. The pellets were suspended and homogenized in 5:1 v/w of extraction buffer containing 2 mM EGTA, 1 mM EDTA, .001 M $MgCl_2$, .06 M KCL, .019 M PO_4, 15 mM DTT, 10 mM ATP (pH 7.4). The extraction, with gentle stirring, proceeded for 30 minutes.

The suspensions were centrifuged at 10,000 xg for 30 minutes and fractionated with ammonium sulphate. The 30-60% cuts were dissolved in .8 M KCL, .1 M PO_4, (pH 7.4), and were dialysed against several volumes of this buffer for three days to remove the ammonium sulphate. The solutions were clarified by centrifugation at 15,000 xg for 30 minutes. An equal volume of glycerol was added and the extracts were stored at -20^{o} C.

The procedures for extracting chicken skeletal and cardiac tissue were slightly different because of the solubility characteristics of their proteins. The extraction solution (5:1 v/w) for these tissues was .8 M KCL, 2 mM EGTA, 1 mM EDTA, 10 mM $MgCl_2$, .019 M PO_4, .5 mM DTT, 10 mM ATP (pH 7.4). All other procedures were the same as those previously stated.

2. Ouchterlony Double Immunodiffusion

The following procedures for making Ouchterlony gels were modified from Garvey et al (1977). Clean slides were precoated with 2.5

ml of molten .5% agarose and dried in an oven (80^{0} F) overnight to increase the adherence of the top gel layer during rinsing and staining. Because of the different solubility properties of the antigens, the precoated slides were layered with 2.5 ml of molten 1% agarose in either high salt buffer (.4 M KCL, .05 M PO_4, pH 7.4), or low salt buffer (.012 M Tris-Cl pH 7.4). When the agarose layer solidified, the plates were refrigerated for 5 minutes and 3 mm wells were punched with a Bio-Rad gel punching kit.

The plates were then placed in a moisture chamber made with damp filter paper inserted in a plastic petri dish. After the reactants were loaded into the wells (8 ul/per well) and were allowed to diffuse into the agarose layer, the moisture chamber was closed and the plates were incubated for two days at room temperature. When the formation of precipitin bands was complete the plates were:

1. rinsed for 2 days with several changes of the same buffer incorporated in the agarose layer
2. rinsed 1 day with H_2O
3. air-dried with filter paper on top
4. stained with either 1% thiazine red in 1% acetic acid or .1% Coomassie brilliant blue in 10% glacial acetic acid, 45% ethanol and 45% distilled H_2O.
5. destained with several changes of 1% acetic acid until the background was clear
6. fixed with 1% glycerol for 10 minutes
7. air-dried in a vertical position.

3. Immunofluorescent Staining

Small cubes (3-4 mm) of fresh tissue were snap frozen by immersion in liquid methane cooled by a bath of liquid nitrogen. The tissues were wrapped in foil, sealed in an airtight container and stored at -20° C until needed. Sections (6 um) were sliced at -20° C and allowed to air dry on coverslips. To standardize staining conditions, each coverslip contained a slice of each tissue to be examined.

All sections were stained using the indirect staining method as described by Groeschel-Stewart (1975). The coverslips were rehydrated for 2 minutes in PBS, fixed for 10 minutes in acetone (22° C) and air dried. In a moist chamber at room temperature, 200 ul of immune sera aDMy (.5 mg/ml), aNMy (.5 mg/ml), aDAc (1 mg/ml), aNAc (1 mg/ml) or preimmune sera (1 mg/ml) were applied to the coverslips for 30 minutes. The coverslips were then washed with six changes of PBS for a total of 30 minutes and incubated for 30 minutes with 200 ul of fluorescein conjugated goat anti-rabbit IgG (1 mg/ml). The wash steps were repeated and the coverslips were mounted in glycerol: glycine buffer pH 8.6 (7.3).

A Leitz Ortholux epifluorescence microscope equipped with a 35 mm Nikon PFM camera was used for examining and photographing the sections. A Ploem illuminator with a Xenon Lamp (HBO-150) was used as the light source. For fluorescein, the light passed through the following filters: 1.) 3 mm BG red suppression filter 2.) KP 500 dichromic mirror and barrier filter and 3.) K-530 barrier filter.

To enable some comparison among the different groups of antisera, the recording procedures were standardized according to Bagby et al (1979). All fluorescent images on each coverslip were recorded on the same roll of Tri-X film, and the prints were made using the same exposures and developing conditions. The printing exposure for a specific group of antisera was adjusted so that the density of the chicken gizzard, the control tissue in each group, was nearly the same for each group.

III. RESULTS

A. Purity of Immunizing Agents

1. Myosin

The results of the myosin purification (see 1. Myosins, p. 7, Materials and Methods) are shown in Figure 1. This figure shows a typical elution profile of the DEAE Sephadex A-50 column. The major peak, which is the myosin peak, appears as a doublet. In the past this has been shown to be caused by aggregated myosin (Richards et al, 1967). SDS PAGE of different areas of the myosin peak gave the same results. A typical gel is shown in Figure 1a. The myosin heavy chains (200,000 daltons) and the light chains (20,000 and 16,000 daltons) show the heaviest staining, while actin (42,000 daltons), tropomyosin (38,000 daltons), and a few high molecular weight contaminants (approximately 130,000 daltons) show lighter staining. Thus, the column chromatography has reduced but not eliminated the amount of actin and tropomyosin contaminants present in the 55-60% ammonium sulphate cut. However, after the addition of ATP and $MgCl_2$ and high speed centrifugation, the presence of actin and tropomyosin has been eliminated (Figure 1b). A few high molecular weight bands remain below the myosin heavy chains, which may be proteolytic breakdown products. From 1 kg of fresh tissue approximately 80 mg of pure myosin were obtained.

Figure 1. Column Purification of Chicken Gizzard Myosin on DEAE Sephadex A-50. Approximately 400 mg of crude actomyosin from the 55-60% ammonium sulphate cut were applied to a 2.5 x 90 cm column equilibrated with 40 mM sodium pyrophosphate buffer (pH 7.5). A linear KCL gradient (1 L of 40 mM sodium pyrophosphate, 1 L of .1 M KCL in 40 mM sodium pyrophosphate, both at pH 7.5) was used to elute the myosin (main peak) at a rate of 25 ml/hr. The gradient was started after the sample was washed on; an arrow indicates the starting point. Gels a-c: Sodium dodecyl gel electrophoretic analysis of myosin purification (6.5% gels):

a. column purified myosin; myosin heavy chains (MHC); actin (A); tropomyosin (T); light chains (L_1,L_2).
b. myosin after centrifugation at 38,000 x g for 3 hr.
c. chicken gizzard tropomyosin (T) standard.

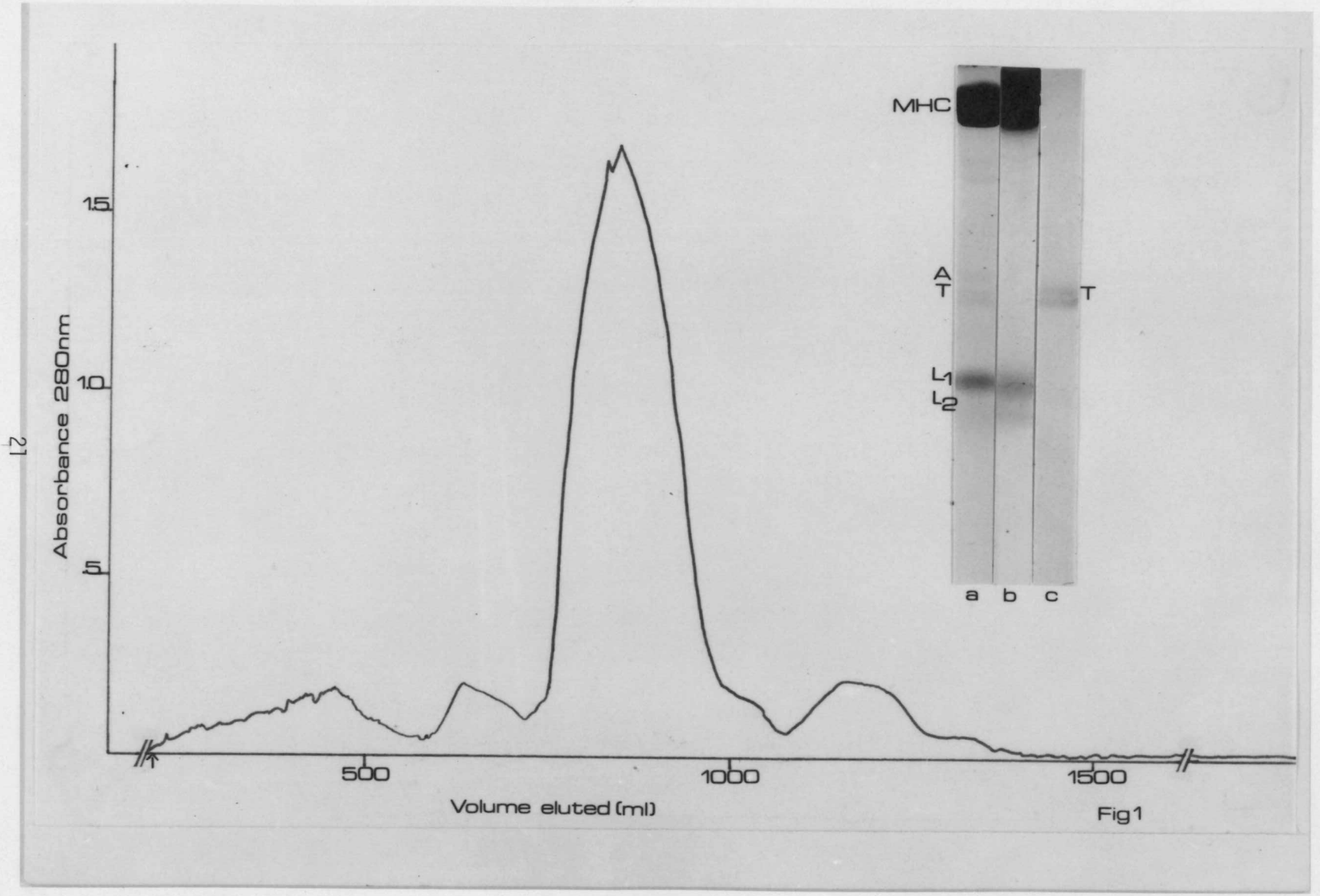

Absorbance 280nm
15
10
5
500
1000
1500
Volume eluted (ml)
MHC
A
T
T
L1
L2
a
b
c

Fig1

2. Actin

The results of the actin purification (see 2. Actins, p. 9, Materials and Methods) are illustrated in Figure 2. After repeated depolymerization and repolymerization, the actin appears homogeneous (Figure 2a). However, when the gel is overloaded (Figure 2b), a slight contaminant of approximately 74,000 daltons appears. From 20 gr of acetone powder, approximately 30 mg of pure actin were obtained.

3. Crude Extracts

SDS PAGE of the crude extracts (see 1. Preparation of Test Antigens, p. 14, Materials and Methods) shows the presence of myosin, αactinin, actin, tropomyosin as well as many other proteins (Figure 3 a-h). Since the extracts were used to detect precipitating antibodies in Ouchterlony plates, it is important to note that all the test antigens (actin, myosin, and many possible contaminants) were present in relative amounts in all the extracts.

B. Production of Precipitating Antibodies

1. Anti-myosins

Three out of three rabbits in each antigen group (DMy, NMy) produced antisera that precipitated native myosin when tested in Ouchterlony double immunodiffusion plates (not shown). Precipitating antibodies were detected 27 days after the initial boost.

Figure 2. Sodium Dodecyl Sulphate Gel Electrophoretic Analysis of Purified Actin. The following samples (gels a-c) were electrophoresed into 6.5% gels:

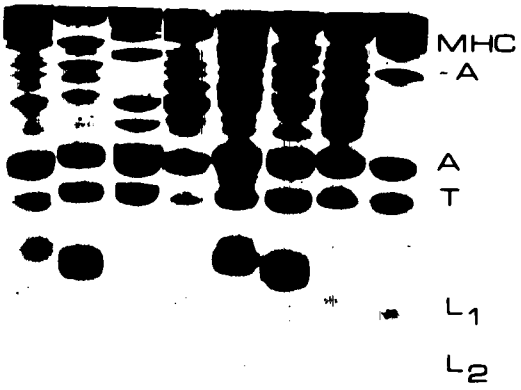
a. 25 ug of purified actin.
b. 50 ug of purified actin; note appearance of slight contaminant.
c. 15 ug chicken gizzard tropomyosin.

Figure 3. Sodium Dodecyl Sulphate Gel Electrophoretic Analysis of Crude Muscle Extracts. The following extracts (gels a-h) were electrophoresed into 10% gels:

a. chicken cardiac
b. chicken skeletal
c. chicken gizzard
d. guinea pig gut
e. pigeon gizzard
f. toad gut
g. turtle gut
h. chicken gizzard standard; myosin heavy chains (MHC); αactinin (A); actin (A); tropomyosin (T); light chains (L_1,L_2).

a b c

Fig2



a b c d e f g h

Fig3

Approximately 2.4 mg of myosin were injected into each rabbit over a period of 36 days.

2. Anti-actins

Three out of three rabbits in the DAc group and two out of three rabbits in the NAc group produced antisera that precipitated globular actin when tested in Ouchterlony double immunodiffusion plates (not shown). Precipitating antibodies were detected 50 days after the initial injection. Approximately 1.8 mg of actin were injected into each rabbit over a period of 58 days.

C. Purity of Antisera

1. Anti-myosins

When each of the six IgG fractions from both groups (aDMy, aNMy) was tested for precipitating antibodies against crude extract (CG), myosin (My), αactinin (αAc), G-actin (gAc), and tropomyosin (T), (all derived from chicken gizzard), the same results were observed. An example from each group of antisera (aDMy, aNMy) is shown in Figure 4. One precipitin band was formed between the IgG and the crude extract that fused completely with the band formed between the IgG and the purified chicken gizzard myosin (Figure 4 a,b). Precipitating antibodies against additional proteins in the crude extract or test antigens listed above were not observed (Figure 4 e,f).

Figure 4. Immunodiffusion Analysis to Test the Purity of the Antisera. The plates were run to check for precipitating antibodies against the "native" form of the homologous antigen as well as possible contaminants. Plate a-d were run in .4 M KCL, .05 M PO_4 (pH 7.4), and e-h in .012 M tris-Cl buffer in the presence of β-mercaptoethanol (pH 7.4). All antigens were run in 50% glycerol to enhance diffusion (Groeschel-Stewart, 1977). Test antigens from chicken gizzard include:

myosin - 1 mg/ml (My);
crude extract - 2 mg/ml (CG);
filamentous actin - 1 mg/ml (Ac);
αactinin - 1 mg/ml (αAc);
tropomyosin - 1 mg/ml (T);
globular actin - 1 mg/ml (gAc).

IgG fractions of the following antisera were tested:

anti-Native Myosin - 5 mg/ml (aNMy);
anti-Denatured Myosin - 5 mg/ml (aDMy);
anti-Native Actin - 10 mg/ml (aNAc);
anti-Denatured Actin - 10 mg/ml (aDAc).

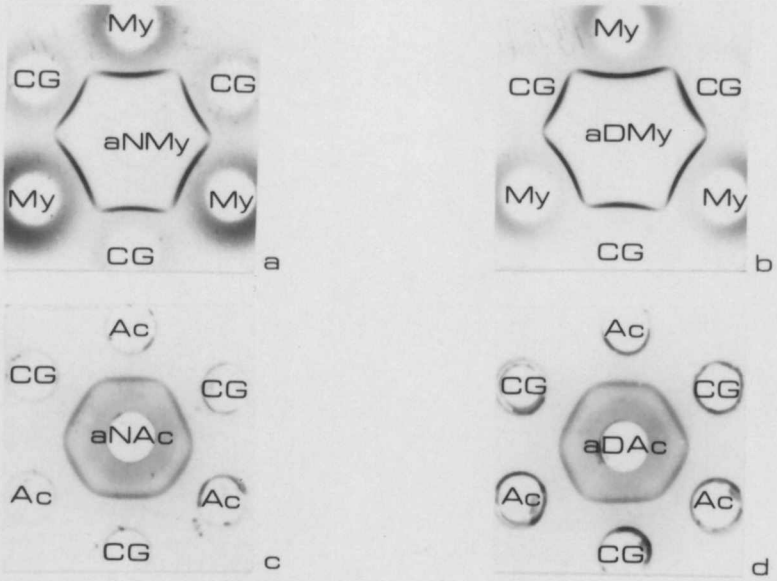

My
CG
CG
aNMy
My
My
CG
a
My
CG
CG
aDMy
My
My
CG
b
Ac
CG
CG
aNAc
Ac
Ac
CG
c
Ac
CG
CG
aDAc
Ac
Ac
CG
d
gAc
-Ac
Tr
aNMy
Tr
-Ac
gAc
e
gAc
-Ac
Tr
aDMy
Tr
-Ac
gAc
f
gAc
-Ac
Tr
aNAc
Tr
-Ac
gAc
g
gAc
-Ac
Tr
aDAc
Tr
-Ac
gAc
h

Fig4

2. Anti-actins

When each of the five IgG fractions from both groups (aDAc, aNAc) was tested for precipitating antibodies against crude extract, pure F-actin, α-actinin, G-actin, and tropomyosin (all derived from chicken gizzard), the same results were observed. An example from each group of antisera (aDAc, aNAc) is shown in Figure 4. One precipitin band was present between the IgG and the crude extract that fused completely with the band that formed between the IgG and the pure chicken gizzard F-actin (Figure 4 c,d). aDAc and aNAc also precipitated the globular form of actin (Figure 4 g,h). Precipitating antibodies against additional proteins in the crude extract or test antigens listed above were not observed (Figure 4 g,h).

Preimmune IgG fractions from each of the eleven rabbits were also tested for precipitating antibodies to the proteins listed above and gave negative results (not shown).

D. Tissue and Species Specificity

1. Ouchterlony Double Immunodiffusion

a. Anti-myosins. Both groups of antisera (aDMy, aNMy) displayed tissue specific antibodies against smooth muscle myosin. An example from each group of antisera (aDMy, aNMy) is shown in Figure 5. Precipitin bands were formed only between the IgG fractions and the "native" form of the homologous antigen;

Figure 5. Immunodiffusion Analysis to Test the Tissue (a,b,c,d) and Species Specificity (e,f,g,h) of the Antisera. All plates were run in .4 M KCL, .05 M PO_4, (pH 7.4). Test antigens include:

myosin - 1 mg/ml (My);
filamentous actin - 1 mg/ml (Ac);
and crude extracts from:
chicken gizzard - 4 mg/ml (CG);
chicken cardiac - 4 mg/ml (CC);
chicken skeletal - 4 mg/ml (CS);
pigeon gizzard - 4 mg/ml (PG);
guinea pig gut - 4 mg/ml (GPG);
toad gut - 4 mg/ml (ToG);
turtle gut - 4 mg/ml (TG).

IgG fractions of the following antisera were analyzed:

anti-Native Myosin - 5 mg/ml (aNMy);
anti-Denatured Myosin - 5 mg/ml (aDMy);
anti-Native Actin - 10 mg/ml (aNAc);
anti-Denatured Actin - 10 mg/ml (aDAc).

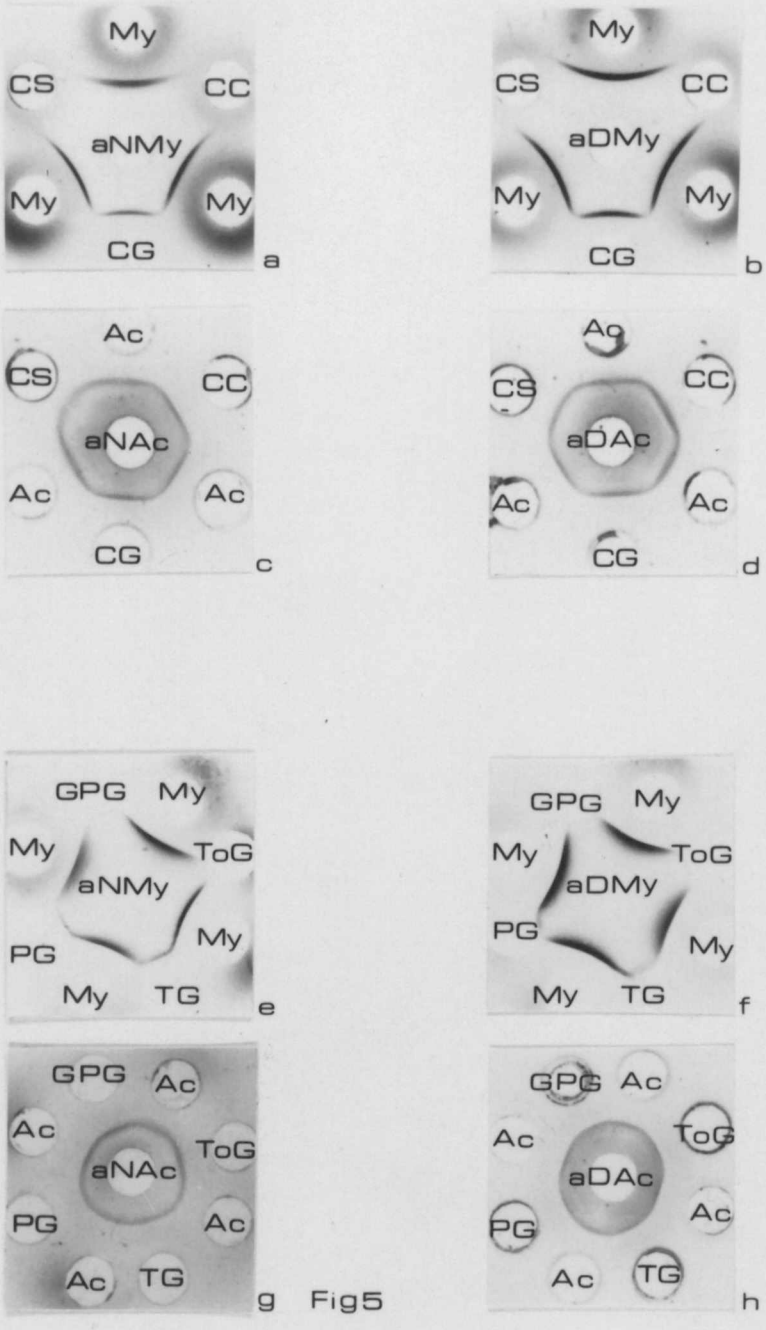

My
CS
CC
aNMy
My
My
CG
a
My
CS
CC
aDMy
My
My
CG
b
Ac
CS
CC
aNAc
Ac
Ac
CG
c
Ac
CS
CC
aDAc
Ac
Ac
CG
d
GPG
My
My
ToG
aNMy
My
PG
My
TG
e
GPG
My
My
ToG
aDMy
PG
My
My
TG
f
GPG
Ac
Ac
ToG
aNAc
PG
Ac
Ac
TG
g
GPG
Ac
Ac
ToG
aDAc
PG
Ac
Ac
TG
h

Fig5

precipitating antibodies against skeletal and cardiac myosins were not observed (Figure 5 a, b).

Some species cross-reactivity was observed; however, no difference was observed between the aDMy IgG fractions and the aNMy IgG fractions. Precipitin bands were formed between the antisera and the homologous antigen that fused with the band formed between the antisera and the pigeon gizzard and turtle gut extracts (Figure 5 e,f). Very light precipitation bands can also be seen between the antisera and the toad gut and guinea pig gut extracts (Figure 5 e,f).

b. Anti-actins. Both groups of antisera (aDAc, aNAc) were tissue and species nonspecific. An example from each group of antisera (aDAc, aNAc) is shown in Figure 5. Precipitation bands were formed between the antisera and the "native" form of the homologous antigen that fused with bands formed between the antisera and the following extracts: chicken gizzard, chicken skeletal, chicken cardiac, pigeon gizzard, turtle gut, toad gut, and guinea pig gut (Figure 5 c,d,g,h).

2. Immunofluorescent Staining

a. Anti-myosins. Immunofluorescence of the test antigens (Figure 6) reinforce the results shown previously with double immunodiffusion analysis. There was no appreciable difference in the ability of either group or antisera (aDMy, aNMy) to react with the fixed form of antigens tested.

Figure 6. Indirect Immunofluorescent Staining of Fixed Frozen Sections with Anti-myosins. Test antigens include:

chicken gizzard (CG);
chicken cardiac (CC);
chicken skeletal (CS);
pigeon gizzard (PG);
turtle gut (TG);
toad gut (ToG);
guinea pig gut (GPG).

IgG fractions of the following antisera were analyzed:

anti-Native Myosin - .5 mg/ml (aNMy);
anti-Denatured Myosin - .5 mg/ml (aDMy);
preimmune serum - 1 mg/ml (Pr).

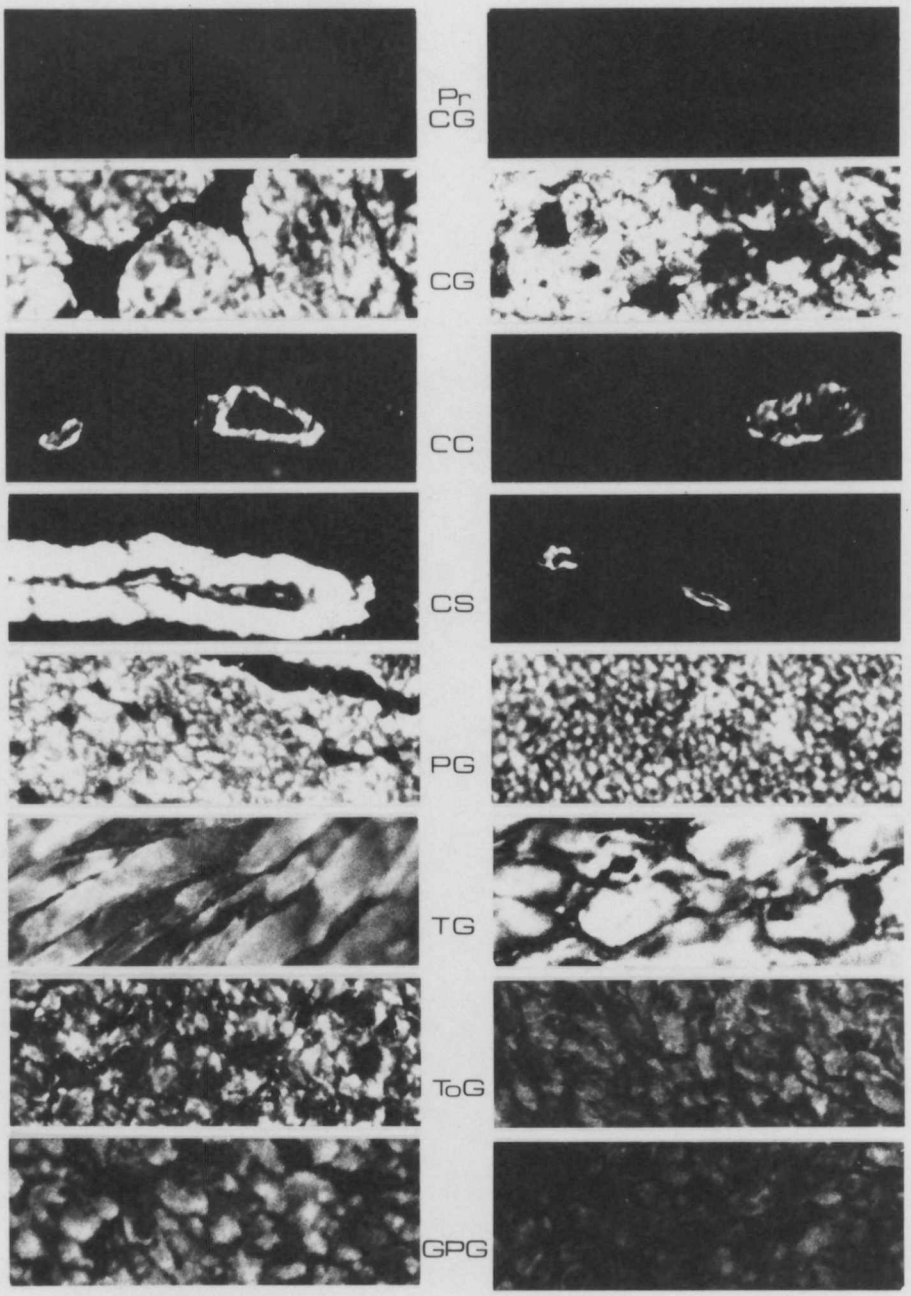


Fig 6

The antisera (aDMy, aNMy) were tissue specific for smooth muscle as they only stained the blood vessels of the cardiac and skeletal tissue (Figure 6 CC and CS). Both groups of antisera were species nonspecific as they stained sections of pigeon gizzard, turtle gut, toad gut, and guinea pig gut (Figure 6 PG, TG, ToG, and GPG); however, the staining of pigeon gizzard and turtle gut was much greater than that of toad gut and guinea pig gut.

The preimmune IgG fractions did not stain the chicken gizzard (PrCG), attesting to the absence of autoantibodies and nonspecific staining.

b. Anti-actins. Once again, the results obtained from the immunofluorescent staining match the precipitation results. Both groups of antisera (aDAc, aNAc) displayed tissue and species nonspecificity as they stained chicken gizzard, chicken skeletal, pigeon gizzard, turtle gut, toad gut, and guinea pig gut tissues (Figure 7 CG, CS, PG, TG, ToG, GPG).

The preimmune IgG did not stain the chicken gizzard (PrCG); however, some nonspecific staining of connective tissue can be seen in the cross sections of the heart and transverse sections of the skeletal tissue.

Figure 7. Indirect Immunofluorescent Staining of Fixed Frozen Sections with Anti-actins. Test antigens include:

chicken gizzard (CG);
chicken cardiac (CC);
chicken skeletal (CS);
pigeon gizzard (PG);
turtle gut (TG);
toad gut (ToG);
guinea pig gut (GPG).

IgG fractions of the following antisera were analyzed:

anti-Native Actin - 1 mg/ml (aNAc);
anti-Denatured Actin - 1 mg/ml (aDAc);
preimmune serum - 1 mg/ml (Pr).

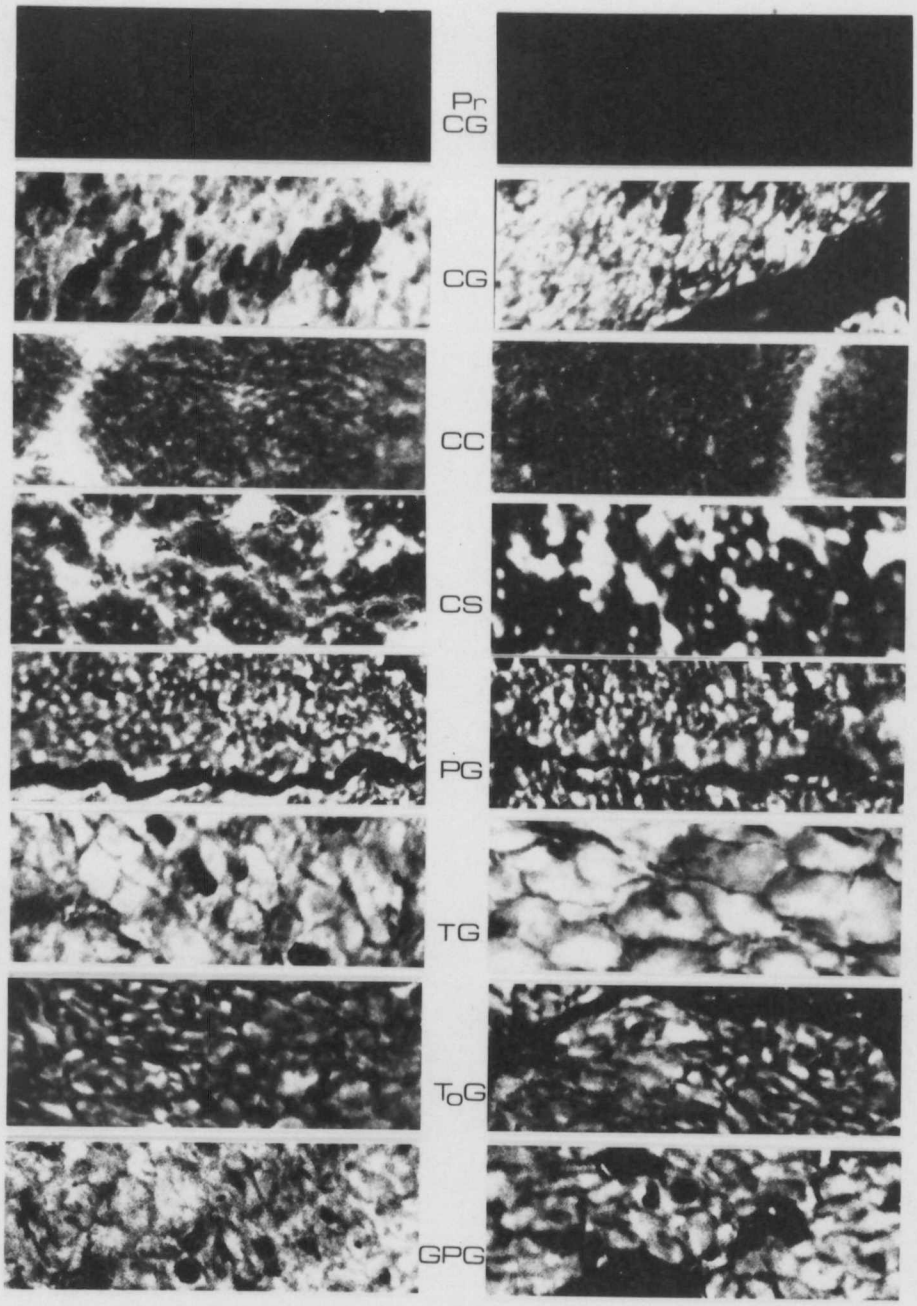


Fig 7

IV. DISCUSSION

A. Purity of Immunizing Agents

Since the purified myosin and actin showed trace contaminants when overloaded onto SDS gels, short immunization schedules with small amounts of the respective proteins were employed to minimize the production of antibodies against these contaminants. Immunodiffusion of the IgG fractions (aDMy, aNMy, aNAc, aDAc) against crude chicken gizzard extract (CG) showed only one precipitin band that fused with the band formed against the "native" form of the homologous antigen (NMy, NAc). The absence of precipitating antibodies against other purified proteins from smooth muscle such as αactinin and tropomyosin also argues for the purity of the antisera.

B. Antigenicity

Since early attempts to produce antibodies against "native" actin failed (Wilson and Finck, 1971) but injections of denatured actin led to detectable antibody production (Herman and Pollard, 1979; Jockusch et al, 1978; Hirabayashi and Hayashi, 1972: Trenchev and Holborrow, 1976; Groeschel-Stewart, 1977; Lazarides, 1974), it could be postulated that denaturation of actin is a requirement for antigenicity. However, in this study two out of three rabbits injected with small amounts of "native" actin (1.2 mg/rabbit) produced antisera that precipitated actin in Ouchterlony double immunodiffusion plates. Thus, these results show that "native" actin can also

be antigenic. In addition, the actin purification procedures used in this study were the same as those followed by others who found poor results when attempting to elicit antisera to "native" actin (Groeschel-Stewart, 1977). However, the total amount of "native" actin injected per rabbit in this study was less than that injected by others (Wilson and Finck, 1971; Groeschel-Stewart, 1977). Thus, it could be speculated that large doses of the "native" antigen are inducing a state of tolerance in the immunized animal. To prove this, however, increasing amounts of actin would have to be injected over a period of time during which the antibody titer should be monitored closely and assayed quantitatively.

Smooth muscle myosin, on the other hand, was immunogenic when injected in the "native" state (Groeschel-Stewart, 1975). In this study, there was no detectable difference in the ability of antisera produced against DMy and NMy to precipitate the "native" form of the homologous antigen in Ouchterlony plates or to stain the "fixed" form of this antigen in immunofluorescence. This, however, does not signify that there is no difference in the specificities of antibody molecules produced against the two forms of myosin. For Elfvin et al (1979) have found that antibodies against "native" myosin will not cross-react with the SDS denatured proteolytic S-1 subfragment of the molecule. Thus, some of the conformational determinants located in the globular portion of the molecule are destroyed by denaturation. This study, however, shows that there are some sequential antigenic determinants located on different portions of the entire mole-

cule which are not destroyed by SDS denaturation. Antisera produced against these determinants are thus able to cross-react with the native molecule.

C. Tissue and Species Specificity

The antisera produced against NAc and DAc were equally tissue and species nonspecific in their abilities to precipitate "native" forms of the antigens in Ouchterlony plates and stain "fixed" forms of the antigens in indirect immunofluorescence. However, it is interesting that a fused precipitation band was observed among all the different actins, indicating a minimum of one antigen antibody system in common; for Vanderchove and Weber (1978) have shown by analyzing the amino acid sequences of N terminal tryptic peptide that there are at least six different actins in one vertebrate species. However, there are similarities in these peptides. It would be interesting to produce antibodies against different tryptic peptides and investigate which antigenic determinants are responsible for the cross-reactivity between these actins.

The antisera produced against DMy and NMy were also equally tissue specific for smooth muscle, but species nonspecific in immunofluorescence and immunoprecipitation tests. However, the avian and reptilian tissues appeared to cross-react more than the amphibian and mammalian tissues by displaying brighter staining in immunofluorescence and heavier fused band formation in immunoprecipitation. This might indicate some type of phylogenetic relationship between

the myosins. However, these differences could be due to greater amounts of protein in the reptilian and avian tissues. Since the exact myosin content of these different tissues is not known and was not quantitated, more quantitative tests would have to be employed to prove this relationship.

D. Biological Significance

Antigenic determinants can be determined by the intrinsic amino acid sequence (sequential determinants) or by conformationally induced juxtapositions of nonsequential amino acids (conformational determinants). Conformational determinants have been shown to participate in the binding of anti-native myosin to the globular S-1 fragment of the myosin molecule (Elfvin, 1979).

Since SDS denatured proteins assume some type of linear form (Payne, 1974), antibodies produced against these proteins are probably directed against sequential determinants. These studies show that antisera produced against SDS denatured actin and myosin are equally species nonspecific as antisera produced against the native forms of these antigens. Thus, some of the determinants governing cross-reactivity must be sequential. This is valuable information since many proteins used in immunochemical research are purified by Preparative SDS PAGE.

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VITA

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