

SPECIES IDENTIFICATION OF FRESH MEAT, HEAT-TREATED MEAT AND  
INTERNAL ORGANS USING ANTISERA TO THERMOSTABLE ANTIGENS

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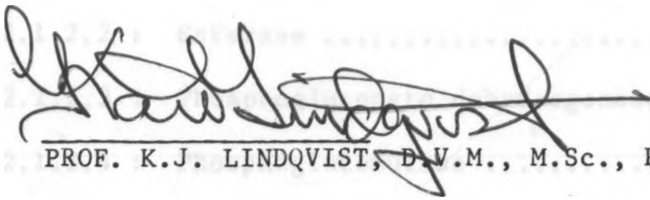
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(iii)  
TABLE OF CONTENTS

	<u>Page</u>
LIST OF FIGURES .....	ix
LIST OF TABLES .....	xiv
ACKNOWLEDGEMENTS .....	xvi
DEDICATION .....	xviii
ABSTRACT .....	xix
1: INTRODUCTION .....	1
2: LITERATURE REVIEW .....	6
2.1 : Raw meats .....	7
✓2.1.1 : Electrophoretic methods .....	7
2.1.2 : Isoenzymes .....	9
2.1.2.1 : Lactate dehydrogenase .....	9
2.1.2.2 : Esterase .....	10
2.1.2.3 : Phosphogluconate dehydrogenase .....	10
2.1.2.4 : Phosphoglucomutase .....	11
2.1.2.5 : Adenylate kinase .....	11
2.1.3 : Serological methods .....	11

TABLE OF CONTENTS

	<u>Page</u>
2.2 : Cooked meats .....	16
2.2.1 : Isoenzyme analyses .....	16
2.2.1.1 : Adenylate kinase .....	17
2.2.1.2 : Creatine kinase .....	17
2.2.2 : Histidine dipeptides .....	18
2.2.3 : Fats .....	19
2.2.4 : Serological methods .....	20
3 : MATERIALS AND METHODS .....	23
3.1 : Antigen extraction procedure .....	24
3.1.1 : Fresh meat antigens .....	24
3.1.2 : Cooked meat antigens .....	25
3.1.3 : Thermostable muscle antigens .....	25
3.1.4 : Thermostable organ antigens .....	28
3.2 : Production of antisera .....	28
3.2.1 : Production of antisera to TMA .....	28
3.2.2 : Production of rabbit antiserum to goat IgG .....	29
3.3 : Absorption of the antisera .....	29
3.3.1 : Absorption of goat and rabbit antisera to TMA for use in immunodiffusion tests .....	29

TABLE OF CONTENTS

	<u>Page</u>
3.3.2 : Absorption of goat antisera to TMA for use in the enzyme immunoassay ...	32
3.3.3 : Regeneration of immunosorbents .....	32
3.4 : Immunodiffusion tests .....	33
3.5 : Enzyme immunoassay .....	34
3.5.1 : Preparation of partially purified thermostable muscle antigens (PTMA) .	34
3.5.2 : Determination of the protein content of the PTMA .....	34
3.5.3 : Preparation of rabbit anti-goat IgG glucose oxidase conjugate .....	36
3.5.4 : Choice of coating buffers .....	37
3.5.5 : Optimisation of PTMA concentration for use in enzyme immunoassay .....	37
3.5.6 : Determination of optimal antiserum and conjugate dilutions .....	38
3.5.7 : Assay for hydroxyproline .....	38
3.5.8 : The effect of gelatin on the adsorption of PTMA to microtitre plates .....	39
3.5.9 : Enzyme immunoassay for species identification of PTMA .....	40

TABLE OF CONTENTS

	<u>Page</u>
3.5.10 : Detection of the species of origin of heated meats in known mixtures ...	41
3.6 : Molecular weight determination of the PTMA by gel filtration .....	41
3.7 : Detection of beef and pork in pork and beef meat products .....	43
3.7.1 : Extraction of antigens from meat products .....	43
3.7.2 : Preparation of pork and beef sausages and pork sausages containing various percentages of beef .....	43
3.7.3 : Effect of milk powder on the detection of beef in pork products ..	45
3.7.4 : Enzyme immunoassay for the detection of beef and pork in beef and pork products .....	45
3.8 : Analysis of enzyme immunoassay data .	45
4 : RESULTS AND DISCUSSION .....	46
4.1 : Immunodiffusion tests .....	47
4.1.1 : Species identification of TMA .....	47
4.1.2 : Species identification of CMA .....	61

TABLE OF CONTENTS

	<u>Page</u>
4.1.3 : Species identification of FMA .....	70
4.1.4 : Species identification of organs ....	72
4.2 : Enzyme immunoassay .....	79
4.2.1 : Purification of TMA .....	79
4.2.2 : Effect of coating buffer on the adsorption of PTMA to microtitre plates .....	82
4.2.3 : Optimal dilutions of PTMA, antiserum and conjugate for use in enzyme immunoassay .....	82
4.2.4 : Effect of gelatin on the adsorption of the antigen to microtitre plates .	82
4.2.5 : Differentiation of PTMA using enzyme immunoassay .....	87
4.2.6 : Determination of the molecular weight of the PTMA using gel filtration ....	90
4.3 : Use of enzyme immunoassay for species identification of meat in meat products .....	93
4.3.1 : Detection of beef and pork in pork and beef products .....	93
4.3.2 : Effect of milk powder on the detection of beef in pork products .....	96

TABLE OF CONTENTS

	<u>Page</u>
5 : CONCLUSIONS .....	99
6 : REFERENCES .....	102
APPENDIX .....	127
APPENDIX I .....	
APPENDIX II .....	
APPENDIX III .....	
APPENDIX IV .....	
APPENDIX V .....	
APPENDIX VI .....	
APPENDIX VII .....	
APPENDIX VIII .....	
APPENDIX IX .....	
APPENDIX X .....	
APPENDIX XI .....	
APPENDIX XII .....	
APPENDIX XIII .....	
APPENDIX XIV .....	
APPENDIX XV .....	
APPENDIX XVI .....	
APPENDIX XVII .....	
APPENDIX XVIII .....	
APPENDIX XIX .....	
APPENDIX XX .....	
APPENDIX XXI .....	
APPENDIX XXII .....	
APPENDIX XXIII .....	
APPENDIX XXIV .....	
APPENDIX XXV .....	
APPENDIX XXVI .....	
APPENDIX XXVII .....	
APPENDIX XXVIII .....	
APPENDIX XXIX .....	
APPENDIX XXX .....	



LIST OF FIGURES

	<u>Page</u>
Fig. 1a : A simplified chart of the zoological relationship of the bovidae animals studied .....	26A
Fig. 1b : Flow chart of the zoological relationship of the non-bovidae animals studied .....	26B
Fig. 1c : Flow chart of the zoological relationship of the non-bovidae animals studied .....	26C
Fig. 2 : Flow chart showing the extraction of FMA, CMA and TMA .....	27
Fig. 3 : Flow chart showing the extraction of PTMA .	35
Fig. 4 : Results of immunodiffusion tests using unabsorbed goat and rabbit antisera to TMA of kongoni .....	50
Fig. 5 : Results of immunodiffusion tests using goat antiserum to TMA of cattle absorbed with copolymerized serum proteins and tested with TMA of various species .....	55
Fig. 6 : Results of immunodiffusion tests using goat antiserum to TMA of cattle absorbed with copolymerized serum proteins and tested with TMA of various species .....	56

LIST OF FIGURES

	<u>Page</u>
Fig. 7 : Results of immunodiffusion tests using goat antiserum to TMA of buffalo absorbed with copolymerized serum proteins and tested with TMA of various species .....	57
Fig. 8 : Results of immunodiffusion tests using goat antiserum to TMA of buffalo absorbed with copolymerized serum proteins and tested with TMA of various species .....	58
Fig. 9 : Results of immunodiffusion tests using goat antiserum to TMA of bushpig absorbed with copolymerized serum proteins and tested with TMA of various species .....	59
Fig. 10 : Results of immunodiffusion tests using goat antiserum to TMA of bushpig absorbed with copolymerized serum proteins and tested with TMA of various species .....	60
Fig. 11 : Results of immunodiffusion tests using goat antiserum to TMA of topi absorbed with copolymerized serum proteins and tested with CMA of various species .....	64

(x1)

LIST OF FIGURES

	<u>Page</u>
Fig. 12 : Results of immunodiffusion tests using goat antiserum to TMA of topi absorbed with copolymerized serum proteins and tested with CMA of various species .....	65
Fig. 13 : Results of immunodiffusion tests using goat antiserum to TMA of warthog absorbed with copolymerized serum proteins and tested with CMA of various species .....	66
Fig. 14 : Results of immunodiffusion tests using goat antiserum to TMA of warthog absorbed with copolymerized serum proteins and tested with CMA of various species .....	67
Fig. 15 : Results of immunodiffusion tests using goat antiserum to TMA of horse absorbed with copolymerized serum proteins and tested with CMA of various species .....	68
Fig. 16 : Results of immunodiffusion tests using goat antiserum to TMA of horse absorbed with copolymerized serum proteins and tested with CMA of various species .....	69

LIST OF FIGURES

	<u>Page</u>
Fig. 17 : Results of immunodiffusion tests using goat antiserum to TMA of Thomson's gazelle absorbed with copolymerized serum proteins and tested with FMA of various species ...	73
Fig. 18 : Results of immunodiffusion tests using goat antiserum to TMA of Thomson's gazelle absorbed with copolymerized serum proteins and tested with FMA of various species ...	74
Fig. 19 : Results of immunodiffusion tests using goat antiserum to TMA of bushpig absorbed with copolymerized serum proteins and tested with FMA of various species .....	75
Fig. 20 : Results of immunodiffusion tests using goat antiserum to TMA of bushpig absorbed with copolymerized serum proteins and tested with FMA of various species .....	76
Fig. 21 : Results of immunodiffusion tests using goat antiserum to TMA of cattle absorbed with copolymerized serum proteins and tested with TOA of cattle .....	78

LIST OF FIGURES

	<u>Page</u>
Fig. 22 : Fractionation of buffalo TMA on Sephadex G-200 column .....	80
Fig. 23 : Fractionation of buffalo TMA on Sephadex G-75 column .....	81
Fig. 24 : Effect of three buffers on the adsorption of the PTMA to microtitre plates .....	83
Fig. 25 : The inhibitory effect of gelatin on the adsorption of PTMA to microtitre plates .....	86
Fig. 26 : Elution profile from Sephadex G-200 of buffalo PTMA and molecular weight markers .....	91
Fig. 27 : Standard curve for the molecular weight determination using Sephadex G-200 .....	92
Fig. 28 : Detection by EIA of beef in experimentally prepared pork sausages using goat antiserum to TMA of cattle absorbed with copolymerized serum proteins .....	97

LIST OF TABLES

	<u>Page</u>
Table 1 : Experimental animals used for the production of antisera to TMA .....	30
Table 2 : Recipe for the preparation of beef and pork sausages .....	44
Table 3 : Results of immunodiffusion tests using unabsorbed rabbit antisera to TMA and TMA of other species .....	48
Table 4 : Results of immunodiffusion tests using unabsorbed goat antisera to TMA and TMA of various species .....	49
Table 5 : Results of immunodiffusion tests using rabbit antisera to TMA absorbed with copolymerized serum proteins and tested with TMA of various species .....	52
Table 6 : Results of immunodiffusion tests using goat antisera to TMA absorbed with copolymerized serum proteins and tested with TMA of various species .....	53
Table 7 : Results of immunodiffusion tests using absorbed rabbit antisera to TMA which could be used to distinguish meat from bovidae and non-bovidae .....	62

LIST OF TABLES

	<u>Page</u>
Table 8 : Results of immunodiffusion tests using goat antisera to TMA absorbed with copolymerized serum proteins and tested with CMA of various species .....	63
Table 9 : Results of immunodiffusion tests using goat antisera to TMA absorbed with copolymerized serum proteins and tested with FMA of various species .....	71
Table 10 : Species identification of internal organs using goat antisera to TMA absorbed with copolymerized serum proteins and tested with TOA in immunodiffusion tests .....	77
Table 11 : Optimal dilutions of PTMA, antiserum and conjugate for use in EIA .....	84
Table 12 : Species differentiation of PTMA in enzyme immunoassay .....	88
Table 13 : Results of EIA for the detection of beef and pork meat in commercial pork and beef products .....	94
Table 14 : Results of EIA, showing detection of beef and pork in meat products from two factories .....	95

ACKNOWLEDGEMENTS

I am very grateful to my supervisors, Professors K.J. Lindqvist and J.M. Gathuma for their guidance, criticism and useful suggestions.

I wish to thank Prof. L. Karstad and Dr. G. Muchemi, formerly of Wildlife Section, Veterinary Laboratories, Kabete for their assistance in collecting the wildlife meat samples. I am also grateful to the Director, Department of Wildlife Conservation and Management, Ministry of Tourism and Wildlife, for allowing me to collect samples from the wild animals used in this study.

My thanks also go to the Chairmen, Departments of Crop Science and Food Science and Technology, Faculty of Agriculture, for allowing me to use their facilities.

I also wish to sincerely thank all the technical staff of the Department of Public Health, Pharmacology and Toxicology for their assistance throughout this study.

I gratefully express my appreciation to my wife, Loise and children Grace, Stephen and Nicholas for their love and support.

I am grateful to Miss Marion Kanyugo for her excellent work so carefully and efficiently done in typing this thesis.



(xvii)

I wish to record my appreciation and gratitude to the Norwegian Agency for International Development (NORAD) for the financial support I received through the Research Support Grant of Professor K.J. Lindqvist and the NORAD KEN 046 project. My appreciation and gratitude also go to the Kenya National Council for Science and Technology for their financial support through the council grant, NCST/SEC/4400-419.

Dedicated to my parents

ABSTRACT

SPECIES IDENTIFICATION OF FRESH MEAT, HEAT-TREATED MEAT AND  
INTERNAL ORGANS USING ANTISERA TO THERMOSTABLE ANTIGENS

Unequivocal identification of fresh meat and heat-treated meat is of special concern to food analysts and game conservation authorities. The food analyst has to provide proof of fraudulent substitution of more expensive meats with cheaper ones. Such adulteration represents unfair trading practices at either international or national level and is objectionable for reasons of culture, health or religion. The game conservation authorities need to differentiate meat of protected species from that of unprotected and domestic animals in their endeavour to curb poaching and protect endangered wildlife species.

It is imperative, therefore, that reliable analytical methods be developed to detect and quantify the adulteration in order to implement legislative measures necessary to achieve these objectives.

In this study, species identification of fresh meat and heat-treated meat was investigated using antisera to thermostable muscle antigens (TMA) obtained from the meat of 13 wild and 7 domestic animal species.

(xx)

Antisera to TMA were raised in rabbits and goats.

Unabsorbed rabbit antisera showed a wider range of cross-reactions than goat antisera. In immunodiffusion tests, rabbit and goat antisera showed two precipitin lines with homologous TMA and reacted also with serum, giving a reaction of identity with one of the two precipitin lines given by TMA of the same species. Thermostable muscle antigens of other species reacted with unabsorbed antisera to TMA, giving a reaction of identity with the precipitin line given by serum. This shows that serum contains a component of TMA which is identical or similar to one present in TMA of several species.

Absorption of rabbit antisera to TMA with copolymerized serum proteins rendered the rabbit antisera to TMA of oryx and camel species-specific, while the other antisera produced in rabbits retained cross-reactivities with TMA of several species. It was concluded that extracts of TMA contain related or similar antigenic components common to several animal species, especially within the bovidae, as shown by the wide range of reactivities of antisera produced in rabbits. It was virtually impossible to render these antisera species-specific by absorptions.

Although rabbit antisera to TMA lacked sufficient specificity to differentiate TMA of closely related species,

the antisera could be used to speciate TMA from distantly related species. In addition, antisera raised in rabbits to TMA of closely related species can be used to screen for the presence or absence of meat from bovidae and non-bovidae species. Rabbit antisera to TMA of topi, warthog and Grant's gazelle were proved to be useful in this regard.

The same absorption procedure rendered antisera produced in goats specific for all species, except the antisera to TMA of kongoni, topi, Thomson's gazelle and Grant's gazelle. Goat antiserum to TMA of kongoni reacted with the TMA of topi and wildebeest, while goat antiserum to TMA of topi reacted only with TMA of kongoni. Goat antisera to TMA of Thomson's gazelle and Grant's gazelle reacted with the TMA of both species. These results demonstrate the importance of selecting the animal species in which the antiserum is to be raised, in order to reduce the cross-reactions with antigens from closely related species.

Goat antisera also identified the species of origin of fresh meat using fresh meat antigens (FMA), and cooked meat using cooked meat antigens (CMA) in immunodiffusion tests. However, goat antiserum to TMA of cattle reacted with CMA and FMA of buffalo, thus failing to differentiate them from those of cattle. Cattle CMA and FMA could be differentiated from those of buffalo by using goat antiserum to TMA of buffalo.

The CMA and FMA of Thomson's gazelle and Grant's gazelle, - topi and kongoni, - pig and bushpig, - were not easily distinguishable using goat antisera to TMA of these species in immunodiffusion tests. While antisera to TMA produced in rabbits possessed limited capability to distinguish between species, goat antisera to TMA were capable either singly or in combination to identify all the FMA, CMA and TMA from the twenty animal species investigated in this study.

The results of immunodiffusion tests using TMA, CMA and FMA of a single species and goat antiserum to TMA, suggest that TMA are naturally occurring antigens of muscle tissue and are not novel antigens arising from heat treatment.

Identification of the species of origin of internal organs (liver, kidney and heart) was easily achieved using thermostable organ antigens (TOA) and goat antisera to TMA in immunodiffusion tests. The reactions of TMA, TOA with goat antisera to TMA, revealed that there exist (i) a species-specific thermostable antigen common to muscles and organs, (ii) a species and striated muscle-specific antigen, found in skeletal and cardiac muscles and (iii) a species and tissue-specific antigen restricted to skeletal muscles.

Hydroxyproline was demonstrated in the TMA extracts, indicating the presence of gelatin in these preparations. Other protein contaminants were also shown to be present in

the TMA extracts. These contaminants inhibited the adsorption of the TMA to microtitre plates. The inhibitory effect of gelatin was demonstrated using commercial gelatin. Five milligrams of gelatin per millilitre resulted in complete inhibition of the adsorption of partially purified TMA (PTMA). Gelatin and the other contaminants are, therefore, undesirable in the enzyme immunoassay (EIA). A modification of the extraction procedure incorporating a partial purification step was adopted and yielded PTMA suitable for use in EIA.

The molecular weight of the PTMA, as determined using buffalo PTMA in gel filtration experiments, was calculated to be 210 kd.

In spite of the apparent high specificity of goat antisera in immunodiffusion tests, several antisera showed cross-reactions with PTMA of several species in quantitative EIA. However, goat antisera to TMA of buffalo, topi, pig, camel and horse were shown to be specific for these species. Nine other antisera showed various degrees of cross-reactions with 2 to 10 heterologous PTMA. Some of these reactions were nearly equal to those given by the homologous systems, thus failing to differentiate homologous from heterologous PTMA.

Goat antisera to TMA of cattle and eland cross-reacted with 13 and 15 heterologous PTMA respectively, giving reactions which did not allow unequivocal differentiation of

the homologous from the heterologous PTMA. In addition, the goat antiserum to TMA of cattle gave only weak reactions with PTMA of topi, pig, horse and Thomson's gazelle, which enabled differentiation between the homologous and these heterologous PTMA at  $P \leq 0.05$ . Similarly, goat antiserum to TMA of eland gave weak reactions with PTMA of horse and cattle which allowed differentiation between the PTMA of eland and those of cattle and horse at  $P \leq 0.05$ .

In instances where a single antiserum failed to distinguish between homologous and heterologous PTMA, species identification could be achieved in enzyme immunoassay by employing a number of goat antisera to TMA of several species. Goat antisera to TMA provided reagents that were capable of correctly identifying the PTMA of eighteen species examined.

Because of their exquisite specificity, monoclonal antibodies to TMA would be more suitable for species identification than the polyclonal antisera used in this study. However, it is conceivable that immunization of additional animals of different species, especially within the bovidae, would yield polyclonal antisera with sufficient specificity for use in enzyme immunoassays.

Indirect EIA was also used to detect the presence of beef in laboratory prepared beef/pork sausages. The test detected beef at 5% level in pork sausages.



Milk powder commonly used as a binder in meat products did not give false positive reactions for beef in EIA, using goat antisera to TMA. Antisera to TMA can therefore be used to detect adulterants in fresh and heat-treated meat products containing milk proteins.

Detection of beef and pork in 90 commercially prepared beef and pork products from two factories were analysed using EIA. Fifty per cent of the pork products from both factories were found to contain beef, while 78.6% of the beef products from factory A and 6.3% from factory B, were found to contain pork. This may not be regarded as adulteration since specifications by the Kenya Bureau of Standards for fresh and heat-treated beef and pork products allow inclusion of flesh from other species, so long as the stipulated content of either beef or pork is maintained in the products. The specifications of the Kenya Bureau of Standards also state that the actual ingredients used in the manufacture of any food product must be shown on the attached label.

This study has shown that antisera to TMA produced in selected animal species possessed sufficient specificity to allow species identification of meat which had been subjected to heat-treatment which destroys the more abundant and easily identifiable heat-labile species-specific antigens. The same antisera also made possible the determination of species of origin of fresh meat as well as of internal organs such as kidney, liver and heart.

The Commission of the European Communities  
has the honor to acknowledge the receipt of your letter  
of the 15th of July 1964, in which you inform it  
that you have decided to withdraw your application  
for the award of a grant under the Community  
scheme for the promotion of research and development  
work in the field of the environment. The Commission  
has no objection to your decision and will therefore  
cancel the grant which has been allocated to you.

I am sure that you will be satisfied with the  
arrangements which have been made for the  
return of the grant which has been allocated to you.  
I am, Sir, very respectfully,  
Yours faithfully,  
The Commission of the European Communities

**I. INTRODUCTION**

The Commission of the European Communities  
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Meat is the edible flesh of animals used as food after the completion of a series of biochemical and biophysical changes initiated in the muscles after the death of the animal. Animal meat is composed of muscle fibres held together by connective tissue through which blood vessels, nerves and fat cells are distributed. Proteins are the most abundant substances in animal tissues and are, without doubt, the most important constituents of meat.

Muscle proteins can be divided broadly into those which are soluble in water or dilute salt solutions (the sarcoplasmic proteins), those which are soluble in concentrated salt solutions (the myofibrillar proteins) and those which are insoluble in the latter, at least at low temperatures (proteins of the connective tissue and other formed substances) (Lawrie, 1979).

Muscles of all animal species contain similar proteins which perform similar biochemical functions. Interspecies differences in muscle composition are very small and differentiation of meat from different species using visual, histological and empirical chemical analysis is therefore almost impossible. Identification of the species of origin of fresh meat, therefore, presents a challenging task to the food analyst and the game conservation authorities whose duties involve prevention of poaching and the protection of endangered species.

A recent trend in the international meat trade is the selling of deboned, frozen and boxed meat. Under these circumstances inclusion of meat from species other than those specified on the label is not uncommon. Recently, there have been reports of substitution of beef with horse meat in the United Kingdom (Anon, 1982), and beef with kangaroo in Australian beef sold to United States (Whittaker et al., 1982). The food analyst is called upon to provide proof that such substitution has occurred and to quantify the level of adulteration. It is therefore, necessary that reliable analytical methods be established to identify the species of origin of meat in the interest of fair trading, either at the international level or simply for the protection of consumers' rights.

Kenya is endowed with a large and varied population of indigenous wild mammals most of which are herbivores. Poaching and substitution of domestic animal meat with wild animal meat is possible. The wildlife conservation authorities are faced with the task of differentiating meat of protected species from that of unprotected and domestic species. Identification of the species of origin of meat is of considerable importance in veterinary forensic medicine involving meats and regulatory control of animal products.

Identification of species of origin of heat-treated meats is a more difficult task because heating denatures most of

the species-specific epitopes in fresh meat. Heating meat at 45°C, results in denaturation of myofibrillar and sarcoplasmic proteins (Lawrie, 1979), while at 65°C the collagen, the abundant protein of connective tissue, forms a gel and dissolves in the meat juices (Bendall, 1946).

Identification of the species of origin of heat-treated meat and meat products depends on the identification of thermostable muscle proteins that withstand the processing temperature, yet retaining their antigenicity and species specificity. It is also conceivable that the heating of meat may result in the emergence of new thermostable antigens with species-specific epitopes for only heated meats. The existence of thermostable antigens would be of great importance in the identification of the species of origin of cooked or heat-treated meat, meat products as well as fresh meat.

The objectives of this project were:

- (i) to demonstrate the existence of species-specific thermostable muscle antigens and use antisera to these antigens in identifying the species of origin of autoclaved, cooked and fresh meat.
- (ii) to identify the species of origin of internal organs using antisera to thermostable antigens.

(iii) to develop an enzyme immunoassay procedure suitable for use in identifying the species of origin of autoclaved meats.

(iv) to evaluate the usefulness of the antisera to thermostable muscle antigens in identifying the species of origin of meat used in commercially prepared fresh and heat-treated meat products.

2. L I T E R A T U R E   R E V I E W

Identification of the species of origin of meats has been accomplished using electrophoresis (Nakkila and Linko, 1955; Scopes, 1968; Kaiser et al., 1980), isoenzyme analyses of electrophoresed muscle extracts (Dilworth and McKenzie, 1970; Slattery and Sinclair, 1983) and serological methods (Pinto, 1961; Kamiyama et al., 1978b; Kang'ethe et al., 1982).

## 2.1 RAW MEAT

### 2.1.1 Electrophoretic methods

The use of electrophoretic techniques for identification of muscle proteins depends mainly upon differences in their electric charge and consequently different migration rates in an electric field. These differences show up as, (i) the number of protein bands formed indicating differences in their physical properties as evidenced by different electric mobilities; and in (ii) the staining intensity of the respective bands denoting differences in their concentration.

Various electrophoretic techniques have been used for identifying the species of origin of raw meats. These include paper electrophoresis (Nakkila and Linko, 1955); starch-gel electrophoresis (Latner and Angel, 1957; Scopes, 1968), polyacrylamide gel electrophoresis (Cowie, 1968; Ebermann and Barna, 1972; Kaiser et al., 1980) and isoelectric focusing (Mackie, 1979; Verbeke, 1983).



Several researchers have shown that identification of the origin of raw meat can be achieved by the analysis of the electrophoretic patterns of serum proteins (Ashton, 1957; Latner and Angel, 1957), sarcoplasmic proteins (Conell, 1953; Nakkila and Linko, 1955; Giles, 1962; Scopes, 1968; Choi et al., 1970; Ebermann and Barna, 1972; Spell, 1974; Lundstrom, 1981; Abdalla et al., 1983), myosin (Champion et al., 1970; Seki et al., 1980), tropomyosin (Parsons et al., 1969), and whole muscle extracts (Cowie, 1968; Matthey et al., 1970; Hoyem and Thorson, 1970; Mackie, 1979; Kaiser et al., 1980).

Addition of non-meat proteins such as soya bean, egg white and milk proteins in meat products during processing is a common practice in many countries. These proteins have been shown not to interfere with species identification using electrophoretic methods since they give characteristic electrophoretic patterns which are different from those of meat proteins (Hofmann, 1974; Hofmann and Penny, 1971; 1973; Penny and Hofmann, 1971; Shiga, 1980; Kaiser et al., 1981).

The concentration of muscle proteins, especially the sarcoplasmic proteins, has been shown to vary not only between species but also within species, and even between muscles of the same animal (Giles, 1962; Lawrie, 1953). Such differences, if not taken into account, would erroneously suggest a sample to be from a different species, though it may be from the same species as the reference sample. The

effects of post-slaughter treatment such as conditioning, electrical stimulation and freezing on the number, mobility and the staining intensity of protein bands formed in an electrophoretic pattern, when compared to the patterns obtained from freshly slaughtered animals, is not well documented. Tiecco (1980) showed that electrophoretic patterns obtained from frozen beef had fewer bands than those of a freshly slaughtered animal. Abdallah et al. (1983) showed that electrophoretic patterns of sarcoplasmic proteins from frozen and fresh beef differed to the extent that species identification based on comparing the electrophoretic patterns of fresh with the frozen samples could not be achieved.

## 2.1.2 Isoenzymes

### 2.1.2.1 Lactate dehydrogenase (LDH)

Dilworth and McKenzie (1970) used the electrophoretic patterns of the LDH isoenzymes to distinguish between various wild animals. These authors found that the LDH isoenzyme patterns could not differentiate between deer and beef. Similar results were obtained by Munday et al. (1974) when they failed to differentiate between the closely related forrester kangaroo and the Bennet wallaby. In their work on closely related ruminant species (cattle, sheep, goat and buffalo meats) Slattery and Sinclair (1983) observed no

significant differences in the LDH isoenzyme patterns and it was therefore not possible to differentiate these species on the basis of LDH patterns alone.

#### 2.1.2.2 Esterase

Dilworth and Mckenzie (1970) and Slattery and Sinclair (1983) used esterase isoenzyme patterns in addition to LDH pattern to differentiate between related ruminant species. Heinert and Klinger (1980) and Slattery and Sinclair (1983) used electrophoretic patterns of muscle extracts together with the esterase isoenzyme patterns in order to differentiate between related species. Esterase isoenzyme patterns alone are not suitable for use in identifying the species of origin of raw meat.

#### 2.1.2.3 Phosphogluconate dehydrogenase (PGD)

King and Kurth (1982) examined the PGD isoenzyme patterns of raw meat in isoelectric focused gels. They were able to differentiate between sheep and goat. However, the use of PGD isoenzyme patterns is limited since it is only a qualitative method. Phosphogluconate dehydrogenase isoenzymes vary in quantity from animal to animal and can therefore not be used for quantitative assays.

#### 2.1.2.4 Phosphoglucomutase (PGM)

King and Kurth (1982) stained for PGM and found that the pattern of this isoenzyme in ruminant species was not affected by postmortem treatment such as electrical 'stimulation' and incubation for 24h at 30°C. They also found that the PGM of beef and kangaroo were different, although the kangaroo PGM was not staining intensely outside the isoelectric point range of beef PGM. This would prevent the use of PGM patterns in detecting kangaroo meat in beef mixtures.

#### 2.1.2.5 Adenylate kinase (AK)

Staining for AK isoenzyme pattern in raw kangaroo and beef samples, King and Kurth (1982) found that the isoelectric point of kangaroo AK isoenzyme was lower than that of beef and stained intensely enough to permit detection of kangaroo meat in beef mixtures.

#### 2.1.3 Serological methods

Serological techniques offer attractive alternatives to electrophoretic methods and isoenzyme analyses. Serological techniques rely upon:

- (i) the production of antisera with adequate titre and sufficient species specificity for the test purposes, and
- (ii) the efficient extraction of the antigen(s) from the test

sample with only minimum alterations of their immunogenicity and in sufficient non-varying quantities to allow quantitative analysis.

Proom (1943) showed that species specificity of an antiserum varies with the route of immunization and with the number of subsequent boosters. He observed that intramuscular and subcutaneous routes of immunization resulted in antisera with greater specificity than antisera produced by intravenous and intraperitoneal routes. Specificity was also shown to decrease with subsequent boosters.

Antisera to serum proteins have been used by Murakami et al. (1969), Shunmugam and Ranganathan (1972), Kamiyama et al. (1978a) and Manz (1980) in agar gel diffusion tests to detect the species of origin of fresh meat. Raising antibodies to serum proteins for the purpose of species identification is based on the fact that current slaughter techniques leave residual blood in the carcass; of the total blood volume, 1.5-2% remains in the musculature (Warriss, 1977).

Katsube and Imaizumi (1968) suggested that serum proteins retained in the muscles might be used to identify the species of origin of meat in precipitin reactions with antiserum to serum proteins. Kamiyama et al. (1978a) and Kang'ethe (1981), using antisera to purified serum albumin, found that the principal antigen(s) in each of the meat extracts of

cattle, goat, sheep, pig and horse detected by the antiserum shared epitopes with serum albumin.

Species-specific epitopes of serum albumin were investigated by Kamiyama (1977 a,b) using cattle, sheep and goat albumins. He observed that albumins cross-reacted with rabbit antisera to albumins. Sheep and goat albumins showed only 10% specificity. Kamiyama (1977 b) raised antisera to N- and C-terminal fragments of cattle, sheep and goat albumins cleaved by cyanogen bromide. Antisera to the C-fragment showed a higher degree of species specificity than the antisera raised to the N-terminal fragment. However, the use of antisera to N- and C-fragments did not offer any added advantage when compared with antisera to intact serum albumin in the identification of the species of origin of meat.

Kamiyama et al. (1978 b) detected pig meat adulterant of beef at the level of 0.2-0.5% using passive haemagglutination test, while Swarts and Wilks (1982), using antiserum to whole blood, were able to detect 5-20% adulteration of beef with kangaroo meat in immunodiffusion tests.

Antisera to whole muscle extracts have been used by Shunmugam and Ranganathan (1972) to differentiate between sheep and goat meat.

Isolation of muscle proteins to serve as antigens of choice in raising antisera for use in species identification has been attempted with some success by Furminger (1964),

Hayden (1977, 1979) and Schweiger et al. (1983). Furminger (1964) used antisera to myosins, but species identification was not possible because of the high degree of cross-reactions of the antisera. Hayden (1977), however, using antisera to chicken troponin, succeeded in detecting chicken meat in beef sausages at the level of 1-5% in immunodiffusion tests. Schweiger et al. (1983) used antiserum to troponin-T to differentiate between turkey and chicken meat. Hayden (1979) produced antisera to isolated myoglobins from horses, pigs and sheep and was able to detect 3% level of adulteration of ground beef with sheep and pig meats in immunodiffusion tests.

During the last two decades, there has been a considerable increase in the number of immunodiagnostic tests available. Immunofluorescence and radioimmunoassay have been used as the methods of choice where high sensitivity was required. Immunofluorescence is a time consuming procedure which can not be easily automated. It can, therefore, only be used conveniently for a small number of samples. In contrast, radioimmunoassay is suitable for large scale operations, but the short half-life of the reagents, the rather expensive equipment and the regulatory control of the isotopes tends to preclude the use of radioimmunoassay in small laboratories. These considerations promoted a search for detection labels for antibodies or antigens and led to

the use of enzymes and the development of enzyme immunoassay (EIA).

Enzyme immunoassays are classified as heterogeneous or homogeneous assays. Homogeneous immunoassays are largely used for assaying small molecular weight substances, while heterogeneous enzyme immunoassays have found use in species identification of meat.

Enzyme linked immunosorbent assay (ELISA) was pioneered by Engvall and Perlmann (1971) and Van Wemmen and Schuurs (1971) based on the original suggestions of Miles and Hales (1968). Several modifications of EIA are now in use.

Patterson et al. (1984), and Jones and Patterson (1985) used the double antibody or 'sandwich' EIA to differentiate unprocessed beef, sheep, horse, kangaroo, pig, camel, buffalo and goat meats to a level of 1% adulteration in mixtures of two species. Jones and Patterson (1985) were able to detect a level of 0.5%.

Kangethe et al. (1982) used the indirect EIA to differentiate between beef, mutton, veal, goat, venison, pork and horse meat using antisera to bovine, sheep and horse serum albumin. Using this technique, they were able to detect 3% level of adulteration of beef with horse meat. Lower percentages of horse in beef (0.2-0.5%) made by mixing appropriate aliquots of pure species extracts gave significant results for horse meat down to 0.5%. Using a similar



technique, Whittaker et al. (1983) obtained similar results to those of Kang'ethe et al. (1982). A modification of indirect EIA where the antibody is incubated with varying amounts of antigen and the free antibody is separated, and then reacted with a sensitized plate was used by Kang'ethe (1981). In this work, it was not possible to quantify precisely the amount of horse meat added to ground beef in known mixtures. This was attributed to the variability in the content of residual blood in various muscles after slaughter. Griffiths and Billington (1984) found that this variation in the amount of blood left in muscles and its deterioration with time hindered accurate determination of the level of adulterant added, thus confirming the observations of Kang'ethe (1981).

## 2.2 COOKED MEATS

Identification of the species of origin of cooked or processed meats presents considerable obstacles to food analysts and forensic scientists because most of the species-specific proteins used in the identification of fresh meats are denatured by the process of heating.

### 2.2.1 Isoenzyme analyses

Differentiation of cooked meat by means of isoenzyme analysis in isoelectric focused gels was described by King (1984). Two isoenzymes namely, adenylate kinase and creatine kinase have been shown to be of some use in the identification of the species of origin of cooked meats.

#### 2.2.1.1 Adenylate kinase (AK)

King (1984) found AK to be useful in the discrimination of possum, buffalo, cat, dog, horse, donkey, red and grey kangaroo meats from beef. The isoelectric points of the AKs' from these species were lower than that of beef. However, the activity of AK isoenzyme decreased in intensity in products heated at 105°C for 30 min. Because of the low recovery rate of AK activity in products heated to 120°C for 30 min, AK isoenzyme patterns are not suitable for use in differentiation of canned or meat products heated above 105°C for 30 min (King, 1984).

#### 2.2.1.2 Creatine kinase (CK)

The CK isoenzyme patterns from beef, pig and sheep showed significant differences in their isoelectric points, thus enabling these species to be identified (King, 1984). Creatine kinase had a higher recovery rate than AK in products heated at 120°C for 30 min. This makes CK a more promising isoenzyme to use in the species identification of meat from canned products than AK.

Creatine kinase has been shown to exist as a dimer of two peptide chains of 41,000 daltons each (Bickerstaff and Price, 1978). Because of existing as a dimer, there is a possibility of a hybrid dimer formation which would complicate interpretation and may preclude identification if a sample

consisted of a complex mixture. Indeed, King (1984) observed such an hybrid dimer formation in mixtures of mutton and pork, mutton and beef, all in the ratio of 1/1.

### 2.2.2 Histidine dipeptides

The histidine dipeptides, carnosine (B-alanyl-L-histidine) and anserine (B-alanyl-L-1-methylhistidine) have long been known to be present in muscle tissue (Briscas and Fromageot, 1953). A third histidine dipeptide, ophidine or belanine (B-alanyl-L-3-methylhistidine) has also been shown to be present in muscle tissue (Rangeley and Lawrie, 1976, 1977; Harris and Milne, 1980, 1981). Carnegie et al. (1982, 1983) have shown pig muscle to contain a higher concentration of ophidine than muscles of kangaroo, cattle and horse. Ophidine has been shown to vary in concentration not only with age (Rangeley and Lawrie, 1976; Harris and Milne, 1981) but also within muscles of the same animal (Carnegie et al., 1982).

The anserine/carnosine ratio and the content of ophidine have been used by Tinbergen and Slump (1976) and Carnegie et al. (1982) to identify the species of origin of meat used in processed luncheon meats and tinned hams. Carnegie et al. (1982) used ophidine/anserine and carnosine/anserine ratios to differentiate between horse, kangaroo, beef and mutton when they were added to pork products in increasing amounts. However, differentiation based on these histidine dipeptide

ratios of other species such as buffalo, kangaroo and rabbit was not possible (Carnegie et al., 1982).

Ophidine is found only in the structure of actin and myosin (Johnson et al., 1967). It has been used in the determination of the lean meat content in meat products (Rangeley and Lawrie, 1976, 1977; Poulter and Lawrie, 1980a, b; Jones et al., 1982).

### 2.2.3 Fats

The curing process and other heating steps in the preparation of processed meat products denature proteins, while fats are resistant to these processing treatments. Fats have, therefore, been used for the identification of the species of origin of cooked meat products.

The pig is efficient in incorporating palmitic acid at position 2 of its triglyceride molecule (Christie and More, 1970; and Brockerhoff et al., 1966). This positional incorporation of palmitic acid has been shown to be species-specific and this allows the differentiation of lard from tallow (Verbeke and Brabander, 1979).

Horse meat can be differentiated from other meats by its linolenic acid content (Payne, 1971). Beef can be differentiated from chicken on the basis of oleic acid proportion in position 2 and the distribution of the stearic acid in the triglyceride fractions (Verbeke and Brabander 1979, 1980).

#### 2.2.4 Serological Methods

Identification of cooked meats rests on the isolation of species-specific thermostable muscle antigens that withstand the processing conditions. At the same time, these antigens must not be lost through juices expressed during cooking or lose their immunogenicity.

Murakami et al. (1969) noticed a decrease in antigenicity of meat heated at various temperatures when tested against antisera raised to unheated meat extracts. Katsube and Imaizumi (1968) had earlier shown that antiserum to boiled serum reacts with raw meat giving better species specificity than antiserum to unheated serum. They also showed that antigens detected in boiled serum were present in raw meat extracts.

Antisera to urea extracts of heat denatured muscle proteins were used by Snell and Mentz (1969) to identify the origin of heat-denatured meats from cattle, pig and horse. Heating of meat at 120°C for 1 h prior to extraction of the antigens with 7M urea did not alter their antigenicity.

Koh (1978) improved the extraction procedure by incorporating 2-mercapto-ethanol in the extraction procedure for denatured muscle proteins from beef skeletal muscle and soya bean proteins. He showed that rabbit antisera to beef skeletal muscle extract reacted only with beef skeletal muscle and cardiac muscle extracts. Cooking of the meat

prior to extraction did not alter the precipitin reactions of the extracts and their homologous antisera. He further demonstrated that soya bean protein (Promine-D) extract did not cross-react with antisera to beef skeletal muscle. Manz (1983) utilized the antisera to urea/mercapto-ethanol extracts of kangaroo meat to detect the presence of kangaroo meat in sausages using enzyme-linked immunosorbent assay. He was able to detect the presence of kangaroo meat in commercial sausages and frankfurters.

Anon (1970) and Karpas et al. (1970) used antisera to heated IgG to determine the species of origin of meat used in the manufacture of sausages. Adulteration to the level of 35% was detected in mixtures containing 3 different meat types. Hirose and Osler (1967) and Henney and Ishizaka (1968) observed that heating of meat resulted in the emergence of new antigenic epitopes. Karpas et al. (1970) noticed an increase in cross-reactions arising from the emergence of these new antigens.

Milgrom and Witebsky (1962) described the presence of thermostable 'adrenal-specific antigens'. Milgrom et al. (1963) further showed that thermostable antigens from horse adrenal glands existed as two distinct antigens, one species-specific and the other cross-reacting with antisera to adrenal extracts from other species. Milgrom et al. (1964) studied the species-specificity of the adrenal

thermostable antigens and showed that antisera to these antigens could be used to identify the species of origin of meat in food products, decayed organ tissues and the species of origin of the food eaten by an animal by testing faeces or urine of an animal fed with those samples. Andersen (1975, 1977a, b) and Hayden (1981) confirmed the results of Milgrom and Witebsky (1962) by using antisera to adrenal thermostable antigens in the identification of the species of origin of cooked meats. Hayden (1981) was able to detect 5-10% adulteration of cooked beef sausages with horse meat in immunodiffusion tests.

Monoclonal antibodies are superior to polyclonal antibodies because they react with single epitopes and are therefore more specific. Murine monoclonal antibodies to heated (70°C) meat from cattle and kangaroo were used by Goerlich and Greuel (1986) in ELISA to differentiate between heated and fresh cattle and kangaroo meats.

The state-of-the-art of meat species identification, is that fresh meat has been easily identified using physico-chemical and immunological methods. Identification of heat-treated or cooked meat has not been successful to the same degree employing the same methods used for fresh meat. The existence of thermostable muscle antigens would simplify species identification, as antisera to these antigens could be used to speciate heat-treated as well as fresh meats.

### 3. MATERIALS AND METHODS



### 3.1 ANTIGEN EXTRACTION PROCEDURE

Extracts of meat samples were prepared from 13 wild and 7 domestic species, namely: buffalo (*Syncerus caffer*), bushbuck (*Tragelaphus scriptus*), bushpig (*Potamochoerus porcus*), eland (*Taurotragus oryx*), Grant's gazelle (*Gazella granti*), impala (*Aepyceros malempus*), kongoni (*Alcelaphus buselaphus cokii*), oryx (*Oryx gazella beisa*), Thomson's gazelle (*Gazella thomsoni*), topi (*Damaliscus lunatus topi*), warthog (*Phacochoerus aethiopicus*), waterbuck (*Kobus spp*), wildebeest (*Connochaetes taurinus*), cattle (*Bos indicus*), dog (*Canis l. familiaris*), goat (*Capra aegagrus hircus*), pig (*Sus s. domestica*), camel (*Camelus dromedarius*), horse (*Equus p. caballus*) and sheep (*Ovis ammon aries*).

Figures 1A, 1B, 1C are flow charts showing the evolutionary relationships of the animals studied.

The method of Milgrom and Witebsky (1962) of extracting adrenal thermostable antigens (BE = boiled ethanol insoluble antigens) was adopted for the extraction of thermostable muscle antigens (TMA), fresh meat antigens (FMA) and cooked meat antigens (CMA).

#### 3.1.1 Fresh meat antigens (FMA)

Meat samples were dissected free of external fat and connective tissue, minced and homogenized in saline in a ratio of 2/1 (w/v). The homogenate was sonicated in an ice

bath at 300 W for 10 min. using a Braunsonic 1510 instrument (B. Braun, Melsungen A.G., Melsungen, W. Germany) with one minute bursts. The sonicated homogenate was centrifuged at 2,000 xg and the supernatant used as the source of FMA (Fig. 2).

### 3.1.2 Cooked meat antigens (CMA)

The samples were treated as in the FMA extraction procedure, except that the sonicated homogenate was placed in a boiling waterbath for 1 h, and rehomogenized. The volume of fluid lost was replaced by adding saline. The rehomogenized samples were centrifuged at 2,000 xg and the supernatant was filtered through a Whatman filter paper number 3 to remove fat. The filtrate was used as the CMA (Fig. 2).

### 3.1.3 Thermostable muscle antigens (TMA)

The samples were treated as in the extraction of CMA but further processed as follows: the filtrate was autoclaved at 121°C for 30 min. After cooling the filtrate was centrifuged at 86,000 xg for 30 min. The TMA were precipitated from the supernatant by the addition of 3 volumes of absolute ethanol and left overnight at room temperature. After centrifugation at 2,000 xg, the remaining ethanol was evaporated from the precipitate in a Rotavapour<sup>(R)</sup> (Buchi, Flawil, Switzerland). The dried precipitate was redissolved

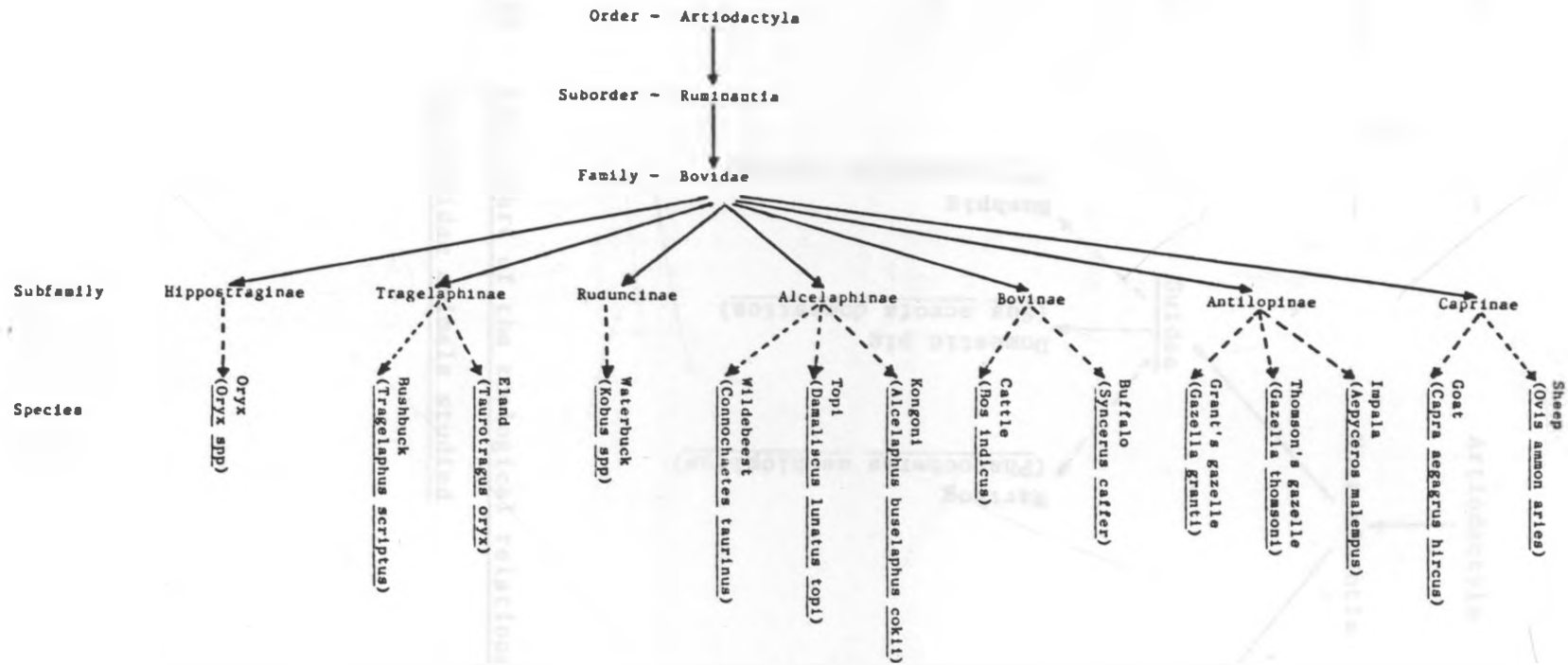


Figure 1A : A simplified chart of the zoological relationship of the bovidae animals studied

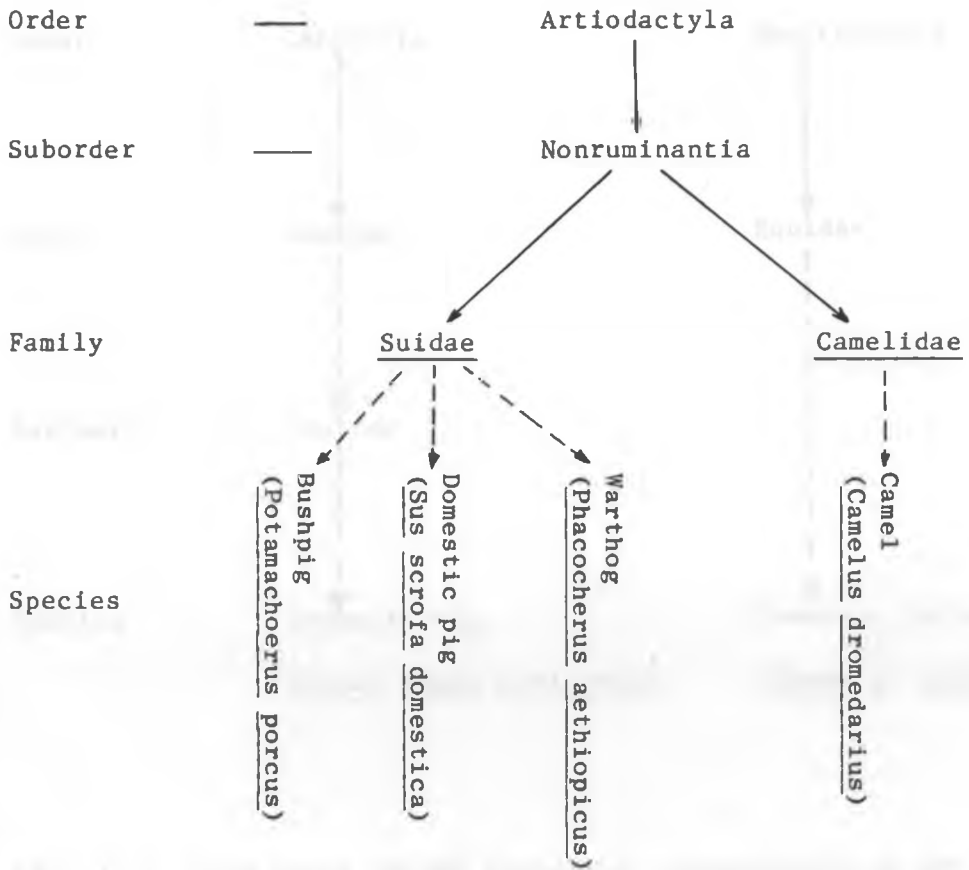


Fig. 1B : Flow chart of the zoological relationship of the non-bovidae animals studied

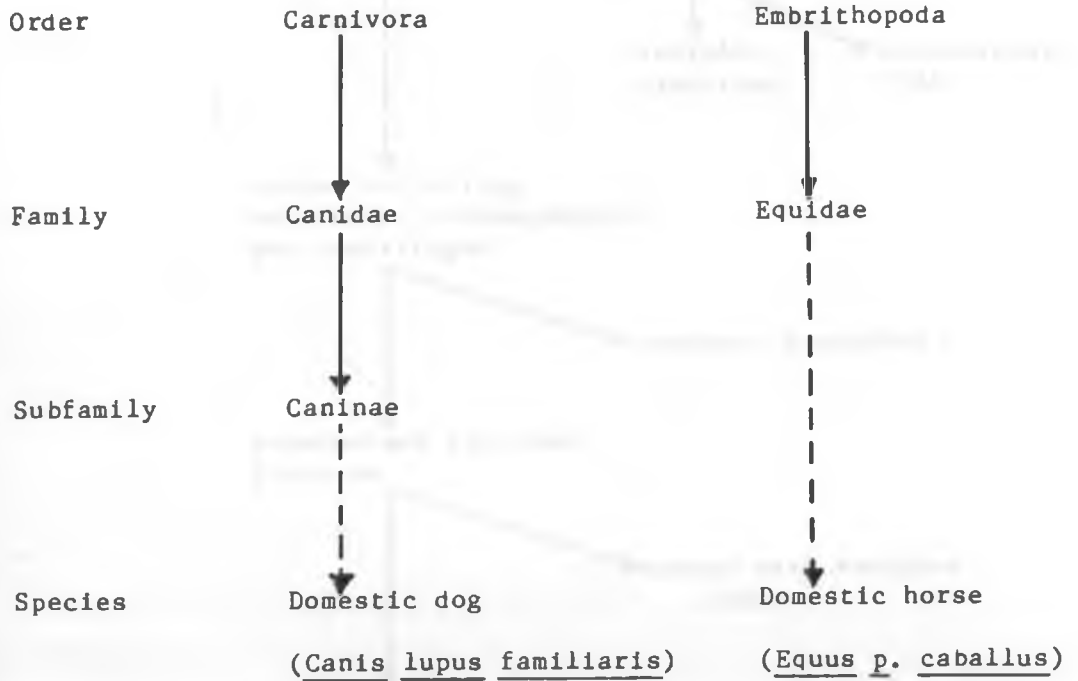


Fig. 1C : Flow chart of the zoological relationship of the non-bovidae animals studied

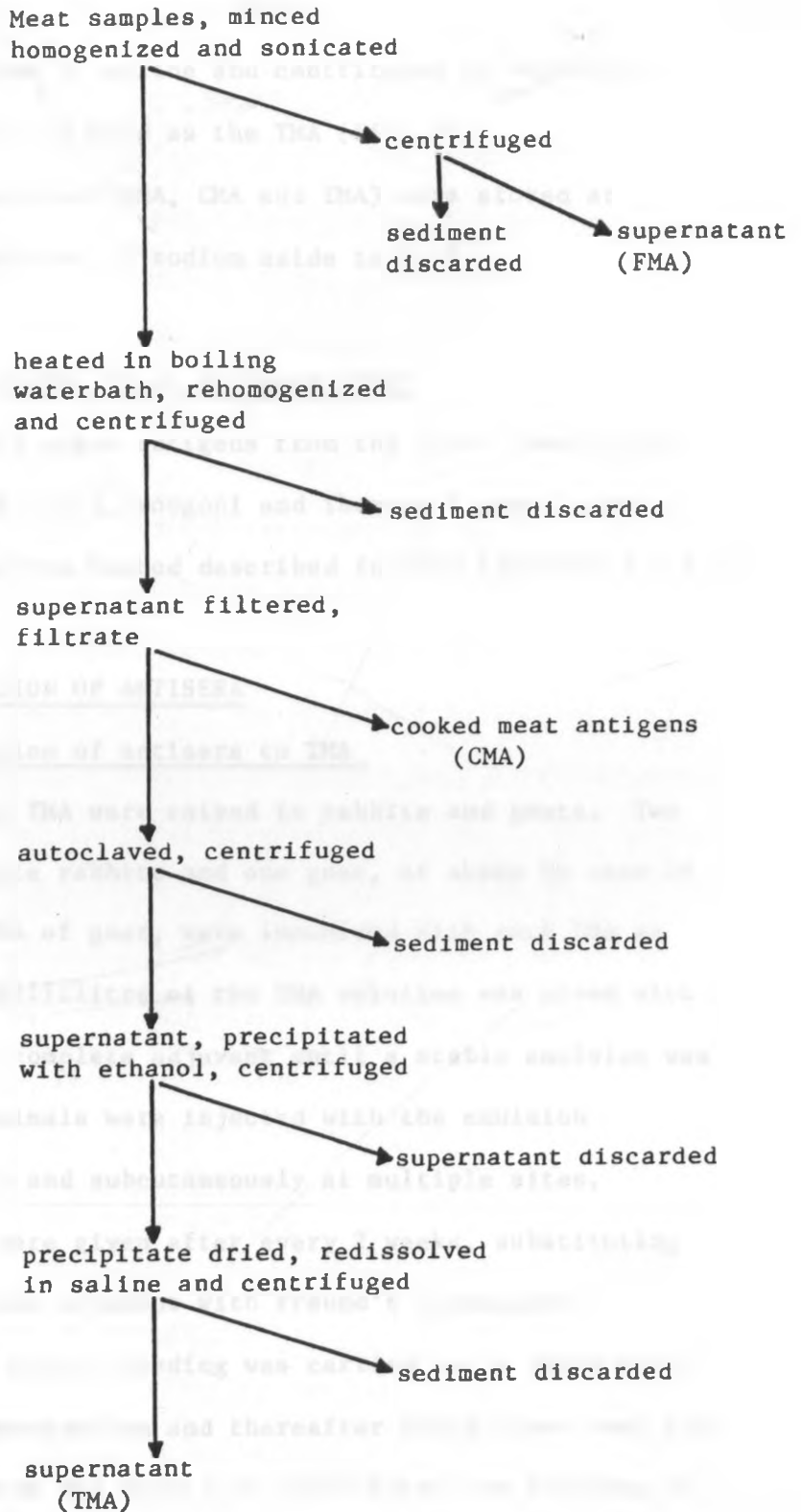


Figure 2 : Flow chart showing the extraction of FMA, CMA and TMA

in a small volume of saline and centrifuged at 86,000 xg. The supernatant was used as the TMA (Fig. 2).

All the antigens (FMA, CMA and TMA) were stored at  $-20^{\circ}\text{C}$  after addition of sodium azide to 0.1%.

#### 3.1.4 Thermostable Organ Antigens (TOA)

Thermostable organ antigens from the liver, heart and kidney of cattle, pig, kongoni and Thomson's gazelle were extracted using the method described for TMA (section 3.1.3.).

### 3.2 PRODUCTION OF ANTISERA

#### 3.2.1 Production of antisera to TMA

Antisera to TMA were raised in rabbits and goats. Two New Zealand white rabbits and one goat, or sheep in case of antiserum to TMA of goat, were immunized with each TMA as follows: one millilitre of the TMA solution was mixed with 2 ml of Freund's complete adjuvant until a stable emulsion was formed. The animals were injected with the emulsion intramuscularly and subcutaneously at multiple sites. Booster doses were given after every 2 weeks, substituting Freund's complete adjuvant with Freund's incomplete adjuvant. The first bleeding was carried out 4 weeks after the primary immunization and thereafter every other week for 11 months. Serum was stored at  $-20^{\circ}\text{C}$  after the addition of sodium azide to 0.1%. The animals used for immunization are listed in Table 1.

### 3.2.2 Production of rabbit antiserum to Goat IgG

Normal sera from 10 goats were pooled and the globulins precipitated using saturated ammonium sulphate solution, pH 7.4, according to the method of Fey et al. (1976).

The globulin fraction was dialysed against several changes of phosphate buffered saline (PBS) for 3 days and 0.02 M phosphate buffer, pH 8.0, for 1 day. The dialysed fraction was passed through a diethyl-aminoethyl cellulose column (BioRad Laboratories, Richmond, California, USA), equilibrated with 0.02M phosphate buffer, pH 8.0. The IgG fraction was eluted using 0.02M phosphate buffer, pH 8.0, and concentrated using a Diaflo ultrafilter with a cut off point of 30,000 daltons (Amicon, Massachusetts, USA). The fraction was shown to contain pure IgG by immunoelectrophoresis using rabbit anti-goat serum. The IgG fraction was used to immunize rabbits. The procedure for immunization was the same as that described in section 3.2.1.

### 3.3 ABSORPTION OF THE ANTISERA

#### 3.3.1 Absorption of goat and rabbit antisera to TMA for use in immunodiffusion tests

Goat and rabbit antisera to TMA were absorbed to remove cross-reacting antibodies using copolymerized serum proteins, according to the ethylchloroformate method of Avrameas and Ternynck (1962). Eighty ml of pooled serum from the 20



Table 1 : Experimental animals used for the production of antisera to TMA

Antigen used (TMA)	Identification number of rabbit used	Identification number of goat or sheep used
Buffalo	305 and LY-694	340
Bushbuck	LY-628 and LY-629	831
Bushpig	885b	892
Camel	886	837
Cattle	302 and 307	343
Dog	882	885
Eland	308 and LY-879	346
Goat	306 and LY-696	345
Grant's gazelle	313 and LY-632	344
Horse	881(b)	897
Impala	312 and LY-881	342
Kongoni	127, 314 and 317	826
Oryx	310 and LY-880	856
Pig	883	863
Sheep	LY-632 and LY-636	841
Thomson's gazelle	315 and 316	347
Topi	303 and LY-690	829
Warthog	884	869
Waterbuck	LY-633 and LY-634	832
Wildebeest	LY-693	827
Goat IgG	887, 889 and 892(b)	

species were mixed with an equal volume of 0.2M acetate buffer, pH 5.0, while stirring. The pH of the mixture was adjusted to pH 5.0. Ethylchloroformate was added dropwise while stirring in amounts of 0.6 ml per 10 ml of serum. The stirring was continued for 15 min. while the pH was maintained between 4.5 and 5.0 by the addition of 1M NaOH. The mixture was allowed to stand for 1 h at room temperature. The gel formed was homogenized using a tissue grinder. The finely ground homogenate was washed 3 times in saline. After the final wash, the immunosorbent was suspended in saline and sodium azide added to 0.1%.

The antiserum was mixed with the insoluble immunosorbent and left on an end-over-end mixer, overnight at 4°C. The antiserum was separated by centrifugation at 2,000 xg for 15 min at 4°C. The absorbed antiserum was tested for cross-reactivity in immunodiffusion tests. If the antiserum was found to be specific, it was stored at 4°C after addition of sodium azide to 0.1%. If the antiserum was found to cross-react with heterologous TMA, it was further absorbed using the same immunosorbent after regeneration until maximum specificity was achieved. The immunosorbents were regenerated as detailed in section 3.3.3.

### 3.3.2 Absorption of goat antisera to TMA for use in the enzyme immunoassay (EIA)

The goat antisera to TMA absorbed with copolymerized serum proteins according to the method of Avrameas and Ternynck (1962) described in Section 3.3.1. were tested for specificity in an enzyme immunoassay (Section 3.5.9). The antisera were absorbed using the appropriate cross-reacting and partially purified thermostable muscle antigens (see Section 3.5.1.) coupled to cyanogen bromide activated Sepharose 4B, and further absorbed with saline extracts of raw meats from wildebeest and warthog copolymerized by the same method.

After absorptions, the antisera were tested for cross-reactions in EIA, and stored at 4°C after adding sodium azide to 0.1%.

### 3.3.3 Regeneration of immunosorbents

The immunosorbents were regenerated by washing 3 times with PBS and once with normal saline. The adsorbed antibodies were eluted with 0.2M glycine-HCl buffer, pH 2.5. The immunosorbents were washed with 0.1M Tris-HCl buffer, pH 8.5, containing 0.5M NaCl. After this final wash, they were suspended in PBS and stored at 4°C after addition of sodium azide to 0.1%.

### 3.4 IMMUNODIFFUSION TESTS

The microtechnique of Ouchterlony's double diffusion test as described by Crowle (1973) was used with slight modifications. One percent (w/v) purified Oxoid agar (Oxoid Ltd., England) in PBS was used as the medium for double diffusion. Sodium azide was added to 0.1%. Three ml of the molten agar was poured onto a microscope slide (75 x 25 mm) on a levelled table to give a depth of approximately 2-3 mm. Wells with a diameter of 4 mm and placed 5 mm apart were punched in a hexagonal pattern. The central well was filled with antiserum (absorbed or unabsorbed rabbit or goat antisera to TMA) while the peripheral wells were filled with the antigen solutions (TMA, CMA, FMA or TOA). The slides were incubated in a humid chamber and diffusion allowed to proceed for 12 - 48 h at room temperature.

The slides were rinsed with distilled water and pressed overnight using absorbent paper. They were then washed in 3% (w/v) trisodium citrate buffer, pH 8.5, for 12 h. The slides were rinsed with distilled water, pressed, rinsed and air dried at room temperature. The slides were stained with either Ponceau 'S' or Coomassie Brilliant Blue protein stains and destained until the background became sufficiently clear (Axelsen, 1973).

### 3.5 ENZYME IMMUNOASSAY (EIA)

#### 3.5.1 Preparation of partially purified thermostable muscle antigens (PTMA)

Partially purified TMA (PTMA) was obtained from meat samples using a modification of the method of Milgrom and Witebsky (1962). Briefly, the following modifications were adopted: The sonicated homogenate was centrifuged at 2,000 xg, filtered, centrifuged at 86,000 xg and the supernatant autoclaved at 121<sup>0</sup>C for 30 min. After this step, other steps were similar to those used in extraction of TMA according to the method of Milgrom and Witebsky (1962) (see Section 3.1.3). After obtaining TMA, the TMA were concentrated on a Diaflo ultrafilter cell with a PM 30 filter. The concentrated TMA was fractionated through either Sephadex G-200 or G-75 column (height 94.5 or 35.5 cm respectively). Fractions of 7 ml were eluted using PBS. The antigen content of each fraction was determined by EIA (section 3.5.9) on the basis of optical density (OD) at 410nm. The tubes containing the antigen(s) were pooled and concentrated by ultrafiltration. Figure 3 is a flow chart showing the extraction procedure for the PTMA.

#### 3.5.2 Determination of the protein content of the PTMA

The protein content of each PTMA was estimated using the Folin-phenol method of Lowry et al. (1951), using bovine serum albumin as the standard.

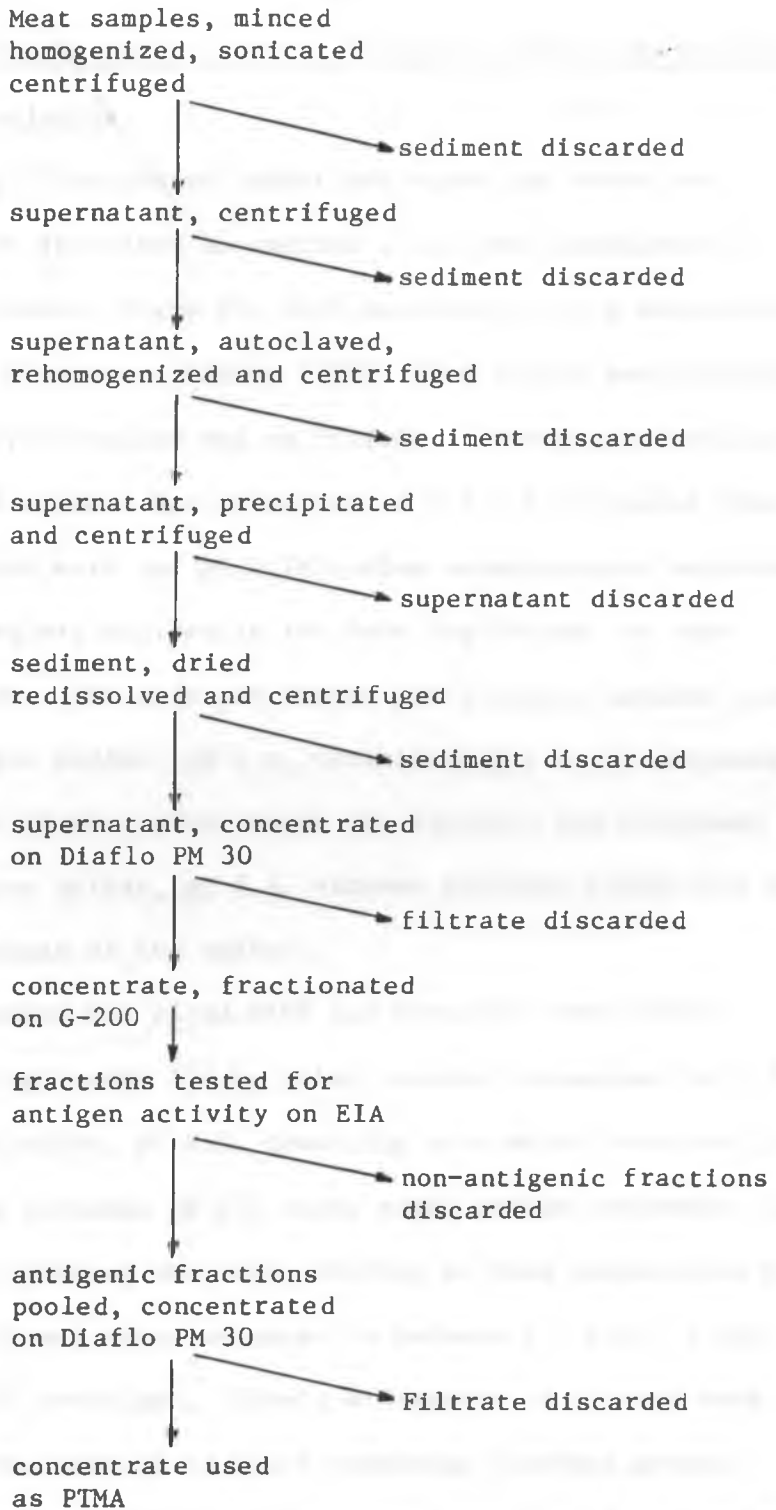


Figure 3: Flow chart showing the extraction of PTMA

### 3.5.3 Preparation of rabbit anti-goat IgG-glucose oxidase conjugate

The IgG fraction of rabbit anti-goat IgG serum was prepared as described in section 3.2.2, and conjugated to glucose oxidase (Sigma Co. Ltd) according to the metaperiodate method of Wilson and Nakane (1978) with slight modifications. The procedure adopted was as follows : Seventy six milligrams of glucose oxidase was dissolved in 9 ml of distilled water and oxidized with 1ml of 0.1M sodium metaperiodate solution. The mixture was stirred in the dark for 30 min. at room temperature. The oxidized enzyme was dialysed against cold 1 mM acetate buffer, pH 4.4, containing 0.1 ml of ethylene glycol for 10 min, after which the dialysis was continued in 1 mM acetate buffer, pH 4.4, without ethylene glycol for 1 h with 4 changes of the buffer.

The enzyme was mixed with IgG fraction from rabbit anti-goat IgG serum (76 mg total protein) dissolved in 0.5 M carbonate buffer, pH 9.0, resulting in a molar ratio of 1/1. The pH was adjusted to 9.0 using solid sodium carbonate. The enzyme IgG mixture was left stirring at room temperature for 2 h, the pH was later adjusted to between 7.5 and 7.8 and left at 4°C overnight. Twenty milligrams of glycine were added to the mixture to block remaining aldehyde groups. Stirring was continued at room temperature for 1 h, and the pH finally adjusted to between 7.4 and 7.8 using 1 M HCl.

After clarification by centrifugation at 2,000 xg for 10 min at 4°C, the conjugate was filtered through 0.45 µm filter (Sartorius, Gottingen, W. Germany) and stored at -20°C after the addition of an equal volume of glycerol.

#### 3.5.4 Choice of coating buffers

Three buffers were tested in EIA (section 3.5.10) for their efficacy in promoting adsorption of the PTMA onto the microtitre plates (Dynatech, M129A, flat bottomed). Buffalo PTMA was used in this assay. Buffalo PTMA was diluted in, 0.05 M carbonate-bicarbonate buffer, pH 9.6, 1:100 PBS + 2% polyethelene glycol 6000 (PEG) + 0.5 M NaCl and in 1:100 PBS + 2% PEG. The plates were incubated overnight in a humid chamber at room temperature. Other steps of EIA were as detailed in section 3.5.9.

#### 3.5.5. Optimisation of PTMA concentration for use in enzyme immunoassay

Each PTMA was diluted 1/2 to 1/2048 in the coating buffer (0.05 M carbonate-bicarbonate, pH 9.6). Each dilution coated 1 column of the wells of a microtitre plate applying 100 µl/well. Using appropriate serum and conjugate dilutions, the optimal antigen dilution for coating the plates was determined by EIA (section 3.5.9).



### 3.5.6 Determination of optimal antiserum and conjugate dilutions

A microtitre plate was coated with 100  $\mu$ l/well of the optimal dilution of each PTMA as determined in section 3.5.6.

Optimum working dilutions of the antiserum and conjugate for each PTMA were determined by checker-board titrations.

Antigen, antiserum, conjugate and substrate controls were included every time.

### 3.5.7 Assay for hydroxyproline

Hydroxyproline was assayed for in the TMA, PTMA and in the Sephadex G-75 and G-200 fractions according to the method of Blumenkrantz and Asboe-Hansen (1975).

Five mls of each sample were hydrolysed in 6 M HCl at 120°C overnight. The hydrolysed samples were dried in a Rotavapour<sup>(R)</sup> and reconstituted to 2 ml using deionized water. To 1 ml of the reconstituted sample was added 1.5 ml of 1/10 borate-alanine buffer and 0.6 ml of 0.2 M chloramine-T solution. The samples were incubated at room temperature for 20 min to allow oxidation. After oxidation, 2 ml of 3.6 M sodium thiosulphate solution was added and the solution saturated with sodium chloride. Three millilitres of toluene were added and the samples placed in a boiling waterbath for 20 min. After cooling, the samples were thoroughly shaken for 15 min and centrifuged at 500 xg for 10 min. The organic phase was separated and 1 ml of the organic

phase was mixed with 0.4 ml of Ehrlich's reagent. The samples were allowed to stand for 30 min at room temperature for colour development. The colour intensity was measured at 565 nm using a Beckman Spectrophotometer, Model 25.

### 3.5.8 The effect of gelatin on the adsorption of PTMA to microtitre plates

Buffalo PTMA was diluted in the coating buffer to contain 42.7 µg/ml, and mixed with 5mg, 500ug and 5ug of commercial gelatin (Matheson Coleman and Bell, Los Angeles, USA). The mixtures were used to coat microtitre plates. The effect of gelatin on the adsorption of the PTMA was determined by EIA using appropriate dilutions of goat antiserum to TMA of buffalo and the rabbit anti-goat IgG-glucose oxidase conjugate. The effect of gelatin in reducing the adsorption of the PTMA was expressed as a percentage inhibition using the equation below:

$$\% \text{ inhibition} = \frac{OD_z - OD_s}{OD_z} \times 100$$

where  $OD_z$  = absorbance at 410 nm of buffalo PTMA  
without gelatin (0% inhibition)

$OD_s$  = absorbance at 410 nm of buffalo PTMA  
plus gelatin at various concentrations

### 3.5.9 Enzyme Immunoassay for species identification of PTMA

The PTMA were initially diluted to contain the protein concentration which had been found to be optimal for each species. The PTMA were then diluted serially in double dilutions in the coating buffer and 100 µl/well of each PTMA dilution was used to coat duplicate wells of the microtitre plates. The plates were incubated in a humid chamber at room temperature overnight. The plates were tapped dry with absorbent paper and washed 5 times at 5 min intervals using the washing solution (PBS + 0.05% Tween 80). The plates were tapped dry, and 100 µl/well of the antiserum diluent (see Appendix 6.2) containing 5% normal rabbit serum was added, and the plates incubated at 37°C for 1 h. The plates were washed as described above, and 100 µl/well of the appropriate antiserum dilution added. The antiserum was diluted in the antiserum diluent (see Appendix 6.2). After incubation at 37°C for 1 h, the plates were washed and 100 µl/well of the appropriate dilution of the rabbit anti-goat IgG glucose oxidase conjugate added. The conjugate diluent was similar to the antiserum diluent except that 0.5% Tween 80 was used instead of 0.1% Tween 80 (see Appendix 6.3). The plates were incubated at 37°C for 1 h, washed, tapped dry and 100 µl/well of the substrate solution (see Appendix 6.4.1) added. The plates were incubated at room temperature for 1 h and the absorbance read at 410 nm using an ELISA Minireader (Dynatech Co. Ltd., Santa Monica, California).

3.5.10 Detection of the species of origin of heated meats in known mixtures

Beef was mixed with buffalo, goat with impala and pork with warthog meat at 1% and 10% levels. The PTMA from these mixtures were extracted according to the procedure described in section 3.5.1. Detection of buffalo, impala and warthog in beef, goat and pork respectively was done in EIA using appropriate goat antisera to TMA.

3.6 MOLECULAR WEIGHT DETERMINATION OF THE PTMA BY GEL FILTRATION

Determination of the molecular weight of the PTMA was carried out on a Sephadex G-200 column by the method of Andrews (1964).

The column was packed using the method described by Hudson and Hay (1980). The column height was 94.2 cm and the internal diameter was 2.6 cm. After equilibration with PBS, the column was calibrated using the following protein markers.

Marker	Molecular weight (daltons)
Blue dextran	2,000,000
Purified IgG	150,000
Bovine serum albumin	67,000

The calibration was repeated twice and the average void and elution volumes of the protein standards were determined. The  $K_{av}$  of each marker was calculated according to the following formula : ("Gel and filtration" : Theory and Practice" Pharmacia Fine chemicals, Uppsala, Sweden).

$$K_{av} = \frac{V_e - V_o}{V_t - V_o} \quad \text{where}$$

$V_e$  = void volume (elution volume of blue dextran)

$V_o$  = elution volume of the other protein marker

$V_t$  = total bed volume

$K_{av}$  = the fraction of the stationary gel volume which is available for diffusion of a given solute

The  $K_{avs}$  were plotted on a semi-logarithmic graph paper against their corresponding molecular weights. The best line which joins the points on the graph paper was drawn to give the molecular weight standard curve using the least squares method.

Three ml of the concentrated buffalo PTMA were applied to the calibrated Sephadex G-200 column. Buffalo PTMA was detected in the eluted fractions by EIA (section 3.5.9). Each eluted fraction was diluted 1/2 in the coating buffer and 100 ul was used to coat two wells of a microtitre plate. The other steps of EIA were as described in section 3.5.9.

The elution volume and the  $K_{av}$  of the PTMA was calculated. From the standard curve, the molecular weight of the PTMA was determined.

3.7 DETECTION OF BEEF AND PORK IN PORK AND BEEF MEAT PRODUCTS

Processed beef and pork products such as sausages, beef burgers, cooked ham, luncheon meat and canned corned beef were purchased from the retail outlets of two leading Kenyan manufacturing firms in Nairobi. Ninety samples bearing different production and batch dates were purchased over a period of 2 months.

3.7.1 Extraction of antigens from meat products

After the removal of the casings and other wrappings, the products were each homogenized in PBS in the ratio 2/1 (w/v). The homogenates were centrifuged at 4,000 xg for 30 min and the supernatant filtered through Whatman filter paper no. 3. The filtrate was stored at 4°C after adding sodium azide to 0.1%.

3.7.2 Preparation of pork and beef sausages and pork sausages containing various percentages of beef

Fresh beef and pork sausages and pork sausages containing 1, 5 and 10% beef were made in 1 kg lots in the laboratory. Table 2 shows the recipe for the preparations. Five percent milk powder was used in the sausage preparation.

Table 2 : Recipe for the preparation of beef and pork sausages

Constituents	Pork sausages containing beef				Beef sausages
	100% Pork	1% beef	5% beef	10% beef	100% beef
Beef lean (g)	-	12	60	120	700
Beef fat (g)	-	-	-	-	200
Pork lean (g)	700	688	640	580	-
Pork fat (g)	200	200	200	200	-
ice/water (g)	100	100	100	100	100
milk powder (g)	50	50	50	50	50
salt	15	15	15	15	15
seasonings	5	5	5	5	5

### 3.7.3 Effect of milk powder on the detection of beef in pork products

The effect of milk powder, frequently used as a binder, on the detection of beef in pork products was examined by coating a microtitre plate with milk powder diluted in the coating buffer. Five, ten and fifteen percent solutions of milk powder was tested in EIA (section 3.5.9).

### 3.7.4 Enzyme Immunoassay for the detection of beef and pork in beef and pork products

A similar procedure to that used for identifying PTMA (section 3.5.9) was adopted, except that the antigen preparations were first screened for either beef or pork using appropriate dilutions of goat antisera to TMA of cattle and pig. Initially an antigen dilution of 1/400 was used. Those products that were negative for either beef or pork at 1/400 were tested starting with an antigen dilution of 1/2 and diluted serially to 1/400 in the coating buffer.

For the differentiation of known beef and pork, the reaction was considered positive when the absorbance exceeded 3 times that of the mean of the negative sample (Savigny and Volier, 1980). Pure beef and pork sausage extracts were included in each test as controls.

## 3.8 ANALYSIS OF ENZYME IMMUNOASSAY DATA

The data of absorbance obtained from the differentiation tests for PTMA were analysed for statistical significance using a paired Student's t-test (Snedecor and Cochran, 1971).



4. RESULTS AND DISCUSSION

#### 4.1 IMMUNODIFFUSION TESTS

##### 4.1.1 Species identification of TMA

Results of species identification of autoclaved meats (TMA) using unabsorbed goat and rabbit antisera to TMA are tabulated in Tables 3 and 4. These results showed that goat and rabbit antisera to TMA gave a wide range of cross-reactions with various TMA. Rabbit antisera to TMA showed a wider range of cross-reactions than goat antisera to TMA. This is exemplified by comparing rabbit antiserum to TMA of kongoni which cross-reacted with all TMA of the bovidae, while goat antiserum to the same TMA cross-reacted only with TMA of topi, oryx, wildebeest, Thomson's gazelle and sheep (Tables 3 and 4). Only goat antisera to TMA of camel, dog, horse, impala, warthog and wildebeest were species-specific without absorptions (Table 4).

In an experiment designed to check whether antigens similar to or identical with TMA were present in serum, it was found that unabsorbed goat and rabbit antisera to TMA of kongoni reacted with kongoni serum, giving a reaction of identity with the inner precipitin line of kongoni TMA (Fig. 4). This shows that kongoni serum shares some antigenic determinants with kongoni TMA. Using unabsorbed rabbit antiserum to TMA of kongoni, it was observed that TMA of goat, Grant's gazelle and Thomson's gazelle also reacted with the same antiserum giving a reaction of identity with the same precipitin line given by kongoni serum. This

Table 3 : Results of immunodiffusion tests using unabsorbed rabbit antisera to TMA and TMA of other species

TMA of	Rabbit antisera to TMA of										
	Buffalo	Cattle	Bushbuck	Eland	Goat	T. gazelle	Impala	Kongoni	Topi	Wildebeest	Oryx
Buffalo	*	*	-	-	+	+	+	+	+	+	+
Cattle	*	*	-	+	+	+	+	+	+	+	*
Bushbuck	+	+	*	+	+	+	+	+	+	+	+
Eland	+	-	-	*	-	+	+	+	+	+	+
Goat	+	-	-	-	*	+	+	+	+	+	*
Sheep	+	+	*	-	+	+	+	+	+	-	*
Impala	+	+	+	+	+	+	*	+	+	+	+
T. gazelle	+	+	-	+	+	*	+	+	+	-	+
G. gazelle	+	+	+	+	+	*	+	+	+	+	+
Kongoni	+	-	-	-	+	+	-	*	*	*	*
Topi	+	+	-	-	+	-	-	*	*	*	*
Wildebeest	+	-	*	+	+	+	+	*	*	*	*
Oryx	+	-	-	-	+	+	+	+	+	+	*
Waterbuck	+	-	-	+	+	+	+	+	+	-	*

Key : + = reaction of partial identity

- = no reaction

\* = reaction of identity

G. gazelle = Grant's gazelle

T. gazelle = Thomson's gazelle

TMA = thermostable muscle antigen

Table 4 : Results of immunodiffusion tests using unabsorbed goat antisera to TMA and TMA of various species

TMA of	Goat antisera to TMA of																	
	Buffalo	Cattle	Bushbuck	Eland	Grant's gazelle	Thomson's gazelle	Impala	Kongoni	Topi	Wildebeest	Oryx	Waterbuck	Warthog	Pig	Bushpig	Horse	Camel	Dog
Buffalo	*	*	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Cattle	+	*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bushbuck	-	-	*	*	-	*	-	-	-	-	-	-	-	-	-	-	-	-
Eland	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Goat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sheep	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
Grant's gazelle	-	-	-	-	*	*	-	-	-	-	-	-	-	-	-	-	-	-
Thomson's gazelle	-	-	-	-	*	*	-	+	-	-	-	-	-	-	-	-	-	-
Impala	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-
Kongoni	-	-	-	-	-	-	*	*	-	-	+	-	-	-	-	-	-	-
Topi	-	-	-	-	-	-	*	*	-	-	+	-	-	-	-	-	-	-
Wildebeest	-	-	-	-	-	-	*	-	*	-	+	-	-	-	-	-	-	-
Oryx	-	-	-	-	-	-	-	+	-	-	*	-	-	-	-	-	-	-
Waterbuck	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-
Warthog	-	-	-	-	-	-	-	-	-	-	-	*	*	-	+	-	-	-
Pig	-	-	-	-	-	-	-	-	-	-	-	-	*	*	-	-	-	-
Bushpig	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	-	-	-
Horse	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-
Camel	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	*	-
Dog	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*

Key :  
 + = reaction of partial identity  
 - = no reaction  
 \* = reaction of identity

TMA = thermostable muscle antigen

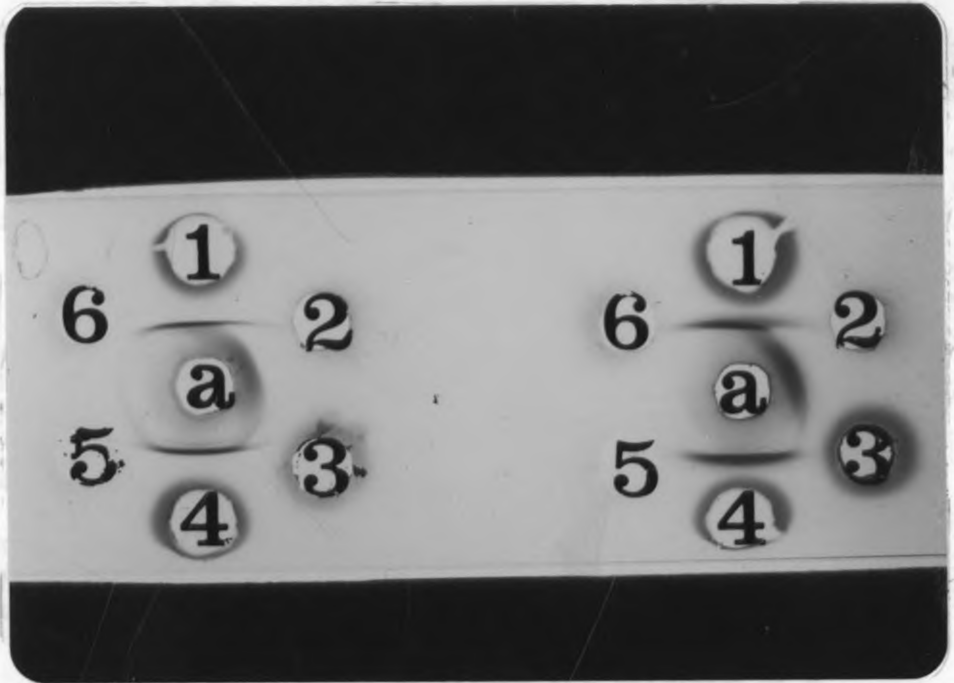


Fig. 4 : Results of immunodiffusion tests using unabsorbed goat and rabbit antisera to TMA of kongoni

- |                           |                           |
|---------------------------|---------------------------|
| (a) = Rabbit antiserum to | (a) = Goat antiserum to   |
| TMA of kongoni            | TMA of kongoni            |
| 1 = Kongoni TMA           | 1 = Kongoni TMA           |
| 2 = Kongoni serum         | 2 = Kongoni serum         |
| 3 = Thomson's gazelle TMA | 3 = Thomson's gazelle TMA |
| 4 = Kongoni               | 4 = Kongoni               |
| 5 = Grant's gazelle       | 5 = Grant's gazelle       |
| 6 = Goat                  | 6 = Goat                  |

means that the epitopes detectable in kongoni serum by the two antisera to TMA of kongoni are similar, if not identical to those that are shared between the TMA of goat, Grant's gazelle, Thomson's gazelle and kongoni.

Since the antisera to the TMA of kongoni raised in goat and rabbit recognized antigenic components of kongoni serum as similar to or identical with those of the TMA of goat, Grant's gazelle and Thomson's gazelle, absorption of the antisera to TMA with homologous serum abolished cross-reactions with TMA of unrelated species.

Milgrom et al. (1963) described the presence of a thermostable adrenal gland antigen that was detectable in homologous and heterologous adrenal gland preparations using rabbit antisera to thermostable adrenal gland preparations of man, pig and horse. The 'TMA-like' antigen demonstrated in kongoni serum and showing reactions of identity with the TMA of heterologous and homologous species, may be compared to the adrenal antigens described by Milgrom et al. (1963) with regard to cross-reactivity.

The results of immunodiffusion tests using absorbed goat and rabbit antisera to TMA of various species are shown in Tables 5 and 6. Absorption of the goat antisera to TMA with copolymerized serum proteins, rendered most of the goat antisera to TMA specific, with the exception of goat antisera to TMA of kongoni, topi, Thomson's gazelle and Grant's

**Table 5 : Results of immunodiffusion tests using rabbit antisera to TMA absorbed with copolymerized serum proteins and tested with TMA of various species**

TMA of	Rabbit antisera to TMA of														
	Buffalo	Cattle	Bushbuck	Eland	Goat	G. gazelle	Impala	Kongoni	Topi	Wildebeest	Oryx	Pig	Warthog	Bushpig	Camel
Buffalo	*	*	+	-	*	+	+	+	+	+	-	-	-	-	-
Cattle	-	*	-	+	+	+	-	+	+	+	-	-	-	-	-
Bushbuck	+	-	*	+	-	+	+	*	*	+	-	-	-	-	-
Eland	+	+	+	*	-	+	+	+	+	+	-	-	-	-	-
Goat	-	-	-	-	*	-	-	-	+	-	-	-	-	-	-
Sheep	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-
G. gazelle	-	-	-	-	+	*	-	+	+	-	-	-	-	-	-
T. gazelle	-	-	-	-	-	*	-	+	+	-	-	-	-	-	-
Impala	-	+	+	-	-	+	*	+	+	-	-	-	-	-	-
Kongoni	-	-	+	-	-	-	-	*	*	*	-	-	-	-	-
Topi	-	-	+	-	-	-	-	*	*	*	-	-	-	-	-
Wildebeest	-	-	+	-	-	-	-	*	*	*	-	-	-	-	-
Oryx	-	-	-	-	-	+	-	-	-	-	*	-	-	-	-
Waterbuck	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Warthog	-	-	-	-	-	-	-	-	-	-	-	*	*	*	-
Pig	-	-	-	-	-	-	-	-	-	-	-	*	*	*	-
Bushpig	-	-	-	-	-	-	-	-	-	-	-	*	*	*	-
Horse	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Camel	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*
Dog	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Key : \* = reaction of identity  
 + = reaction of partial identity  
 - = no reaction

TMA = thermostable muscle antigen  
 G. gazelle = Grant's gazelle  
 T. gazelle = Thomson's gazelle

Table 6 : Results of immunodiffusion tests using goat antisera to TMA absorbed with copolymerized serum proteins and tested with TMA of various species

TMA of	Goat antisera to TMA of																	
	Buffalo	Cattle	Bushbuck	Eland	Grant's gazelle	Thomson's gazelle	Impala	Kongoni	Topi	Wildebeest	Waterbuck	Oryx	Pig	Warthog	Bushpig	Camel	Horse	Dog
Buffalo	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cattle	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bushbuck	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Eland	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Goat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sheep	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Grant's Gazelle	-	-	-	-	*	*	-	-	-	-	-	-	-	-	-	-	-	-
Thomson's Gazelle	-	-	-	-	*	*	-	-	-	-	-	-	-	-	-	-	-	-
Impala	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-
Kongoni	-	-	-	-	-	-	-	*	*	-	-	-	-	-	-	-	-	-
Topi	-	-	-	-	-	-	-	*	*	-	-	-	-	-	-	-	-	-
Wildebeest	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-
Waterbuck	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-
Oryx	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-
Pig	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-
Warthog	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-
Bushpig	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-
Camel	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-
Horse	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-
Dog	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*

Key : \* = reaction of identity  
 - = no reaction

TMA = thermostable muscle antigen



gazelle (Table 6). The majority of the rabbit antisera to TMA retained a wide range of cross-reactions even after extensive absorptions (Table 5). Only rabbit antisera to the TMA of oryx and camel were rendered species-specific using the same absorption procedure. Goat antisera to TMA of cattle, buffalo and bushpig demonstrated species specificity when tested with TMA of closely related species (Fig. 5 and 6, 7 and 8, 9 and 10).

Doberstein and Greuel (1982) using rabbit antisera to serum of eland, springbok and impala failed to differentiate between the meat of the closely related eland and kudu in immunodiffusion tests. Kamiyama et al. (1978b) were unable to differentiate between the meat of sheep and goat using rabbit antiserum to goat albumin in passive haemagglutination tests. Similarly, Kang'ethe et al. (1982) using rabbit antiserum to sheep albumin failed to differentiate between the meat of sheep and goat in an enzyme-linked immunosorbent assay.

These results show that antisera to TMA of bovidae raised in rabbits are less specific while antisera to the same raised in related species show a higher degree of specificity. These results are comparable to those reported by Lindqvist et al. (1982).

Although absorbed rabbit antisera to TMA proved to be less species-specific for the purpose of species identification of

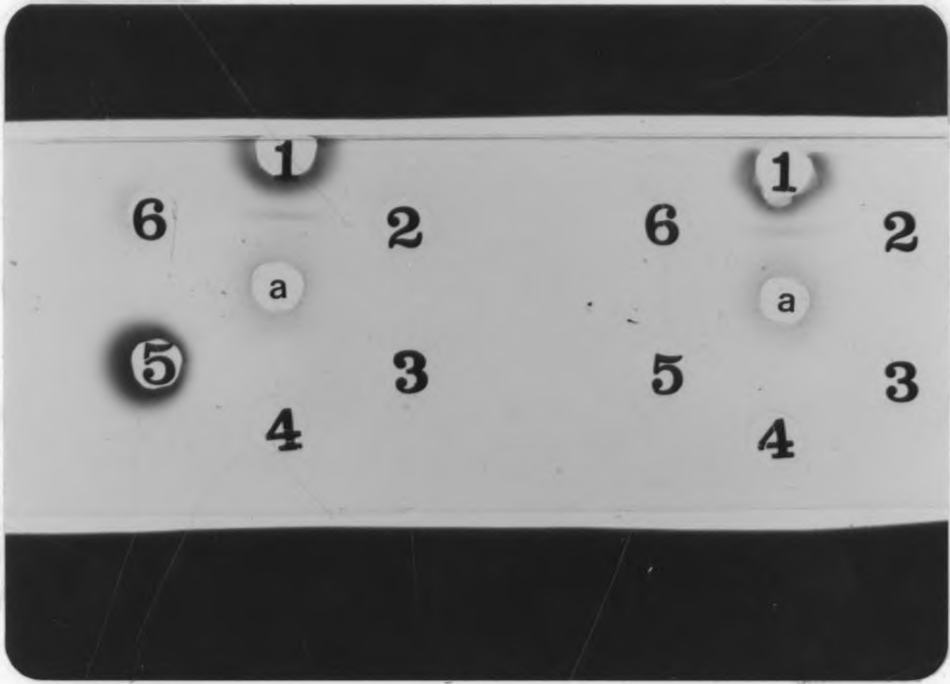


Fig. 5 : Results of immunodiffusion tests using goat antiserum to TMA of cattle absorbed with copolymerized serum proteins and tested with TMA of various species

(a) = Goat antiserum to

TMA of cattle

1 = Cattle TMA	1 = Cattle TMA
2 = Sheep "	2 = Waterbuck "
3 = Goat "	3 = Bushbuck "
4 = Buffalo "	4 = Impala "
5 = Eland "	5 = Thomson's gazelle "
6 = Oryx "	6 = Grant's gazelle "

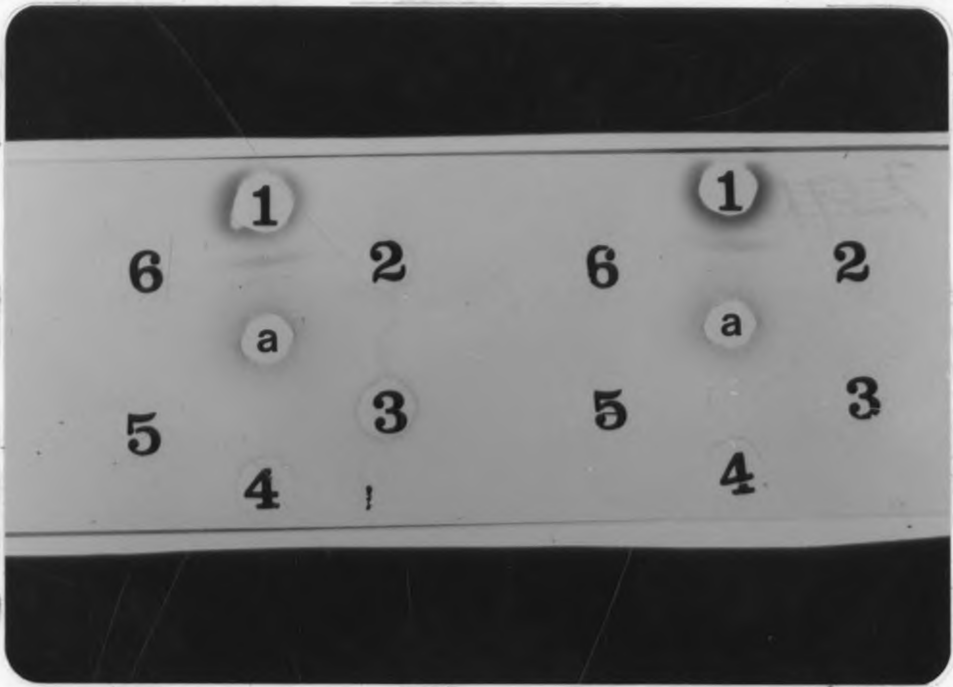


Fig. 6 : Results of immunodiffusion tests using goat antiserum to TMA of cattle absorbed with copolymerized serum proteins and tested with TMA of various species

(a) = Goat antiserum to  
TMA of cattle

- |                  |                |
|------------------|----------------|
| 1 = Cattle TMA   | 1 = Cattle TMA |
| 2 = Kongoni "    | 2 = Pig "      |
| 3 = Topi "       | 3 = Warthog "  |
| 4 = Wildebeest " | 4 = Camel "    |
| 5 = Dog "        | 5 = Bushpig "  |
| 6 = Horse "      | 6 = Buffalo "  |

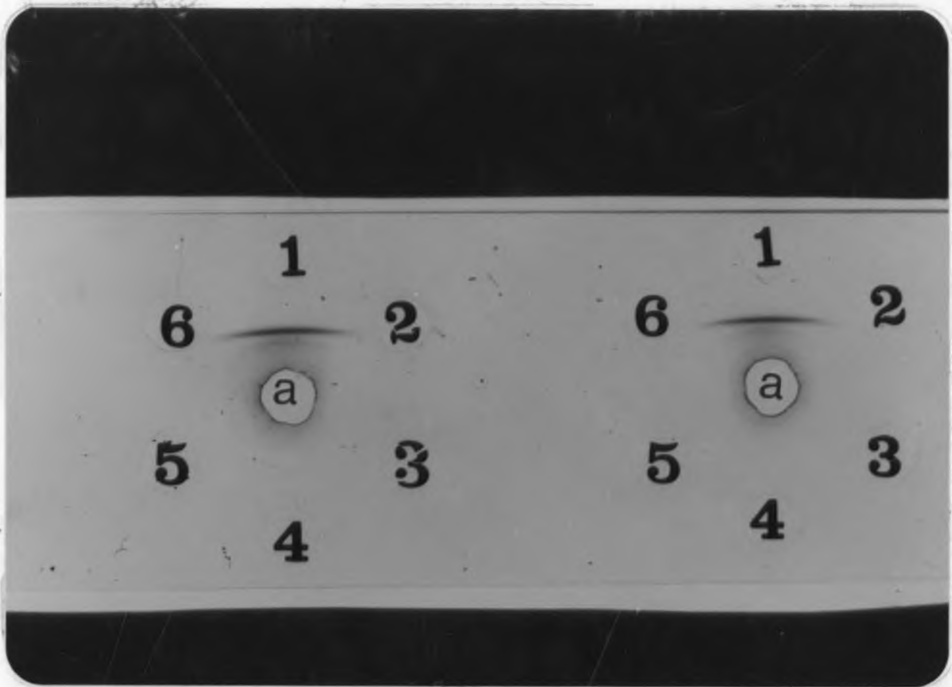


Fig. 7 : Results of immunodiffusion tests using goat antiserum to TMA of buffalo absorbed with copolymerized serum proteins and tested with TMA of various species

(a) = Goat antiserum to

TMA of buffalo

- |                 |                       |     |
|-----------------|-----------------------|-----|
| 1 = Buffalo TMA | 1 = Buffalo           | TMA |
| 2 = Sheep "     | 2 = Bushbuck          | "   |
| 3 = Goat "      | 3 = Waterbuck         | "   |
| 4 = Cattle "    | 4 = Impala            | "   |
| 5 = Eland "     | 5 = Thomson's gazelle | "   |
| 6 = Oryx "      | 6 = Grant's gazelle   | "   |

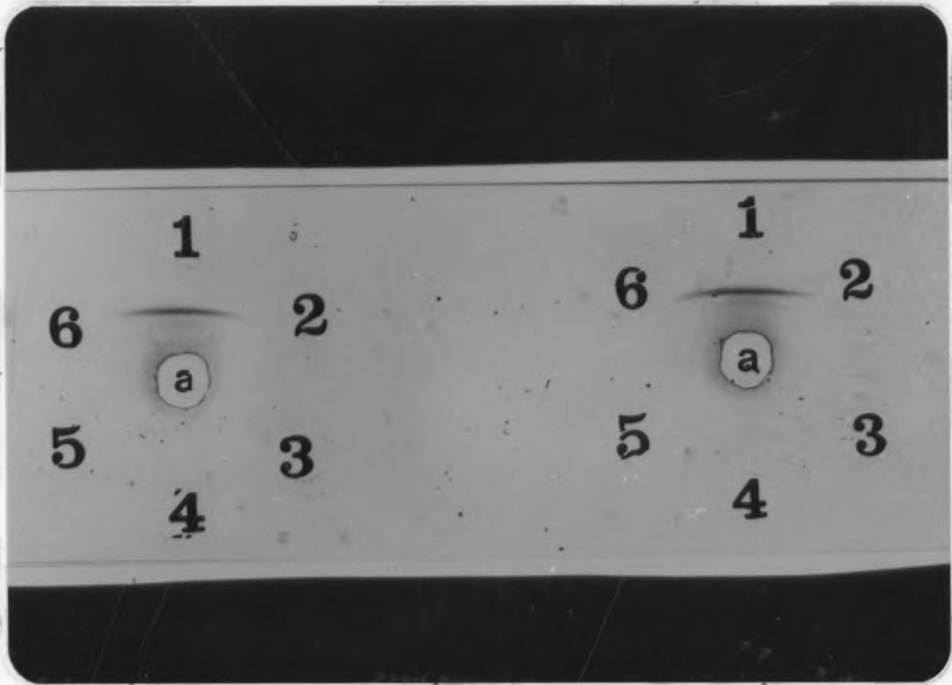


Fig. 8 : Results of immunodiffusion tests using goat antiserum to TMA of buffalo absorbed with copolymerized serum proteins and tested with TMA of various species

(a) = Goat antiserum to  
TMA of buffalo

1 = Buffalo	TMA	1 = Buffalo	TMA
2 = Kongoni	"	2 = Pig	"
3 = Topi	"	3 = Warthog	"
4 = Wildebeest	"	4 = Bushpig	"
5 = Dog	"	5 = Camel	"
6 = Horse	"	6 = Cattle	"

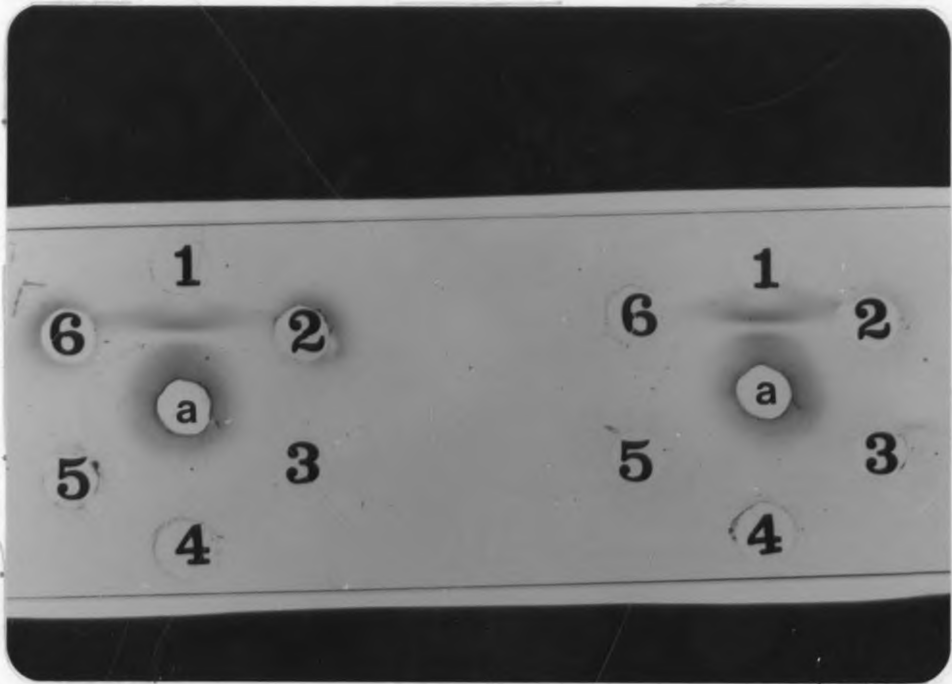


Fig. 9 : Results of immunodiffusion test using goat antiserum to TMA of bushpig absorbed with copolymerized serum proteins and tested with TMA of various species

(a) = Goat antiserum to

TMA of bushpig

1 = Bushpig TMA	1 = Bushpig	TMA
2 = Sheep "	2 = Waterbuck	"
3 = Goat "	3 = Bushbuck	"
4 = Buffalo "	4 = Impala	"
5 = Eland "	5 = Thomson's gazelle	"
6 = Oryx "	6 = Grant's gazelle	"

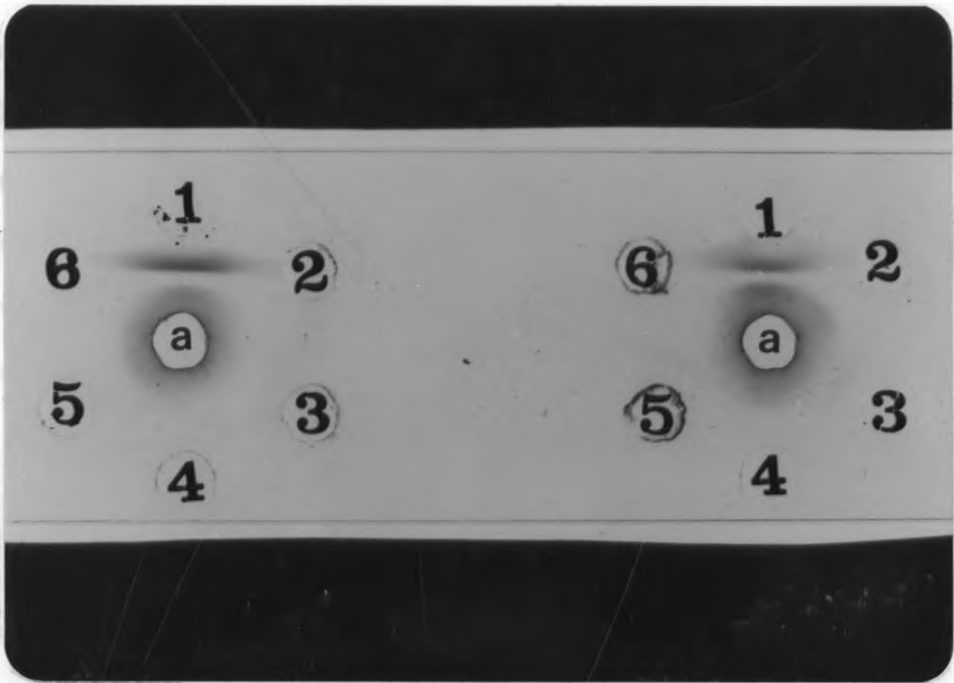


Fig. 10 : Results of immunodiffusion test using goat antiserum to TMA of bushpig absorbed with copolymerized serum proteins and tested with TMA of various species

(a) = Goat antiserum to

TMA of bushpig

- |                |     |             |     |
|----------------|-----|-------------|-----|
| 1 = Bushpig    | TMA | 1 = Bushpig | TMA |
| 2 = Kongoni    | "   | 2 = Pig     | "   |
| 3 = Topi       | "   | 3 = Warthog | "   |
| 4 = Wildebeest | "   | 4 = Camel   | "   |
| 5 = Dog        | "   | 5 = Cattle  | "   |
| 6 = Horse      | "   | 6 = Cattle  | "   |

meat, the rabbit antisera could be used to differentiate reliably meats of distantly related species (Table 5). In addition, the antisera to TMA of topi, Grant's gazelle and warthog could be used to check initially for the presence of bovidae and non-bovidae meats in samples presented for analysis (Table 7).

#### 4.1.2 Species identification of CMA

Most of the absorbed goat antisera to TMA were shown to be species-specific in their reactions with CMA, except goat antisera to TMA of cattle, kongoni, bushpig, pig, topi, Thomson's gazelle and Grant's gazelle. Absorbed goat antiserum to TMA of cattle reacted with buffalo CMA, while goat antiserum to TMA of buffalo did not react with TMA or CMA of cattle (Tables 6 and 8). Similar results were observed with goat antiserum to TMA of pig and the TMA and CMA of bushpig and warthog (Tables 6 and 8).

Reactions observed with goat antisera to TMA of topi, bushpig and horse with CMA of other species are shown in Fig. 11 and 12, 13 and 14, 15 and 16. These results show that absorbed goat antiserum to TMA of horse was species-specific, but antisera to TMA of topi and bushpig cross-reacted with CMA of related species.

Absorbed goat antisera to TMA of kongoni, topi, Thomson's gazelle and Grant's gazelle reacted with TMA and CMA of other



Table 7 : Results of immunodiffusion tests using absorbed rabbit antisera to TMA, which could be used to distinguish meat from bovidae and non-bovidae

Absorbed rabbit antisera to TMA of			
TMA of	Grant's Gazelle	Topi	Warthog
Buffalo	+	+	-
Cattle	+	+	-
Bushbuck	+	*	-
Eland	+	+	-
Goat	-	+	-
Sheep	-	-	-
Grant's gazelle	*	+	-
Thomson's gazelle	*	+	-
Impala	+	+	-
Kongoni	-	*	-
Topi	-	*	-
Wildebeest	-	*	-
Waterbuck	+	-	-
Oryx	+	-	-
Pig	-	-	*
Bushpig	-	-	*
Warthog	-	-	*
Camel	-	-	*
Horse	-	-	-
Dog	-	-	-

Key : \* = reaction of identity  
 + = reaction of partial identity  
 - = no reaction  
 TMA = thermostable muscle antigen

Table 8 : Results of immunodiffusion tests using goat antisera to TMA absorbed with copolymerized serum proteins and tested with CMA of various species

CMA of	Goat antisera to TMA of																		
	Buffalo	Cattle	Bushbuck	Eland	Grant's gazelle	Thomson's gazelle	Impala	Kongoni	Topi	Wildebeest	Waterbuck	Oryx	Pig	Warthog	Bushpig	Horse	Camel	Dog	
Buffalo	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cattle	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bushbuck	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Eland	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Goat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sheep	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Grant's gazelle	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Thomson's gazelle	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Impala	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Kongoni	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Topi	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Wildebeest	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Waterbuck	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Oryx	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Pig	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-
Warthog	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-
Bushpig	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-
Horse	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Camel	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Dog	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+

Key : + = reaction of identity  
 - = no reaction

CMA = cooked meat antigen  
 TMA = thermostable muscle antigens

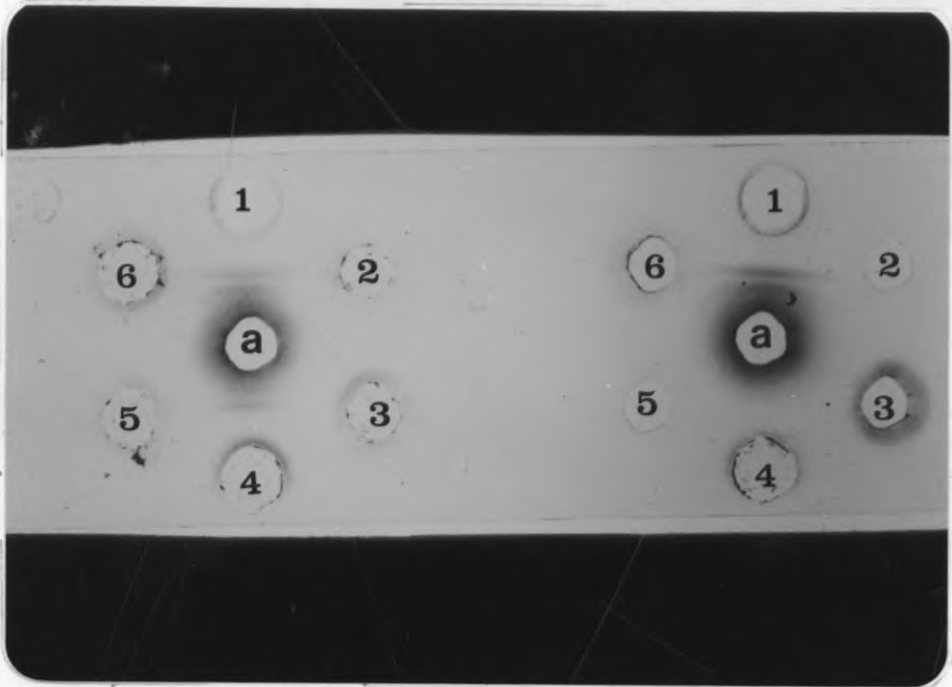


Fig. 11 : Results of immunodiffusion tests using goat  
antiserum to TMA of topi absorbed with copolymerized  
serum proteins and tested with CMA of various species

(a) = Goat antiserum to

TMA of topi

1 = Topi	CMA	1 = Topi	CMA
2 = Sheep	"	2 = Eland	"
3 = Goat	"	3 = Wildebeest	"
4 = Kongoni	"	4 = Waterbuck	"
5 = Cattle	"	5 = Bushbuck	"
6 = Oryx	"	6 = Kongoni	"

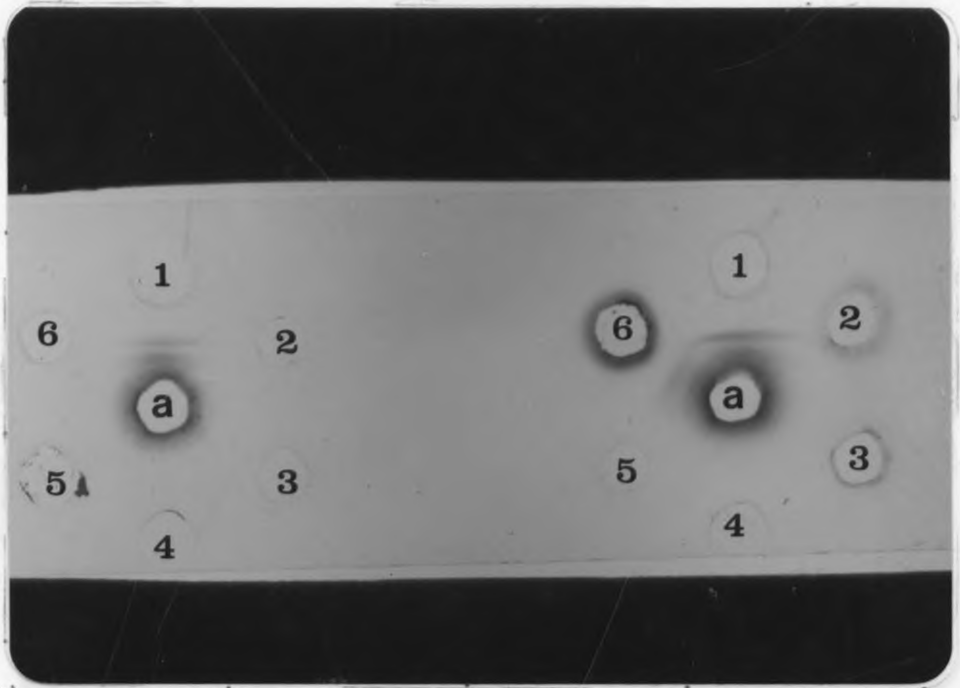


Fig. 12 : Results of immunodiffusion tests using goat antiserum to TMA of topi absorbed with polymerized serum proteins and tested with CMA of various species

(a) = Goat antiserum to

TMA of topi

1 = Topi	CMA	1 = Topi	CMA
2 = Thomons's gazelle	"	2 = Warthog	"
3 = Grant's gazelle	"	3 = Buffalo	"
4 = Dog	"	4 = Bushpig	"
5 = Pig	"	5 = Camel	"
6 = Horse	"	6 = Kongoni	"

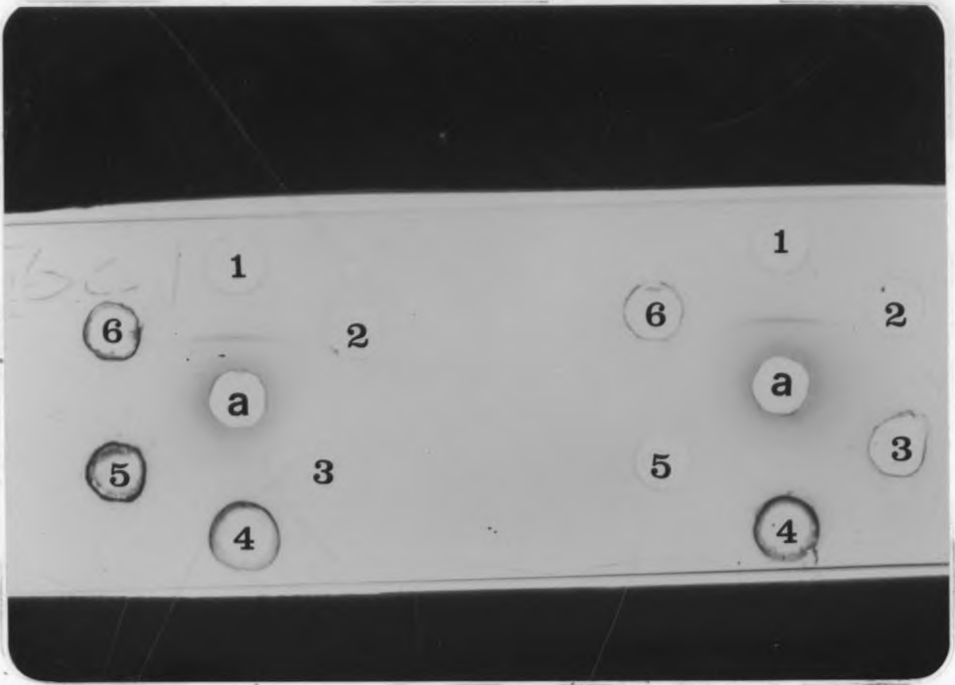


Fig. 13 : Results of immunodiffusion tests using goat antiserum to TMA of warthog absorbed with copolymerized serum proteins and tested with CMA of various species

(a) = Goat antiserum to

TMA of warthog

- |                 |                  |
|-----------------|------------------|
| 1 = Warthog CMA | 1 = Warthog CMA  |
| 2 = Sheep "     | 2 = Eland "      |
| 3 = Goat "      | 3 = Wildebeest " |
| 4 = Kongoni "   | 4 = Waterbuck "  |
| 5 = Cattle "    | 5 = Bushbuck "   |
| 6 = Oryx "      | 6 = Impala "     |

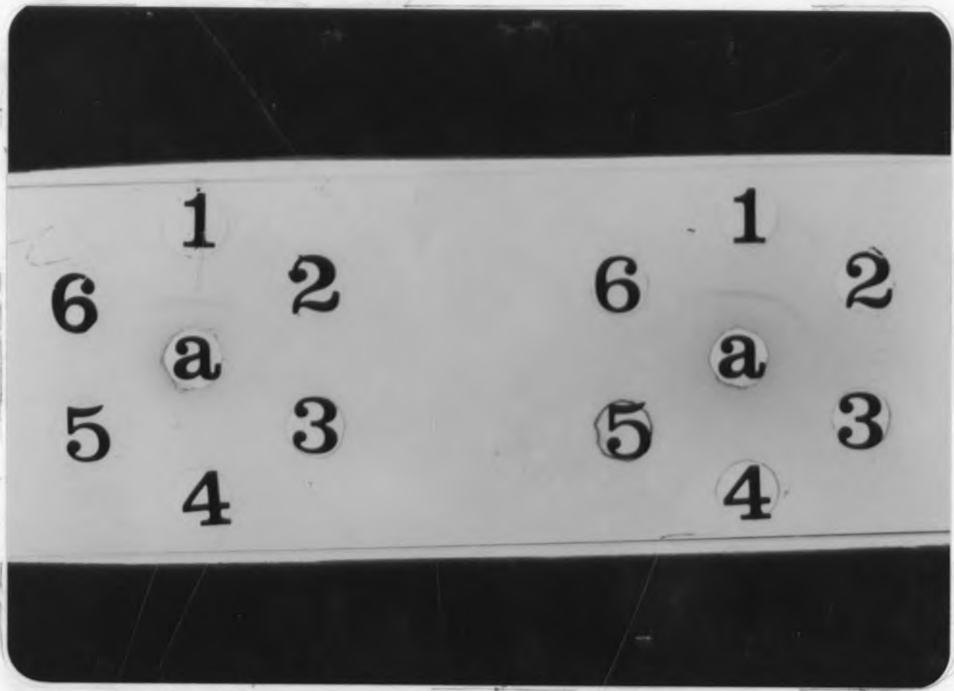


Fig. 14 : Results of immunodiffusion tests using goat antiserum to TMA of warthog absorbed with copolymerized serum proteins and tested with CMA of various species

(a) = Goat antiserum to

TMA of warthog

1 = Warthog	CMA	1 = Warthog	CMA
2 = Thomson's gazelle	"	2 = Pig	"
3 = Grant's gazelle	"	3 = Buffalo	"
4 = Dog	"	4 = Topi	"
5 = Camel	"	5 = Kongoni	"
6 = Dog	"	6 = Bushpig	"

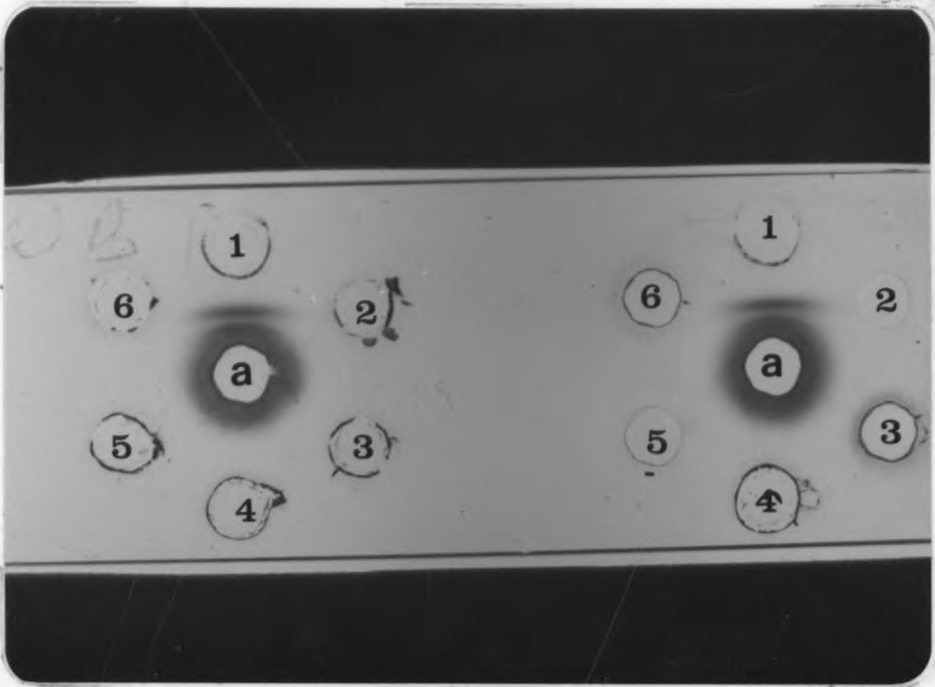


Fig. 15 : Results of immunodiffusion tests using goat antiserum to TMA of horse absorbed with copolymerized serum proteins and tested with CMA of various species

(a) = Goat antiserum to

TMA of horse

1 = Horse	CMA	1 = Horse	CMA
2 = Sheep	"	2 = Eland	"
3 = Goat	"	3 = Wildebeest	"
4 = Kongoni	"	4 = Waterbuck	"
5 = Cattle	"	5 = Bushbuck	"
6 = Oryx	"	6 = Impala	"

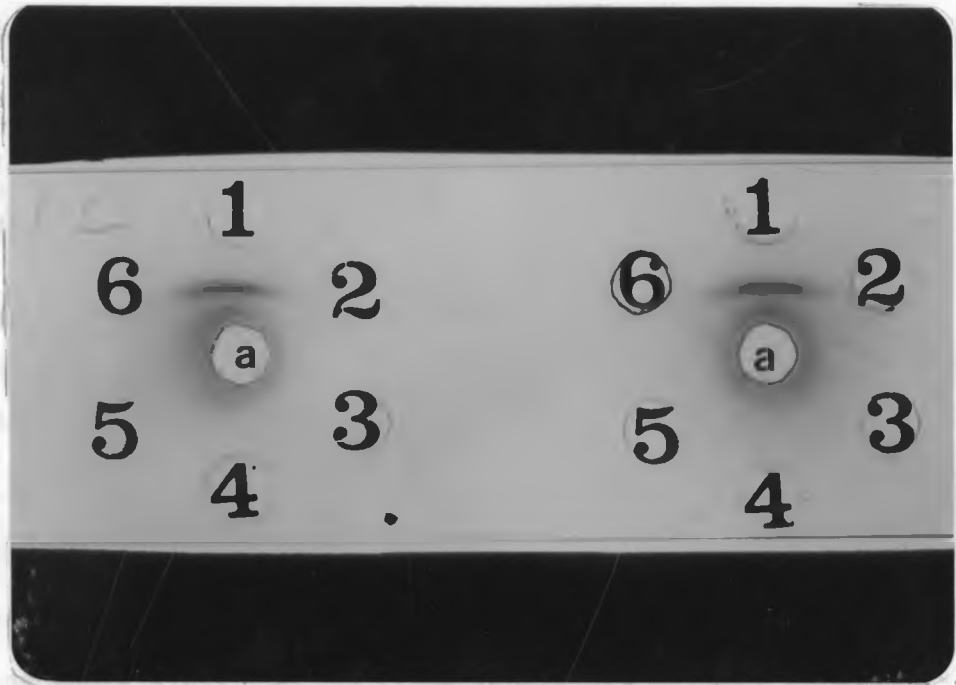


Fig. 16 : Results of immunodiffusion tests using goat antiserum to TMA of horse absorbed with copolymerized serum proteins and tested with CMA of various species

(a) = Goat antiserum to

TMA of horse

- |                       |     |             |     |
|-----------------------|-----|-------------|-----|
| 1 = Horse             | CMA | 1 = Horse   | CMA |
| 2 = Thomson's gazelle | "   | 2 = Pig     | "   |
| 3 = Grant's gazelle   | "   | 3 = Buffalo | "   |
| 4 = Warthog           | "   | 4 = Bushpig | "   |
| 5 = Camel             | "   | 5 = Topi    | "   |
| 6 = Dog               | "   | 6 = Kongoni | "   |



species (Table 6 and 8), while goat antisera to TMA of cattle, pig and bushpig reacted only with CMA and not with TMA of closely related species (Table 6 and 8).

These results indicate that the species-specificity observed when goat antisera to TMA is tested with TMA of various species may possibly be a result of emergence of novel antigens due to the heat treatment. Hirose and Osler (1967), Henney and Ishizaka (1968) and Karpas et al. (1970) reported the emergence of new antigens arising during the heating process. However, if this were true, one would have expected a wider species cross-reactivity between goat antisera to TMA of bovidae and CMA because the TMA precursors present in fresh meat of these species could be similar if not identical. It is possible that the autoclaving step may produce conformational changes which expose the species-specific epitopes masked by non-specific determinants common in CMA of closely related species.

#### 4.1.3 Species identification of FMA

The results given in Table 9, show that absorbed goat antisera to most of the TMA were species-specific, except antisera to TMA of cattle, bushpig, kongoni, warthog, Grant's gazelle and Thomson's gazelle which reacted with FMA of closely related species.

Absorbed goat antisera to TMA of Thomson's gazelle cross-reacted with FMA of Grant's gazelle but not with the

Table 9 : Results of immunodiffusion tests using goat antisera to TMA absorbed with copolymerized serum proteins and tested with FMA of various species

FMA of	Goat antisera to TMA of																	
	Buffalo	Cattle	Bushbuck	Eland	Grant's gazelle	Thomson's gazelle	Impala	Kongoni	Topi	Wildebeest	Waterbuck	Oryx	Pig	Warthog	Bushpig	Camel	Horse	Dog
Buffalo	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cattle	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bushbuck	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Eland	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Goat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sheep	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Grant's gazelle	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Thomson's gazelle	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Impala	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Kongoni	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
Topi	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
Wildebeest	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Waterbuck	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Oryx	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Pig	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-
Warthog	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
Bushpig	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
Camel	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Horse	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Dog	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

Key : + = reaction of identity  
 - = no reaction

FMA = fresh meat antigen  
 TMA = thermostable muscle antigens

FMA of goat, sheep and impala (Fig. 17 and 18) which are closely related. Absorbed goat antisera to TMA of bushpig cross-reacted with FMA of pig and warthog but not with FMA of other species (Fig. 19 and 20).

Reactions of goat antisera to TMA with FMA revealing a high degree of species-specificity strongly suggests that TMA are naturally occurring antigens of muscle tissue and are not new antigens arising from heat denaturation.

#### 4.1.4 Species identification of organs

Results of immunodiffusion tests using absorbed goat antisera to TMA of cattle, pig, kongoni and Thomson's gazelle with TOA of liver, heart and kidney are shown in Table 10. These results show that antisera to TMA can also be used to identify the species of origin of organs.

Results of immunodiffusion tests using absorbed goat antiserum to TMA of cattle and TOA of cattle liver, heart and kidney are shown in Fig. 21. These results show that species-specific TMA of cattle exist as three distinct antigens. One antigen that is species-specific, and found in the striated muscles (skeletal and cardiac) and in other organs (kidney and liver). The second antigen was found to be restricted only to the striated muscles (skeletal and cardiac) while a third antigen was found to be specific for skeletal muscle. Similar results were obtained when goat antisera to TMA of kongoni, pig and Thomson's gazelle reacted with their respective TOA.

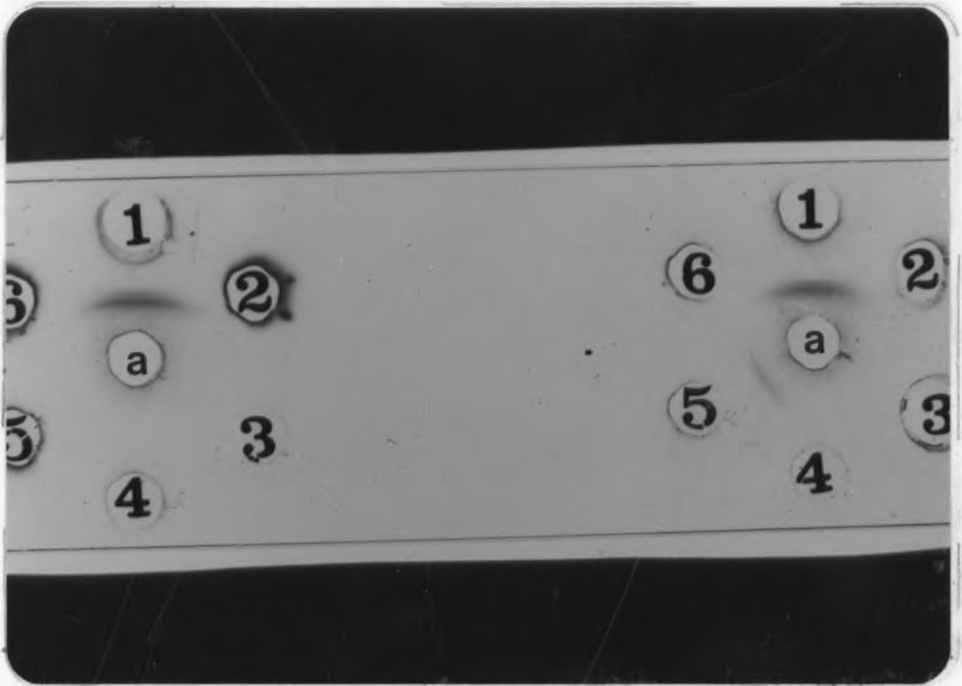


Fig. 17 : Results of immunodiffusion tests using goat antiserum to TMA of Thomson's gazelle absorbed with copolymerized serum proteins and tested with FMA of various species

(a) = Goat antiserum to

TMA of Thomson's gazelle

1 = Thomson's gazelle FMA		1 = Thomson's gazelle FMA	
2 = Sheep	"	2 = Waterbuck	"
3 = Goat	"	3 = Bushbuck	"
4 = Cattle	"	4 = Impala	"
5 = Eland	"	5 = Grant's gazelle	"
6 = Oryx	"	6 = Buffalo	"

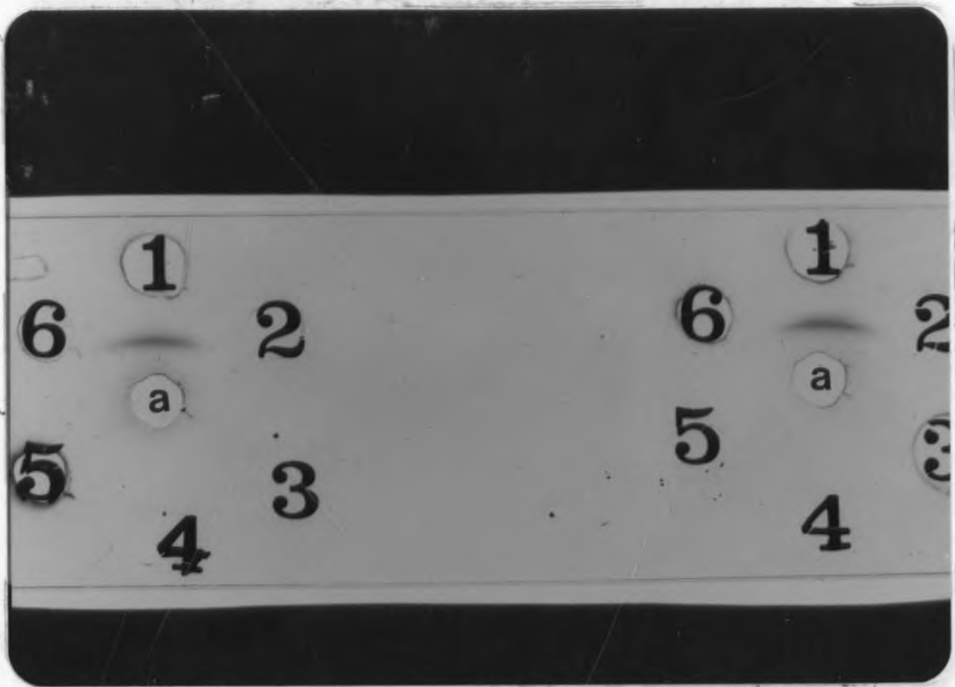


Fig. 18 : Results of immunodiffusion tests using goat antiserum to TMA of Thomson's gazelle absorbed with copolymerized serum proteins and tested with FMA of various species

(a) = Goat antiserum to

TMA of Thomson's gazelle

- |                           |                           |
|---------------------------|---------------------------|
| 1 = Thomson's gazelle FMA | 1 = Thomson's gazelle FMA |
| 2 = Kongoni "             | 2 = Pig "                 |
| 3 = Topi "                | 3 = Warthog "             |
| 4 = Wildebeest "          | 4 = Camel "               |
| 5 = Dog "                 | 5 = Bushpig "             |
| 6 = Pig "                 | 6 = Cattle "              |

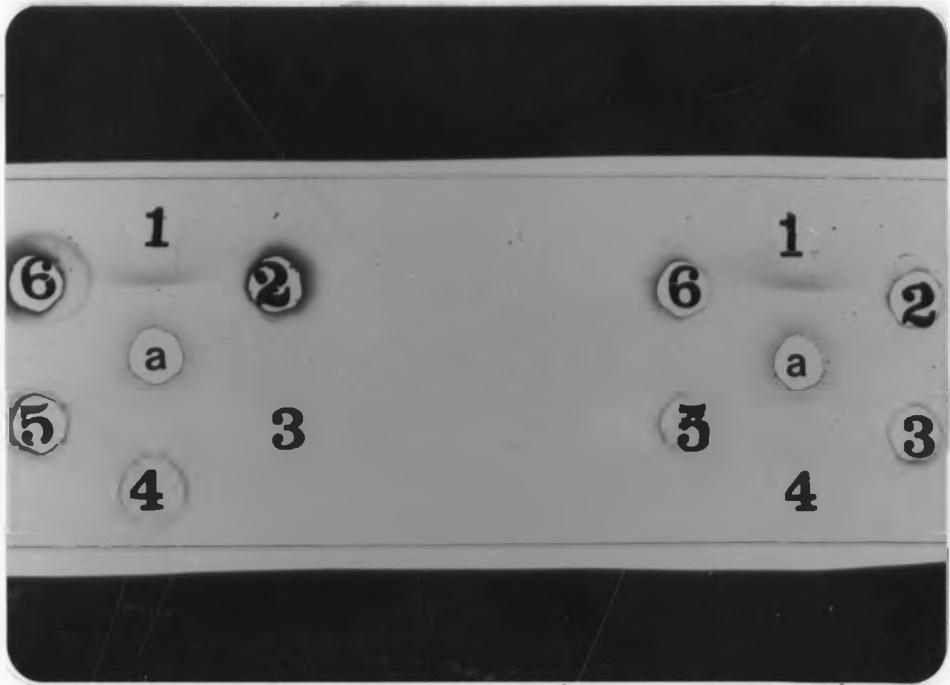


Fig. 19 : Results of immunodiffusion tests using goat antiserum to TMA of bushpig absorbed with copolymerized serum proteins and tested with FMA of various species

(a) = Goat antiserum to

TMA of bushpig

1 = Bushpig FMA	1 = Bushpig	FMA
2 = Sheep "	2 = Waterbuck	"
3 = Goat "	3 = Bushbuck	"
4 = Cattle "	4 = Impala	"
5 = Eland "	5 = Grant's gazelle	"
6 = Oryx "	6 = Thomson's gazelle	"

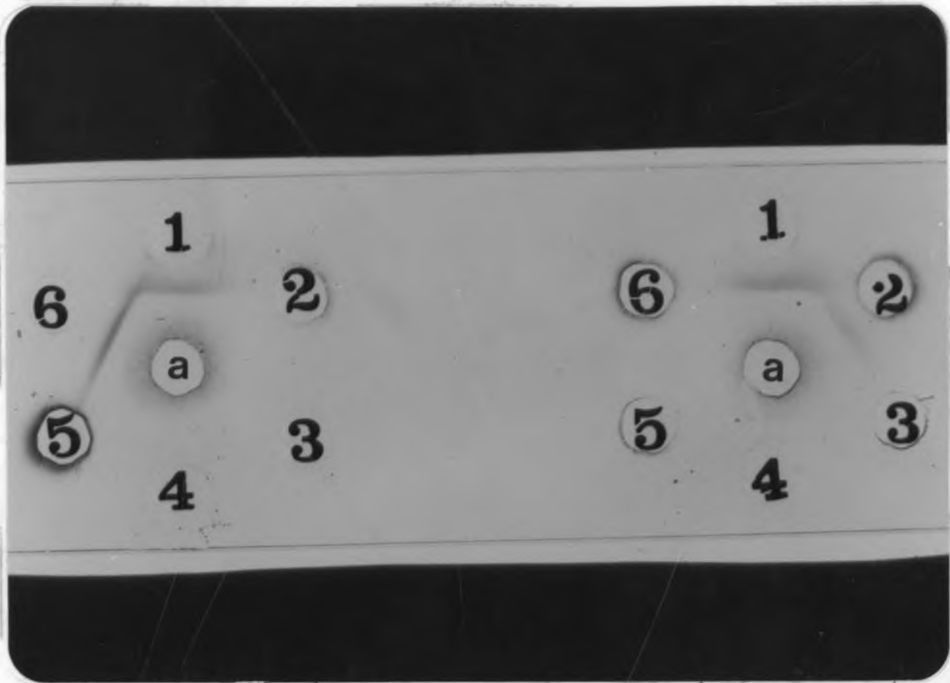


Fig. 20 : Results of immunodiffusion tests using goat antiserum to TMA of bushpig absorbed with copolymerized serum proteins and tested with FMA of various species

(a) = Goat antiserum to

TMA of bushpig

1 = Bushpig	FMA	1 = Bushpig	FMA
2 = Kongoni	"	2 = Warthog	"
3 = Topi	"	3 = Horse	"
4 = Wildebeest	"	4 = Camel	"
5 = Dog	"	5 = Buffalo	"
6 = Pig	"	6 = Cattle	"

Table 10 : Species identification of internal organs using goat antisera to TMA absorbed with copolymerized serum proteins and tested with TOA in immunodiffusion tests

Organ	TOA of	Goat antisera to TMA of			
		Cattle	Kongoni	Thomson's gazelle	Pig
Heart	Cattle	*	-	-	-
	Kongoni	-	*	-	-
	Thomson's gazelle	-	-	*	-
	Pig	-	-	-	*
Kidney	Cattle	*	-	-	-
	Kongoni	-	*	-	-
	Thomson's gazelle	-	-	*	-
	Pig	-	-	-	*
Liver	Cattle	*	-	-	-
	Kongoni	-	*	-	-
	Thomson's gazelle	-	-	*	-
	Pig	-	-	-	*

Key :      \* = reaction of identity  
              - = no reaction  
              TOA = thermostable organ antigens  
              TMA = thermostable muscle antigens



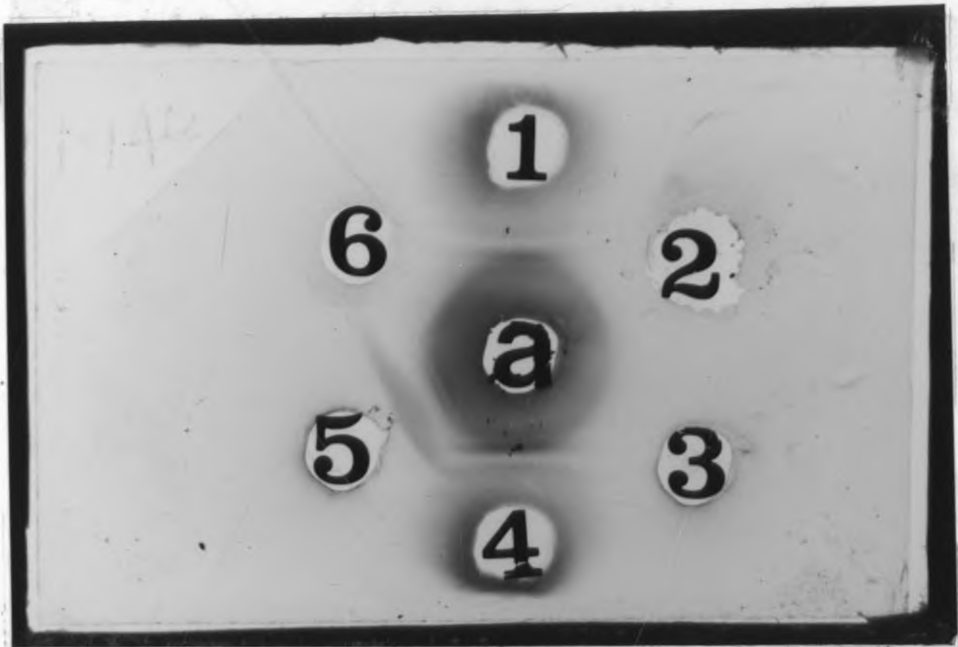


Fig. 21 : Results of immunodiffusion tests using goat antiserum to TMA of cattle absorbed with copolymerized serum proteins and tested with TOA of cattle

(a) = Goat antiserum to TMA of cattle

1 = cattle TMA

2 = cattle liver TOA

3 = cattle kidney "

4 = cattle TMA

5 = cattle heart TOA

6 = cattle lung "

Milgrom et al. (1963) described the presence of two adrenal thermostable antigens, which were species and adrenal gland specific. In this study, species-specific TMA has been shown to consist of at least three antigens. The adrenal gland specific thermostable antigen may be compared to the skeletal muscle specific antigen.

#### 4.2 ENZYME IMMUNOASSAY (EIA)

##### 4.2.1 Purification of TMA

The fractions from the Sephadex G-200 and G-75 columns that contained the PTMA as detected by EIA, gave low absorbances at 280 nm. The fractions with high absorbances at 280nm were shown to contain no specific activity to goat antiserum to TMA of buffalo (Figs. 22 and 23).

The proteins represented by the high absorbances at 280 nm in the fractions from both Sephadex G-75 and G-200 columns and shown not to be antigenic, would compete for binding to the microtitre plate with the PTMA and result in inconsistent and irregular adsorptions observed using TMA extracted according to the method of Milgrom and Witebsky (1962). While these TMA may provide antigens suitable for use in immunodiffusion tests, such antigens are unsuitable for use in EIA. Although ultrafiltration alone using PM30 is capable of some degree of purification, it is not recommended as the only method for the preparation of PTMA for use in EIA.

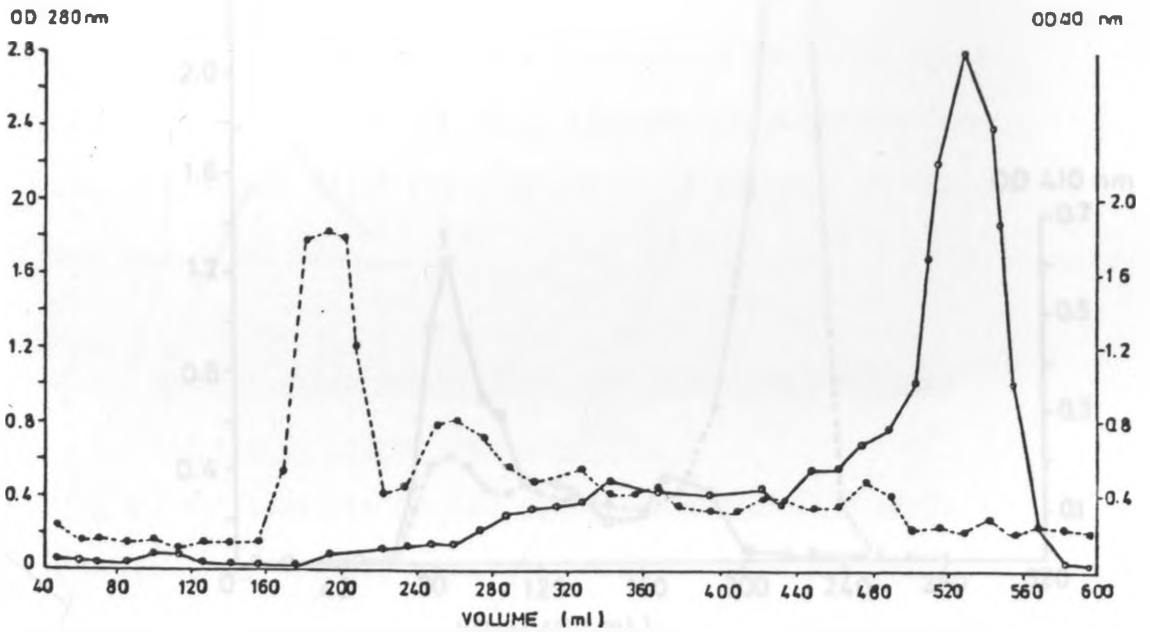


Fig. 22 : Fractionation of buffalo TMA on Sephadex G-200 column

Three ml of buffalo TMA (11.50 mg protein/ml) were fractionated on Sephadex G-200 column, <sup>length</sup> 94.4 cm and 2.6 cm diameter. The optical density of fractions was read at 280 nm (—○—) and their antigen contents determined by a standard EIA (OD 410 nm ---●---).

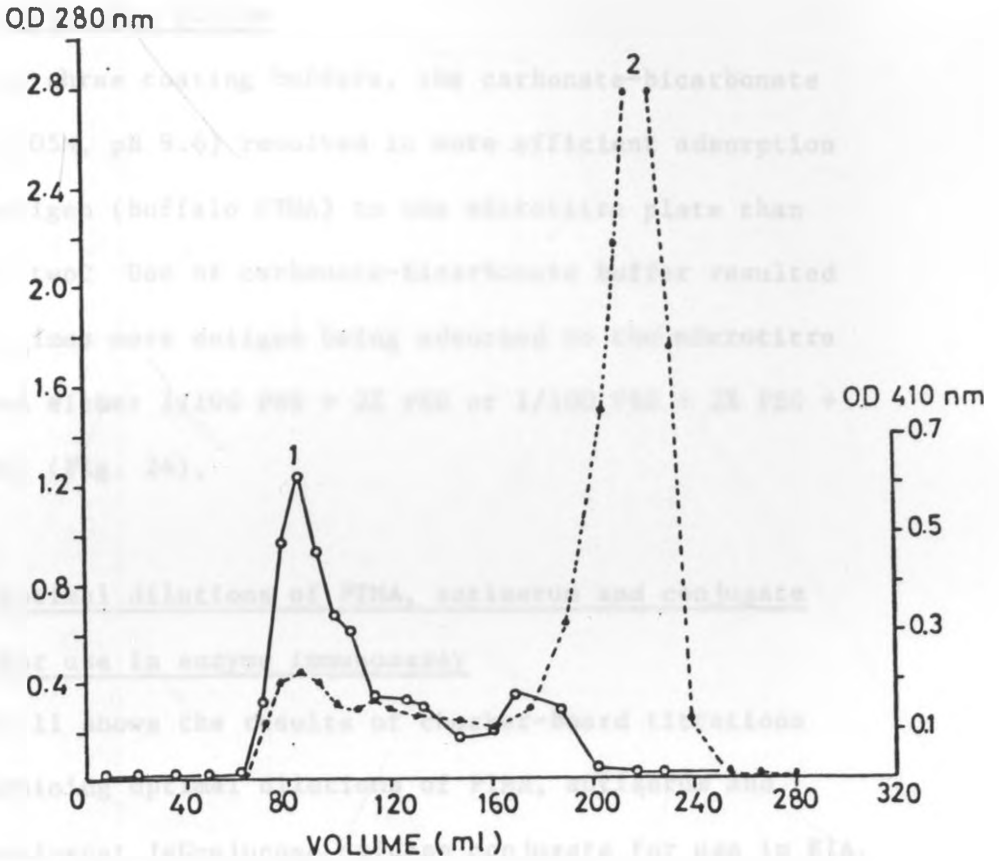


Fig. 23 : Fractionation of buffalo TMA on Sephadex G-75  
column

Three ml of buffalo TMA (11.50 mg protein/ml) were fractionated on Sephadex G-75 column, length 35.5 cm and 2.6 cm diameter. The optical density of the fractions was read at 280 nm (---●-----●-) and their antigen contents determined by a standard EIA (OD 410 nm —○—○—)

#### 4.2.2 Effect of coating buffer on the adsorption of PTMA to microtitre plates

Of the three coating buffers, the carbonate-bicarbonate buffer (0.05M, pH 9.6) resulted in more efficient adsorption of the antigen (buffalo PTMA) to the microtitre plate than the other two. Use of carbonate-bicarbonate buffer resulted in forty times more antigen being adsorbed to the microtitre plate than either 1/100 PBS + 2% PEG or 1/100 PBS + 2% PEG + 0.5 M NaCl (Fig. 24).

#### 4.2.3 Optimal dilutions of PTMA, antiserum and conjugate for use in enzyme immunoassay

Table 11 shows the results of checker-board titrations for determining optimal dilutions of PTMA, antiserum and rabbit anti-goat IgG-glucose oxidase conjugate for use in EIA.

#### 4.2.4 Effect of gelatin on the adsorption of the antigen to microtitre plates

Thermostable muscle antigens when used to coat microtitre plates resulted in inconsistent and irregular adsorption to the plates. These antigens were also observed to gel at 4°C, indicating that they contained high amounts of gelatin. This gelatin was thought to be the cause of poor adsorption of the PTMA on the plates.

Qualitative assay of hydroxyproline in the TMA extracted according to the method of Milgrom and Witebsky (1962), PTMA,

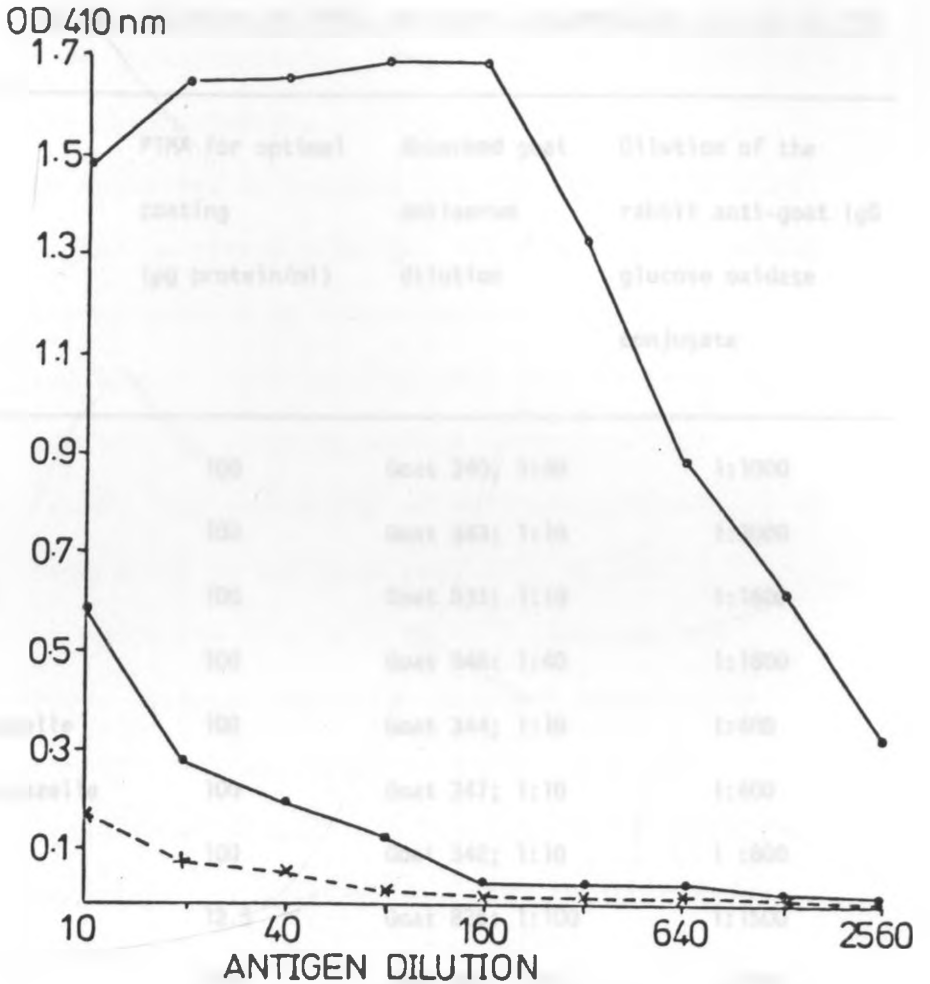


Fig. 24 : Effect of three buffers on the adsorption of the PTMA to microtitre plates

Buffalo PTMA (100 µg protein/ml) was diluted serially in 0.05 M carbonate-bicarbonate buffer, pH 9.6 (—○—○—), 1/100 PBS + 2% PEG + 0.5 M NaCl (—●—●—) and 1/100 PBS + 2% PEG (—\*—\*—). The dilutions were applied to microtitre plates and the adsorption of antigen determined by a standard EIA.

Table 11 : Optimal dilutions of PTMA, antiserum and conjugate for use in EIA

PTMA	PTMA for optimal coating ( $\mu\text{g}$ protein/ml)	Absorbed goat antiserum dilution	Dilution of the rabbit anti-goat IgG glucose oxidase conjugate
Buffalo	100	Goat 340; 1:40	1:1000
Cattle	100	Goat 343; 1:10	1:3000
Bushbuck	100	Goat 831; 1:10	1:1600
Eland	100	Goat 346; 1:40	1:1600
Grant's Gazelle	100	Goat 344; 1:10	1:400
Thomson's gazelle	100	Goat 347; 1:10	1:400
Impala	100	Goat 342; 1:10	1 :800
Kongoni	12.5	Goat 826; 1:100	1:1500
Topi	100	Goat 829; 1:80	1:1500
Wildebeest	100	Goat 827; 1:10	1:3000
Waterbuck	12.5	Goat 832; 1:20	1:1500
Oryx	100	Goat 856; 1:40	1:800
Pig	6.25	Goat 863; 1:640	1:3000
Warthog	100	Goat 869; 1:80	1:1500
Horse	12.5	Goat 897; 1:40	1:3000
Camel	100	Goat 837; 1:40	1:3000

fractions of Sephadex G-200 that showed no antigenic activity in EIA, and the concentrated extracts of the modified procedure before chromatography, showed that TMA, concentrated extracts before chromatography and the Sephadex G-200 fractions that contained no antigen contained hydroxyproline while the PTMA had no hydroxyproline. This was an indication of the presence of gelatin in these preparations.

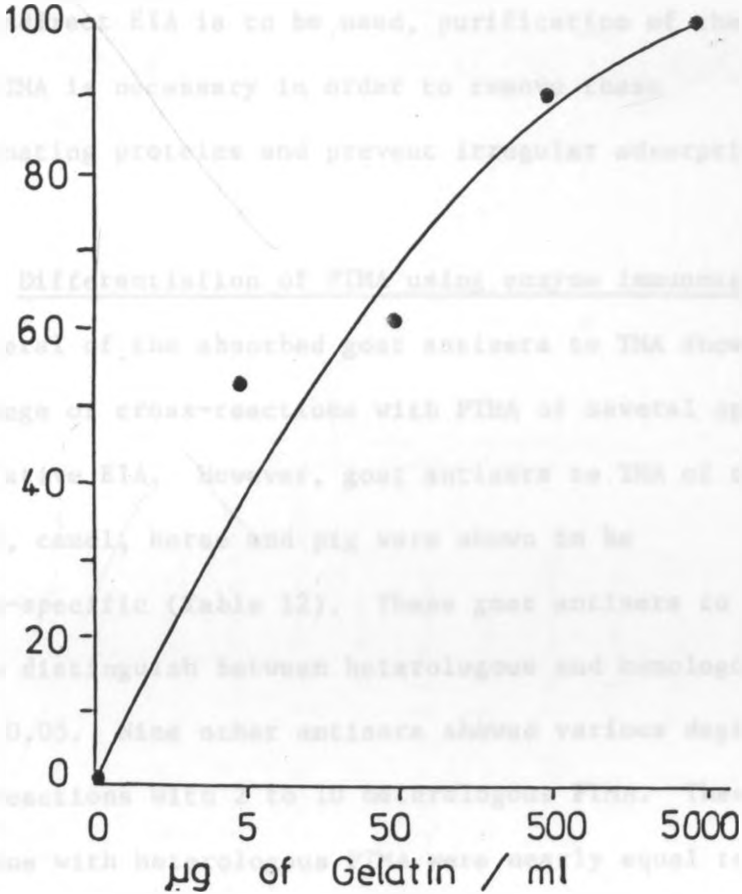
Machlik and Draudt (1963) showed that when collagen is heated to about 60°C it shortens and is converted into a more soluble form. The percentage of collagen solubilized by heating has been shown to increase gradually as the temperature is increased from 60°C to 90°C (Snowden and Weidemann, 1978). Gelatin formation has been shown to be swift with pressure cooking (Bendall, 1946). The TMA extraction procedure of Milgrom and Witebsky (1962) provides favourable conditions for gelatin formation as it includes cooking and autoclaving steps.

Increasing the amount of gelatin in the buffalo PTMA, resulted in an increased inhibitory effect on the adsorption of the PTMA to the microtitre plate. A gelatin concentration of 5 mg/ml resulted in 100% inhibition of the antigen adsorption to the plate (Fig. 25).

The presence of gelatin in TMA extracts and Sephadex G-200 fractions as demonstrated by the assay for hydroxyproline and the contaminating proteins present in TMA would inhibit the adsorption of the PTMA to the plates.



% INHIBITION



+ 42.7 µg of protein of buffalo PTMA

Fig. 25 : The inhibitory effect of gelatin on the adsorption of PTMA to microtitre plates

A fixed amount of buffalo PTMA (42.7 µg protein/ml) was mixed with increasing amounts of commercial gelatin and applied to microtitre plates. A standard EIA was performed and the percent inhibition calculated.

Where indirect EIA is to be used, purification of the TMA to yield PTMA is necessary in order to remove these contaminating proteins and prevent irregular adsorptions.

#### 4.2.5 Differentiation of PTMA using enzyme immunoassay

Several of the absorbed goat antisera to TMA showed a wide range of cross-reactions with PTMA of several species in quantitative EIA. However, goat antisera to TMA of topi, buffalo, camel, horse and pig were shown to be species-specific (Table 12). These goat antisera to TMA were able to distinguish between heterologous and homologous PTMA at  $p \leq 0.05$ . Nine other antisera showed various degrees of cross-reactions with 2 to 10 heterologous PTMA. These reactions with heterologous PTMA were nearly equal to those given by the homologous PTMA. Absorption of the antisera with the highly cross-reacting PTMA resulted in abolition of the homologous reaction.

Goat antiserum to TMA of cattle and eland cross-reacted with 13 and 15 heterologous PTMA respectively. Reactions with these PTMA did not allow unequivocal differentiation of the homologous from the heterologous PTMA. However, goat antiserum to TMA of cattle gave weak reactions with PTMA of topi, pig, horse and Thomson's gazelle which enabled differentiation between homologous and these heterologous PTMA at  $p \leq 0.05$ . Similarly, goat antiserum to TMA of eland

Table 12 : Species differentiation of PTMA in enzyme immunoassay

PTMA of	Goat antisera to TMA of															
	Buffalo	Cattle	Bushbuck	Eland	Grant's Gazelle	Thomson's Gazelle	Impala	Kongoni	Topi	Wildebeest	Waterbuck	Oryx	Pig	Warthog	Camel	Horse
Buffalo	A	NS	NS	NS	NS	NS	NS	NS	**	NS	NS	NS	**	*	*	**
Cattle	**	A	**	*	**	***	*	NS	*	**	**	*	**	**	**	***
Bushbuck	**	NS	A	NS	**	***	*	**	*	**	*	*	**	**	**	***
Eland	**	NS	NS	A	*	NS	**	**	**	NS	**	*	**	**	**	***
Goat	**	NS	**	NS	*	NS	*	**	**	**	***	*	**	NS	**	**
Sheep	**	NS	**	NS	**	*	*	**	**	**	***	**	**	NS	**	**
Grant's Gazelle	**	NS	NS	NS	A	NS	*	NS	*	NS	NS	*	**	*	**	***
Thomson's Gazelle	**	*	**	NS	**	A	*	NS	*	**	**	*	**	*	**	***
Impala	**	NS	NS	NS	NS	NS	A	**	**	**	*	*	**	**	*	**
Kongoni	**	NS	NS	NS	NS	**	NS	A	*	**	***	*	**	**	**	***
Topi	**	*	*	NS	NS	**	NS	NS	A	**	**	*	**	NS	**	***
Wildebeest	**	NS	NS	NS	NS	NS	NS	NS	**	A	NS	*	**	**	*	**
Waterbuck	**	NS	*	NS	*	**	*	NS	**	**	A	*	**	*	*	**
Oryx	**	NS	NS	NS	*	NS	*	*	*	NS	**	A	**	**	*	***
Pig	**	*	NS	NS	NS	NS	*	*	**	NS	**	*	A	**	*	***
Warthog	**	NS	NS	NS	*	NS	*	*	**	NS	**	*	***	A	*	**
Camel	**	NS	NS	NS	NS	NS	NS	NS	**	NS	**	NS	**	*	A	**
Horse	*	*	**	**	NS	***	*	*	**	**	***	*	**	**	*	A

Key: Significance level  
 \*\*\* = P<0.001  
 \*\* = P<0.01  
 \* = P<0.05  
 TMA = thermostable muscle antigen  
 PTMA = partially purified thermostable muscle antigen  
 NS = not significant  
 A = homologous system

reacted with PTMA of cattle and horse giving reactions that allowed differentiation between the PTMA of eland from those of cattle and horse at  $p \leq 0.05$ .

While goat antisera to TMA of eland and cattle reacted with most of PTMA of other species giving reactions that did not allow differentiation of homologous from heterologous PTMA, the PTMA of cattle and eland reacted with most of the goat antisera to TMA of other species, giving reactions that allowed these PTMA to be differentiated from the PTMA of other species (Table 12).

In instances where a single antiserum failed to distinguish between homologous and heterologous PTMA, species identification could be achieved in EIA by employing a number of goat antiserum to TMA of several species. Using this technique, goat antisera to TMA provided reagents that were capable of correctly identifying the PTMA of all the eighteen species examined.

Because of their exquisite specificity, monoclonal antibodies to TMA would be more suitable for species identification than the polyclonal antisera used in this study. Monoclonal antibodies to heated ( $70^{\circ}\text{C}$ ) meat extracts of cattle and kangaroo have been used successfully to identify the species of origin of fresh and heated meat in EIA by Georlich and Greuel (1986). However, it is conceivable that immunization of additional animals and

animals of different species especially within the bovidae would yield polyclonal antisera with sufficient specificity for use in EIA. Such polyclonal antisera has been raised to whole serum of vertebrate species by Rurangirwa et al. (1986).

In an experiment to determine the lowest detectable level of buffalo PTMA in beef, impala in goat and warthog in pig, the EIA failed to detect 1% and 10% buffalo in cattle, impala in goat and warthog in pig at  $p < 0.05$ . Lower level of adulteration 0.5% has been detected in fresh meats using sandwich EIA by Jones and Patterson (1985). These authors used fresh meats from remotely related animal species, cattle and pig. In this study, heat-treated meats (PTMA) of unrelated species were easily differentiated (Table 12). If such species were chosen, lower levels of admixtures could have been detected.

#### 4.2.6 Determination of the molecular weight of the PTMA using gel filtration

The buffalo PTMA was eluted just after the blue dextran representing the void volume of the Sephadex G-200 column (Fig. 26). Using the molecular weight standard curve (Fig. 27), the molecular weight of the PTMA was calculated to be 210 kd.

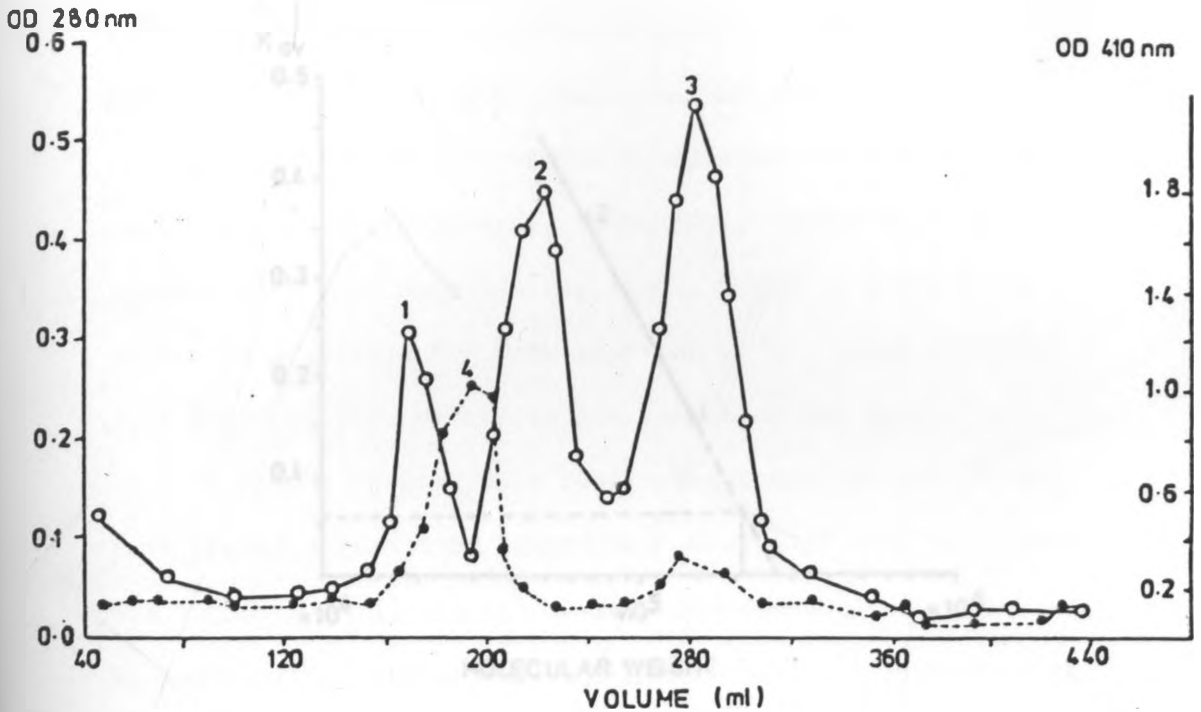


Fig. 26 : Elution profile from Sephadex G-200 of buffalo PTMA and molecular weight markers

Three ml of buffalo TMA (11.5 mg protein/ml) was fractionated on a Sephadex G-200 (94.4 cm long and 2.6 cm diameter) which had been calibrated with a mixture of blue dextran<sup>(1)</sup> (1 mg, approx. 2,000,000 D); human IgG<sup>(2)</sup> (20 mg, approx. 150,000 D) and bovine serum albumin<sup>(3)</sup> (40 mg, approx. 67,000 D) on the basis of OD 280 nm (—○—○—). PTMA was detected in the fractions by a standard EIA (OD 410 nm--●--●--).

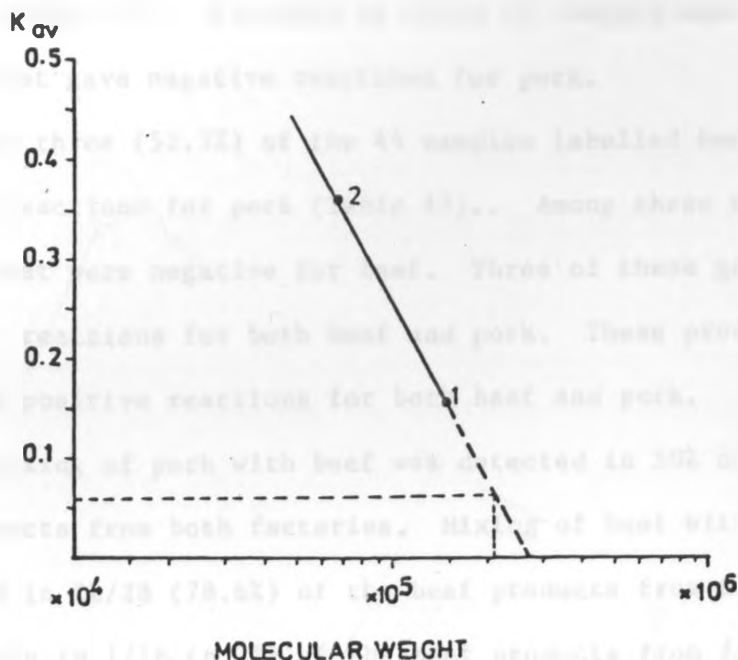


Fig. 27 : Standard curve for molecular weight determination  
using Sephadex G-200 column

The  $K_{av}$  of the molecular weight markers (1 = human IgG, 150,000 D,  $K_{av}$  = 0.15 and 2 = bovine serum albumin, 67,000 D,  $K_{av}$  = 0.36) were plotted against their corresponding molecular weight on a semilogarithmic graph paper. The molecular weight of buffalo PTMA (-----,  $K_{av}$  = 0.06) was read off the curve.

4.3 USE OF ENZYME IMMUNOASSAY FOR SPECIES IDENTIFICATION  
OF MEAT IN MEAT PRODUCTS

4.3.1 Detection of beef and pork in pork and beef products

Of the 46 samples labelled pork, 23 (50%) were positive for beef (Table 13). Included in these 23 samples were 8 samples that gave negative reactions for pork.

Twenty three (52.3%) of the 44 samples labelled beef gave positive reactions for pork (Table 13).. Among these were 6 samples that were negative for beef. Three of these gave negative reactions for both beef and pork. These products gave weak positive reactions for both beef and pork.

The mixing of pork with beef was detected in 50% of the pork products from both factories. Mixing of beef with pork was found in 22/28 (78.6%) of the beef products from factory A, and only in 1/16 (6.3%) of the beef products from factory B (Table 14).

This high rate of addition of pork to beef and vice-versa in products from both factories cannot be referred to as adulteration, since in this country specifications for the manufacture of beef and pork sausages allows inclusion of other unspecified meats. For beef products, the allowed minimum lean beef should at least be 32.5% while pork products should contain at least 52% lean pork (Kenya Bureau of Standards, 1978).

In an experiment to determine the lowest detectable level of beef in pork sausages, the EIA detected 5% beef in pork at



Table 13 : Results of EIA for the detection of beef and pork  
meat in commercial pork and beef products

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Product label	Number tested	No. positive for pork (%)	No. positive for beef (%)	No. positive for beef and pork (%)
Beef	44	23 (52.3)	38 (86.4)	23 (52.3)
Pork	46	38 (82.6)	23 (50)	23 (50)

---

Table 14 : Results of EIA showing detection of beef and pork in meat products from two factories

Factory	Product label	Number of products examined	Tested for	Number positive (%)
A	beef	28	Pork	22 (78.6)
	pork	24	Beef	12 (50)
B	Beef	16	Pork	1 (6.3)
	Pork	22	Beef	11 (50)

$P < 0.01$  but not 1% beef in pork even at  $P < 0.05$  (Fig. 28). Kang'ethe et al. (1982) detected 3% horse meat in beef, while Patterson and Spencer (1985), and Jones and Patterson (1985) reported that they could detect 0.1% kangaroo meat in beef and 0.5% horse meat in beef using a sandwich EIA. The methods described earlier by Kang'ethe et al. (1982), Patterson and Spencer (1985) and Jones and Patterson (1985) were designed to identify the species of origin of unheated meats and are therefore unsuitable for heat-treated products. Use of antisera to TMA allows application of the EIA to be used for the examination of raw, unheated products and also for commercially heat-treated products.

#### 4.3.2. Effect of milk powder on the detection of beef in pork products

Milk powder 5% (same level as the one incorporated in the sausage) when diluted in the coating buffer did not result in false positive reactions for beef. Increasing the quantity of milk powder to 15% in the coating buffer did not produce significant differences in the absorbance at 410 nm when compared to those obtained with 5% milk powder.

Di Antonio et al. (1984) were unable to differentiate between pork sausages containing bovine milk powder with those that contained bovine meat in immunodiffusion tests using anti-bovine serum obtained from a commercial firm. The

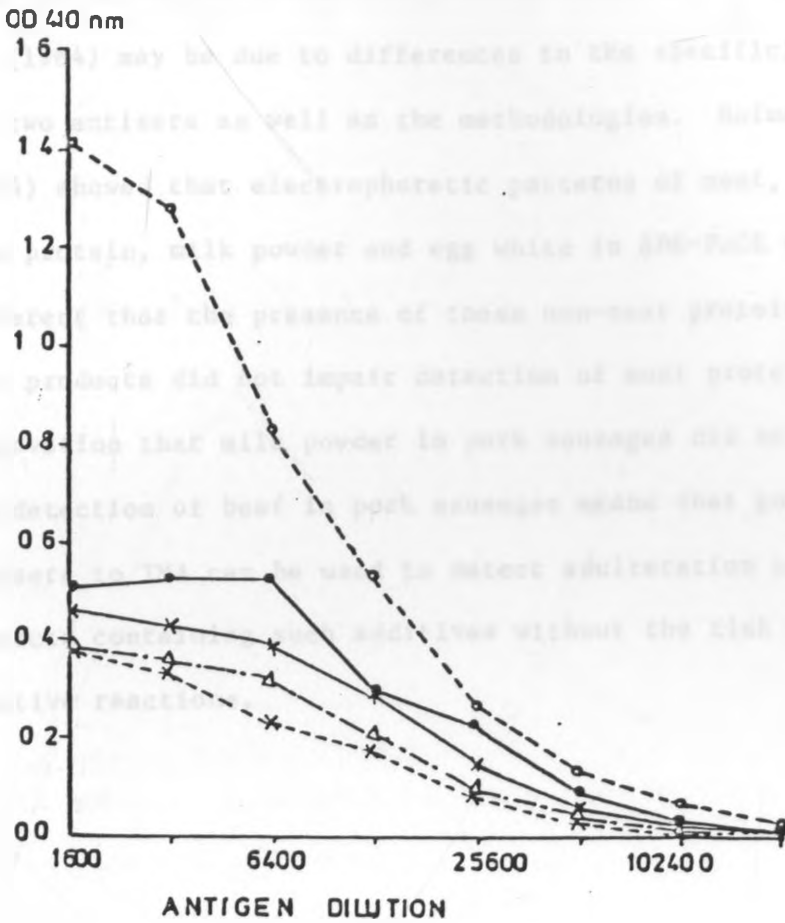


Fig. 28 : Detection by EIA of beef in experimentally prepared pork sausages using goat antiserum to TMA of cattle absorbed with copolymerized serum proteins

Dilutions of phosphate buffered saline extracts of pure beef sausage ( --○--○-- ) pure pork sausage ( --△--△-- ) 10% beef in pork sausage ( ●—●— ), 5% beef in pork sausage ( —x—x— ) 1% beef in pork sausage ( ---x---x-- ) and were tested for the presence of beef using a standard EIA.

discrepancy between these results and those of Di Antonio et al. (1984) may be due to differences in the specificities of the two antisera as well as the methodologies. Hofmann (1974) showed that electrophoretic patterns of meat, soya bean protein, milk powder and egg white in SDS-PAGE were so different that the presence of these non-meat proteins in meat products did not impair detection of meat proteins. The observation that milk powder in pork sausages did not impair the detection of beef in pork sausages means that goat antisera to TMA can be used to detect adulteration of meat products containing such additives without the risk of false positive reactions.



Identification of the species of origin of fresh, cooked and autoclaved meats is a challenging and a complex problem for the food analyst and forensic scientist. While species identification of fresh meat has been achieved using a variety of techniques, identification of the species of origin of heat-treated meat and meat products has remained a persistent problem in the food industry. The objectives of this study were to demonstrate the existence of TMA and use the same to identify the species of origin of autoclaved, cooked and fresh meat and meat products using serological techniques.

The following conclusions can be drawn based on the results obtained in this study.

1. By selection of zoologically related animals for the production of antisera to TMA, it is possible to produce antisera with adequate specificity for the purpose of identification of the species of origin of cooked and fresh meat as well as of organs.
2. Antisera to TMA gave reactions of identity with homologous TMA, CMA, FMA and TOA demonstrating that TMA are not novel antigens but are present in both heat-treated and fresh meats and also in organs.
3. Thermostable muscle antigens exist as three clearly distinct components.

- (a) a species-specific antigen, found in striated muscle and organs of homologous species.
  - (b) species and striated muscle specific antigen, found in skeletal and cardiac muscles.
  - (c) species and tissue specific antigen, found only in skeletal muscle.
4. Although rabbit antisera to TMA lacked sufficient specificity, antisera to TMA of topi, warthog and Grant's gazelle could be used to distinguish between meats from bovidae or non-bovidae.
  5. Gelatin and other contaminating proteins found in the TMA extracted according to the method of Milgrom and Witebsky (1962) are undesirable in EIA as they inhibit adsorption of the PTMA to microtitre plates. A modified procedure for the extraction of PTMA yielded antigens suitable for use in EIA.
  6. Differentiation of PTMA by EIA could be achieved using a selection of antisera produced against TMA of several species.
  7. Antisera to TMA have been shown to be specific for meat proteins. They can, therefore, be used to identify the species of origin of meat in meat products which may contain non-meat proteins in EIA without the risk of false positive reactions.
  8. Antisera to TMA are and will be indispensable in species identification of heat-treated as well as fresh meats.



6. REFERENCES

1. ABDALLAH, N.M.; AMER, H.A.; ABD-EL-MEGID, M.M. AND HASSAN, E.M. (1983).  
Meat species identification by electrophoretic pattern of sarcoplasmic proteins in fresh and frozen conditions. J. Egyptian Vet. Medical Ass. 43: 383-391.
  
2. ANDERSEN, A.E. (1975).  
Species specificity in thermostable and ethanol insoluble tissue antigens. I. Immunizations of rabbits and goats with bovine antigens.  
Acta Vet. Scand. 16: 251-257.
  
3. ANDERSEN, A.E. (1977a).  
Species specificity in thermostable and ethanol insoluble tissue antigens. II. Further immunization of rabbits with bovine kidney preparations.  
Acta Vet. Scand. 18: 559-560.
  
4. ANDERSEN, A.E. (1977b).  
Species specificity in thermostable and ethanol insoluble tissue antigens. III. Further immunization of ruminants with antigens from other ruminants and whale (Balenoptera acutorostrata).  
Acta Vet. Scand. 18: 561-562.

5. ANDREWS, P. (1964).  
Estimation of the molecular weight of proteins by  
Sephadex gel-filtration.  
Biochem. J. 91: 222-233.
  
6. ANON, (1970).  
Testing pig meat in sausages.  
Agric. Research, Washington 18: 14.
  
7. ANON (1982).  
Kangaroo meat.  
British Food Journal 84: 115.
  
8. ASHTON, G.C. (1957).  
Zone electrophoresis of mammalian sera in starch gels.  
Nature Lond. 179: 824-825.
  
9. AVRAMEAS, S. AND TERNYNCK, T. (1962).  
Biologically active water-insoluble proteins polymers,  
their use for isolation of antigens and antibodies.  
J. Biol. Chem. 242: 1651-1659.
  
10. AXELSEN, N.H. (1973).  
A manual of quantitative immunoelectrophoresis method  
and application.  
(Ed. by Axelsen, N.H.; Kroll, J. and Weeke, B.  
Universitetsforlaget, Oslo) pp. 71-79.

11. BENDALL, J.R. (1946).  
The effect of cooking on the creatine-creatinine, phosphorus, nitrogen and pH values of raw lean beef.  
J. Soc. Chem. (London) 65: 226-230.
  
12. BICKERSTAFF, G.F. AND PRICE, N.C. (1978).  
Properties of matrix-bound dimer and monomer derivatives of immobilized creatine kinase from rabbit skeletal muscle.  
Biochem. J. 173: 85-93.
  
13. BLUMENKRANTZ, N. AND ASBOE-HANSEN, G. (1975).  
An assay for hydroxyproline and proline on one sample and simplified method for hydroxyproline.  
Anal. Chem. 63: 331-340.
  
14. BRISCAS, E. AND FROMAGEOT, C. (1953).  
Naturally occurring peptides.  
Adv. in Protein Chem. 8: 1-125.
  
15. BROCKERHOFF, H.; HOYLE, R.J. AND WOOLMARK, N. (1966).  
Positional distribution of fatty acid in triglycerides of animal depot fats.  
Biochim. Biophys. Acta 116: 67-72.

16. CARNEGIE, P.R.; HEE, K.P. AND BELL, A.W. (1982).  
Ophidine (B alanyl-L-3-methylhistidine, belanine) and other histidine dipeptides in pig muscles and tinned hams.  
J. Sci. Food Agric. 33: 795-801.
17. CARNEGIE, P.R.; ILIC, M.Z.; ETHERIDGE, M.O. AND COLLINS, M.G. (1983).  
Improved high performance liquid chromatography method for analysis of histidine dipeptides anserine, carnosine and belanine present in fresh meat.  
J. Chrom. 261: 153-157.
18. CHAMPION, A.; LAWRIE, R.A. AND PARSONS, A.L. (1970).  
Note on differentiation of myofibrillar proteins.  
J. Sci. Food Agric. 21: 7-8.
19. CHOI, H.M.; HAN Y.I. AND LEE J.J. (1970).  
Studies of disc electrophoretic patterns of fish muscle proteins for species identification.  
Korean J. of Food Sci. and Technol. 2: 30-33.
20. CHRISTIE, W.W. AND MORE, J.H. (1970).  
A comparison of the structure of triglycerides from various pig tissues.  
Biochim. Biophys. Acta 210: 46-56.

21. CONELL, J.J. (1953).  
Electrophoretic analysis of low ionic strength extracts of several species of fish.  
Biochem. J. 55: 378-388.
  
22. COWIE, W.P. (1968).  
Identification of fish species by thin slab polyacrylamide gel electrophoresis of muscle myogens.  
J. Sci. Food Agric. 19: 226-229.
  
23. CROWLE, A.J. (1973).  
Immunodiffusion.  
Academic Press, New York 2nd Ed. 286-294.
  
24. DI ANTONIO, E.; AVELLINI, P.; SEVERINI, M. AND VIZZANI, A. (1984).  
Detection of beef in the presence of powdered milk in sausages declared to consist of pure pork. In:  
Biochemical Identification of Meat Species.  
(Ed. R.L.S. Patterson). Elsevier Applied Science Publishers, London pp. 86-89.
  
25. DILWORTH, T.G. AND MCKENZIE, J.A. (1970).  
Attempts to identify meat of game animals by starch gel electrophoresis.  
J. Wildl. Mgnt. 34: 917-921.

26. DOBERSTEIN, K-H. AND GREUEL, E. (1982).  
Preparation of specific antisera for the identification of meat of African antelope species in agar gel precipitin test.  
Fleishwirtschaft 62: 1011-1013.
27. EBERMANN, R. AND BARNA, J. (1972).  
Polyacrylamide gel electrophoresis of press juice from meat extract of cattle, pigs, calves, sheep, horses and chicken and protein extracted from beef tallow and pork lard.  
Z. Lebensm. Unters. Forsch. 148: 341-343.
28. ENGVALL, E. AND PERLMANN, P. (1971).  
Enzyme-linked immunosorbent assay for quantitative assay of IgG.  
Immunochem. 8: 871-874.
29. FEY, H.; PFISTER, H.; MESSERLI, J.; STURZENEGGER, N. AND GROLIMUND, E. (1976).  
Method of isolation and quantitation of bovine immunoglobulins.  
Zentbl. Vet. Med. Series B. 23: 269-300.

30. FURMINGER, I.G.S. (1964).  
Species specificity of bovine, pig, sheep and horse  
myosins.  
Nature (London) 202:1332-1333.
  
31. GEORLICH, R. AND GREUEL, E. (1986).  
Identification of raw and heat denatured muscle protein  
from cattle and kangaroos by using monoclonal  
antibodies of murine origin.  
Archiv fur Lebensmittelhygiene 37: 87-90.
  
32. GILES, B.G. (1962).  
Species differences in sarcoplasmic proteins of  
mammalian muscle.  
J. Sci. Food Agric. 13: 264-268.
  
33. GRIFFITHS, N.M. AND BILLINGTON, M.J. (1984).  
Evaluation of an ELISA for beef blood serum to  
determine indirectly the apparent beef content in beef  
joints and model mixtures.  
J. Sci. Food Agric. 35: 909-914.
  
34. HARRIS, C.I. AND MILNE, G. (1980).  
Occurrence of the N<sup>T</sup> methylhistidine containing  
dipeptides, belanine in muscle extracts of various  
mammals.  
Biochem. Soc. Trans. 8: 552.



35. HARRIS, C.I. AND MILNE, G. (1981).  
The inadequacy of urinary N<sup>T</sup> methylhistidine excretion  
in the pig as a measure of muscle protein breakdown.  
Brit. J. Nutr. 45: 411-422.
36. HAYDEN, A.R. (1977).  
Detection of chicken flesh in beef sausages.  
J. Food Sci. 42: 1189-1192.
37. HAYDEN, A.R. (1979).  
Immunochemical detection of ovine, porcine and equine  
flesh in beef products with antisera to species  
myoglobin.  
J. Food Sci. 44: 494-500.
38. HAYDEN, A.R. (1981).  
Use of antisera to heat-stable antigens of adrenals for  
species identification in thoroughly cooked beef  
sausages.  
J. Food Sci. 46: 1810-1813.
39. HEINERT, H.H. AND KLINGER, A. (1980).  
Species-specific protein differentiation. Protein and  
enzyme pattern in roe deer and red deer.  
Fleishwirtschaft 60: 1683-1685.

40. HENNEY, C.S. AND ISHIZAKA, K. (1968).  
Antigenic determinants specific for aggregated  
gammaglobulins.  
J. Immunol. 100: 718-725.
41. HIROSE, S.I. AND OSLER, A.G. (1967).  
Interaction of rabbit anti-human H-chain with denatured  
gammaglobulin and its subunits.  
J. Immunol. 98: 618-627.
42. HOFMANN, K. (1974).  
Identification and determination of meat and non-meat  
protein by means of sodium dodecylsulphate  
polyacrylamide gel electrophoresis.  
Anal. Chem. 267: 355-361.
43. HOFMANN, K. AND PENNY, I.F. (1971).  
Identification of soya bean protein and meat proteins  
by SDS-PAGE.  
Fleishwirtschaft 51: 577-578.
44. HOFMANN, K. AND PENNY, I.F. (1973).  
A method for identification and quantitative  
determination of meat and foreign proteins using  
SDS-PAGE.  
Fleishwirtschaft 53: 252-254.

45. HOYEM, T. AND THORSON, B. (1970).  
Myoglobin electrophoretic patterns in identification of  
meat from different animal species.  
J. Agric. Food Chem. 18: 737-739.
  
46. HUDSON, L. AND HAY, F.C. (1980).  
Practical Immunology, 2nd Edition. Blackwell  
Scientific Publishers, Oxford pp, 161-165.
  
47. JOHNSON, P.; HARRIS, C.I. AND PERRY, S.V. (1967).  
3-methylhistidine in Actin and other muscle proteins.  
Biochem. J. 105: 361-370.
  
48. JONES, D.; SHORLEY, D. AND HITCHCOCK, C.H.S. (1982).  
The fluorometric determination of 3-methylhistidine in  
meat and meat products.  
J. Sci. Food Agric. 33: 671-685.
  
49. JONES, S.J. AND PATTERSON, R.L.S. (1985).  
Double-antibody ELISA for detection of trace amounts of  
pig meat in raw meat mixtures.  
Meat Sci. 15: 1-13.

50. KAISER, K.P.; MATHEIS, G.; KMITA-DUERMAN AND BELITZ, H.D. (1980).  
Identification of animal species of meat, fish derived products by protein differentiation with electrophoretic methods.  
Z. Lebensm. Unters. Forsch. 170: 334-342.
51. KAISER, K.P.; MATHEIS, G.; KMITA-DUERMAN AND BELITZ, H.D. (1981).  
Qualitative and quantitative analysis of binary mixtures of raw meat and fish by isoelectric focusing in agarose gels.  
LKB Instrument J. Science Tools 28: 5-7.
52. KAMIYAMA, T. (1977a).  
Immunological cross-reactions and species specificities of bovine, goat and sheep serum albumins.  
Immunochem. 14: 85-90.
53. KAMIYAMA, T. (1977b).  
Immunological cross-reactions and species specificities of cyanogen bromide cleaved fragments of bovine, goat and sheep serum albumins.  
Immunochem. 14: 91-95.

54. KAMIYAMA, T.; KATSUBE, Y. AND IMAIZUMI, K. (1978a).  
Serum albumin contents in meat extracts in relation to  
serological identification of animal species of meat.  
Jap. J. Vet. Sci. 40: 559-563.
55. KAMIYAMA, T.; KATSUBE, Y. AND IMAIZUMI, K. (1978b).  
Serological identification of animal species of meat by  
passive haemagglutination inhibition test using  
cross-reacting antiserum albumin antiserum.  
Jap. J. Vet. Sci. 40: 653-661.
56. KANG'ETHE, E.K. (1981).  
Serological identification of the species of origin of  
animal meats.  
M.Sc. Thesis, University of Bristol.
57. KANG'ETHE, E.K.; JONES, S.J. AND PATTERSON, R.L.S.  
(1982).  
Identification of the species of origin of fresh meat  
using an enzyme-linked immunosorbent assay procedure.  
Meat Sci. 7: 229-240.
58. KATSUBE, Y. AND IMAIZUMI, K. (1968).  
Serological identification of animal meats.  
Jap. J. Vet. Sci. 30: 218-232.

59. KARPAS, A.B.; SEGRE, D. AND MEYERS, W.L. (1970).  
Serological identification of species origin of sausage  
meat.  
J. Food Sci. 35: 150-155.
60. KENYA BUREAU OF STANDARDS (1978).  
Specification for fresh or cooked pork and beef  
sausage, pork and beef sausage meat.  
Kenya Bureau of Standards publication number KS-05-59.  
UDC 637-524.
61. KING, N.L. (1984).  
Species identification of cooked meats by enzyme  
staining in isoelectric focusing gels.  
Meat Sci. 11: 59-72.
62. KING, N.L. AND KURTH, L. (1982).  
Analysis of raw beef samples for adulterant meat  
species by enzyme staining in isoelectric focusing gels.  
J. Food Sci. 47: 1608-1612.
63. KOH, T.Y. (1978).  
Immunochemical method for the identification and  
quantitation of cooked or uncooked beef and soya bean  
protein in mixtures.  
J. Inst. Canadian Sci. Technol. Aliment. 11: 124-128.

64. LATNER, A.L. AND ANGEL, H.Z. (1957).  
Starch gel electrophoresis of animal sera.  
Nature (London) 179: 1366-1367.
65. LAWRIE, R.A. (1953).  
The activity of the cytochrome system in muscle and its  
relation to myoglobin.  
Biochem. J. 55: 298-305.
66. LAWRIE, R.A. (1979).  
Meat Science.  
3rd Ed. Pergamon Press, p. 77.
67. LINDQVIST, K.J.; GATHUMA, J.M. AND KABURIA, H.F.A.  
(1982).  
Analysis of blood meals of haematophagus insects by  
haemagglutination inhibition and enzyme immunoassay.  
In: Proceedings of 3rd Medical Scientific Conference.  
Current Medical Research in Eastern Africa (Eds. Tukei,  
P.M. and Njogu, A.R.) AfricaScience International, pp.  
122-133.
68. LOWRY, O.H.; ROSEBROUGH, N.J.; FARR, A.L. AND RANDALL,  
R.J. (1951).  
Protein measurement with Folin phenol reagent.  
J. Biol. Chem. 193: 265-275.

69. LUNDSTROM, R.C. (1981).  
Rapid fish species identification by agarose gel  
isoelectric focusing of sarcoplasmic proteins.  
J. Assoc. Off. Anal. Chem. 64: 38-43.
70. MACHLIK, S.M. AND DRAUDT, H.N. (1963).  
The effect of heating time on the shear of beef  
semitendinosus muscle.  
J. Food Sci. 28: 711-718.
71. MACKIE, I. (1979).  
A review of some recent applications of electrophoresis  
and isoelectric focusing in identification of species  
of fish and fish products.  
Adv. in Fish Science & Technology (Proc. of Jubilee  
Conf. of Tory Res. Station) pp. 444-450.
72. MANZ, J. (1980).  
Serological determination of species specific antigens  
in extracts of the musculature of rabbits and hares.  
Fleishwirtschaft 60: 763-765.
73. MANZ, J. (1983).  
Detecting heat-denatured muscle protein by means of an  
enzyme-linked immunosorbent assay. Determining  
kangaroo muscle proteins.  
Fleishwirtschaft 63: 1767-1769.



74. MATTEY, M.; PARSONS, A.L. AND LAWRIE, R.A. (1970).  
Qualitative identification of meat species after  
heating.  
J. Food Technol. 5: 41-46.
75. MILES, L.E.M. AND HALES, C.N. (1968).  
Labelled antibodies and immunological assay systems.  
Nature 219: 186-189.
76. MILGROM, F. AND WITEBSKY, E. (1962).  
Immunological studies on adrenal glands. Immunization  
with adrenals of foreign species.  
Immunol. 5: 46-66.
77. MILGROM, F., TUGGAC, Z.M. AND WITEBSKY, F. (1963).  
Immunological studies on adrenal glands. Interspecies  
relations of thermostable adrenal species antigens.  
Immunol. 6: 105-118.
78. MILGROM, F.; TUGGAC, Z.M. AND WITEBSKY, E. (1964).  
Studies on species specificity.  
J. Immunol 93: 902-909.
79. MUNDAY, B.L.; GOLBOULD, A. AND GOODSALL, G. (1974).  
Identification of muscle tissue from animals.  
J. Wildl. Mgnt. 38: 884-886.

80. MURAKAMI, H.; UMEKI, F.; NAKAMURA, T. AND HIRUTA, M.  
(1969).

Application of a micro-electrophoresis and  
micro-gel-diffusion technique in differentiation of  
household meat.

J. Food Hyg. Soc. 10: 434-439.

81. NAKKILA, O.E. AND LINKO, R.R. (1955).

Paper electrophoresis analysis of protein extracted at  
low ionic strength from fish skeletal muscle.

Biochem. J. 60: 242-247.

82. PARSONS, A.L.; BLANSHARD, J.M.; PARSONS, A.J. AND  
LAWRIE, R.A. (1969).

Electrophoretic differentiation of myofibrillar  
proteins in the pig.

Biochem. J. 112: 673-678.

83. PATTERSON, R.M. AND SPENCER, T.L. (1985).

Differentiation of raw meat from phylogenically related  
species by enzyme-linked immunosorbent assay.

Meat Sci. 15: 119-123.

84. PATTERSON, R.M.; WHITTAKER, R.G. AND SPENCER, T.L. (1984).  
Improved species identification of raw meat by double sandwich enzyme-linked immunosorbent assay.  
J. Sci. Food Agric. 35: 1018-1023.
85. PAYNE, E. (1971).  
The use of the fatty acid composition of lipids in the identification of horse and kangaroo meat.  
J. Sci. Food Agric. 22: 520-522.
86. PENNY, I.F. AND HOFMANN, K. (1971).  
Detection of soybean protein in meat products.  
In: Proceedings of the 17<sup>th</sup> European Meeting of Meat Research Workers. Bristol. 5, 809.
87. PINTO, F.G. (1961).  
Serological identification of ox, buffalo, goat and deer flesh.  
Brit. Vet. J. 117: 540-544.
88. POULTER, N.H. AND LAWRIE, R.A. (1980a).  
Rapid determination of the 3-methyl histidine content of meat using 1-fluoro-2-4-dinitrobenzene.  
Meat Sci. 4: 15-20.

89. POULTER, N.J. AND LAWRIE, R.A. (1980b).  
The practical application of 3-methyl histidine in  
determining the lean meat content of food products.  
Meat Sci. 4: 21-31.
90. PROOM, H. (1943).  
Preparation of precipitin sera for the identification  
of animal species.  
J. Path. Bact. 55: 419-426.
91. RANGELEY, W.R.D. AND LAWRIE, R.A. (1976).  
Methylamino acid indices in meat products.  
J. Food Technol. 11: 143-159.
92. RANGELEY, W.R.D. AND LAWRIE, R.A. (1977).  
Methylated amino acids as indices in meat products.  
J. Food Technol. 12: 9-26.
93. RURANGIRWA, F.R.; MINJA, S.H.; MUSOKE, A.J.; NANTULYA,  
V.M.; GROOTENHUIS, J. AND MOLOO, S.K. (1986).  
Production and evaluation of species-specific antisera  
against sera of various vertebrate species for  
identification of bloodmeals of Glossina morsitans  
centralis.  
Acta Tropica 43: 379-389.

94. SAVINGY, De. D. AND VOLLER, A.J. (1980).  
Communication of ELISA data from laboratory to  
clinician.  
J. of Immunoassay. 1: 105-128.
95. SCHWEIGER, A., BAUDER, S. AND GUNTHER, H. (1983).  
Isolation by free-flow electrophoresis and immunological  
detection of Troponin-T from turkey muscle. An  
application in food industry.  
Electrophoresis. 4: 158-163.
96. SCOPES, R.K. (1968).  
Methods for starch gel electrophoresis of sarcoplasmic  
proteins.  
Biochem. J. 107: 139-150.
97. SEKI, N.; TAKAYASU, M. AND KOKURYO, H. (1980).  
Preparation of myosin light chains as an index for  
identification of fish species from raw muscle and  
cooked products.  
Bull. of the Jap. Soc. of Sci. Fisheries. 46: 1001-1006.
98. SHIGA, K. (1980).  
Interaction between meat proteins and soya bean  
proteins. Effect of heating on SDS-PAGE patterns.  
J. of Fac. of Appl. Biol. Sci., Hiroshima University,  
19: 183-170.

99. SHUNMUGAM, A.M. AND RANGANATHAN, M. (1972).  
Differentiation of mutton and beef by precipitation tests.  
Indian Vet. J. 49: 1024-1028.
100. SLATTERY, W.J. AND SINCLAIR, A.J. (1983).  
Differentiation of meat according to species by the electrophoretic separation of muscle lactate dehydrogenase and esterase isoenzyme and isoelectric focusing of soluble muscle proteins.  
Aust. Vet. J. 60: 47-51.
101. SNELL, H.J. AND MENTZ, I. (1969).  
Serological identification of species of origin of heat denatured muscle proteins by means of urea extraction.  
Berl. Munch. Tierartztl. Wschr. 82: 55-58.
102. SNEDECOR, G.W., AND COCHRAN, W.G. (1971).  
Statistical methods, 6th Ed. Iowa State University Press, Ames.
103. SNOWDEN, J. Mck. AND WEIDEMANN, J.F. (1978).  
A morphological and biochemical examination of the hydrothermal determination of collagen.  
Meat Sci. 2, 1-18.

104. SPELL, E. (1974).  
Electrophoretic methods for distinguishing different kinds of meat.  
Fleishwirtschaft 54: 533-535.
  
105. SWARTS, K.S. AND WILKS, C.R. (1982).  
An immunodiffusion method for the identification of the species of origin of meat samples.  
Aust. Vet. J. 59: 21-22.
  
106. TIECCO, G. (1980).  
Electrophoretic patterns of fresh and frozen meat.  
Archivio Veterinario Italiano 31: 108-110.
  
107. TINBERGEN, B.J. AND SLUMP, P. (1976).  
The detection of chicken meat in meat products by means of anserine : carnosine ratio.  
Z. Lebensm. Unters. Forch. 161: 7-11.
  
108. VAN WEEMEN, B.K. AND SCHUURS, A.H.W.M. (1971).  
Immunoassays using enzyme conjugates.  
FEBS Letters 15: 232-236.

109. VERBEKE, R. (1983).  
Quantitative analysis of raw meat mixtures of pork and beef by isoelectric focusing in agarose gels. In: Proceedings of 29th European Meeting of Meat Research Workers, Italy. 2: 676-683.
110. VERBEKE, R. AND De. BRABANDER, H.F. (1979).  
An alternative method for the detection of pork fat adulteration with beef tallow. In: Proceedings of 25th European Meeting of Meat Research Workers, Budapest, 2: 767-772.
111. VERBEKE, R. AND DE BRABANDER, H.F. (1980).  
Identification of animal fat species. In: Proceedings of the 26th European Meeting of Meat Research Workers, Colorado Springs, pp. 150-153.
112. WARRISS, P.D. (1977).  
The residual blood content of meat.  
J. Sci. Food Agric. 28: 457-462.
113. WHITTAKER, R.G.; SPENCER, T.L. AND COPLAND, J.W. (1982).  
Enzyme-linked immunosorbent assay for meat species testing.  
Aust. Vet. J. 59: 125.



114. WHITTAKER, R.G.; SPENCER, T.L. AND COPLAND, J.W. (1983).  
An enzyme-linked immunosorbent assay for species  
identification of raw meat.  
J. Sci. Food Agric. 34: 1143-1148.
115. WILSON, M.K. AND NAKANE, P.K. (1978).  
Recent development in the periodate method of  
conjugating horseradish peroxidase (HRPO) to  
antibodies. In: Immunofluorescence and related  
staining techniques. (Eds. Knapp, W., Holubar, K.,  
Wick, G.). Elsevier/North Holland Biomedical Press,  
Amsterdam, 1978, pp. 215-224.

INDEX

LIST OF TABLES AND FIGURES

Table 1. Summary of data for the study area.

1.1. 1.1.1. Summary of data for the study area

1.1.1.1. Summary of data for the study area	1.1.1.1
1.1.1.2. Summary of data for the study area	1.1.1.2
1.1.1.3. Summary of data for the study area	1.1.1.3

1.2. 1.2.1. Summary of data for the study area

1.2.1.1. Summary of data for the study area	1.2.1.1
1.2.1.2. Summary of data for the study area	1.2.1.2
1.2.1.3. Summary of data for the study area	1.2.1.3

A P P E N D I X

1.3. 1.3.1. Summary of data for the study area

1.3.1.1. Summary of data for the study area	1.3.1.1
1.3.1.2. Summary of data for the study area	1.3.1.2
1.3.1.3. Summary of data for the study area	1.3.1.3

APPENDIX 1

1 List of buffers and solutions used for immunodiffusion tests

1.1 0.15M Phosphate buffer, pH 7.4

Di-sodium hydrogen phosphate (anhydrous)	21.3 g
Di-sodium dihydrogen phosphate	23.4 g
Distilled water upto	1000 ml

1.2 Trisodium citrate buffer for washing slides

Trisodium citrate	3 g
Sodium azide	1 g
Distilled water made upto	1000 ml

1.3 1% agar in phosphate buffered saline pH 7.4, for immunodiffusion tests

Purified Oxoid agar	2 g
Sodium azide	0.2 g
Phosphate buffered saline	200 ml

APPENDIX 2

2 Protein staining and destaining solutions

2.1 Coomassie Brilliant Blue 250R solution

Coomassie Brilliant Blue 250R	10 g
Ethanol	900 ml
Glacial acetic acid	200 ml
Distilled water	900 ml

2.2 Ponceau S solution

Ponceau S	2 g
1 M Acetic acid	1000 ml
0.1 M Sodium acetate	1000 ml

2.3 Destaining solution for Coomassie Blue

Ethanol	900 ml
Glacial acetic acid	200 ml
Distilled water	900 ml

2.4 Destaining solution for Ponceau S procedure

3% v/v glacial acetic acid in distilled water

APPENDIX 3

3 Buffers and solutions used in the preparation of insoluble immunosorbents

3.1 1 mM hydrochloric acid solution for washing the freeze dried cyanogen bromide activated Sepharose 4B

This solution was prepared by diluting 35% concentrated acid Sp. gr. 1.18

3.2 0.1M Sodium bicarbonate solution pH 8.3 with 0.5M sodium chloride (protein coupling buffer)

Sodium hydrogen carbonate	8.40 g
Sodium chloride	29.22 g

8.4 g of sodium hydrogen carbonate were dissolved in 200 mls of distilled water, the pH was adjusted to 8.3 with 1 M NaOH. 29.22 of NaCl was added and stirring continued until the salt had dissolved. The pH was checked again and the volume made to 1000 ml.

3.3 0.2M glycine, pH 8.0 (blocking agent)

Glycine	15.01 g
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15.01 g of glycine were dissolved in 200 ml of distilled water and the pH adjusted to 8.0, using 1M HCl. Distilled water was added up to 1000 ml.

3.4 0.1M acetate buffer pH 4.0, containing 0.5M sodium chloride

Sodium acetate	8.2 g
Sodium chloride	29.22 g
Acetic acid	5.77 ml

dissolved in 1000 ml distilled water

3.5 0.1M glycine-HCl buffer pH 2.5 - for regeneration of immunosorbents

Glycine	7.51 g
HCl	0.2 M

7.51 g of glycine were dissolved in 500 ml distilled water and the pH titrated to 2.5 using 0.2 M HCl, then made up to 1000 ml.

3.6 0.1M Tris-HCl buffer, pH 8.5, containing 0.5M NaCl for regenerating immunosorbents

Tris (hydroxymethyl)aminomethane	12.1 g
Sodium chloride	29.22 g

12.1 g Tris and 29.22 g Sodium chloride were dissolved in 500 ml distilled water. The pH was adjusted to 8.5 using 1M HCl, and then made up to 1000 ml.

3.7 0.2M acetate buffer, pH 5.0 - for copolymerizing serum proteins

Sodium acetate	16.41 g
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dissolved in 500 ml distilled water

Glacial acetic (17.5 M)	11.42 ml
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and the volume made up to 1000 ml

APPENDIX 4

4 Reagents and solutions used in the Folin-Ciocalteu's method for determination of protein content

4.1 Solution 1

2% sodium carbonate in 0.1N sodium hydroxide.

Solution 2

0.5% copper sulphate in 1% sodium tartrate prepared freshly by mixing equal volumes of double strength reagents.

Solution 3

Alkaline copper solution, prepared by mixing 50 parts of solution 1 with 1 part of solution 2.

4.2 Phenol reagent

Commercial phenol reagent (Fisher Scientific Company, New York) was diluted in the ratio of 5 parts reagent to 4.7 parts of distilled water.

4.3 Bovine serum albumin (BSA)

BSA (No. A-4503, lot no. 107C - 0307 Fraction V, Sigma Chemical Company, St. Louis, USA) was used to prepare the standard curve for the determination of protein content.

APPENDIX 5

5 Buffers and reagents used for the determination of hydroxyproline

5.1 L-hydroxyproline (BDH, Chemicals Ltd., Poole, England). Concentrations ranging from 1-6 ug/ml were prepared in distilled water.

5.2 Borate buffer pH 8.7 (1M buffer in terms of boric acid)

Boric acid	61.84 g
Potassium chloride	225g

dissolved in 800 ml distilled water.

The pH was adjusted to 8.7 using 1 M KOH and the volume adjusted 1000 ml.

5.3 0.112M Alanine solution

DL-Alanine (BDH Chemicals Ltd., Poole, England) 10 g dissolved in 90 ml distilled water, and pH adjusted to 8.7 and the volume adjusted to 1000 ml.

5.4 Borate-alanine buffer

100 ml of the borate buffer was mixed with 50 ml alanine solution. The resulting solution was used in 1:10 concentration (0.066 M boric acid).



5.5 0.2M Chloramine-T Solution

Chloramine-T 56.34 g  
dissolved in 1000 ml distilled water

5.6 3.6M Sodium thiosulphate solution

Sodium thiosulphate 89.34 g  
dissolved in 100 ml distilled water

5.7 Toluene - analytical grade (BDH Chemicals Ltd.,  
Poole, England).

5.8 Ehrlich's Reagent

p-dimethylaminobenzaldehyde (BDH Chemicals Ltd.,  
Poole, England) 120 g

dissolved in 200 ml absolute ethanol

Concentrated sulphuric acid was added slowly to 200 ml  
absolute ethanol in a beaker and the mixture cooled.

The acid ethanol mixture was added to the

p-dimethylaminobenzaldehyde solution in an ice-bath.

APPENDIX 6

6 Reagent and buffers for enzyme immunoassay

6.1 0.05M carbonate-bicarbonate buffer, pH 9.6 (coating buffer)

Sodium carbonate	1.59 g
Sodium hydrogen carbonate	2.93 g
Distilled water	1000 ml

This buffer was prepared fresh every day

6.2 Buffer used for diluting antiserum

0.05M phosphate buffer pH 8.0	1000 ml
Potassium chloride	75 g
Ethylenediaminetetraacetic acid disodium salt	1 g
Benzoic acid	2.5 g
Tween 80	1 ml

The pH was adjusted to 7.5 using 4M NaOH. Normal rabbit serum was added to 5%.

6.3 Buffer used for diluting conjugate

0.05M phosphate buffer pH 8.0	1000 ml
Potassium chloride	75 g
Ethylenediaminetetraacetic acid disodium salt	1 g
Benzoic acid	2.5 g
Tween 80	5 ml

The pH was adjusted to 7.5 using 4 M NaOH. Normal rabbit serum was added to 5%.

6.4 Substrate buffers

6.4.1 0.05 M ammonium citrate buffer pH 5.0

Citric acid	10.5 g
Benzoic acid	1 g
Glacial acetic acid	3 ml
Distilled water	approx. 800 ml

The pH was adjusted to 5.0 with concentrated ammonia solution.

The volume was made up to 1000 ml with distilled water.

6.4.2 Substrate solution for glucose oxidase conjugate for one microtitre plate

0.05 M ammonium citrate buffer, pH 5.0	10 ml
d-Glucose solution (20%)	1 ml
Horseradish peroxidase type II (1 mg/ml) in ammonium citrate buffer, pH 5.0	0.1 ml

ABTS (2'2'-azinobis(3 ethylbenzthiazoline sulfonic acid) disodium salt (25 mg/ml) in distilled water	0.1 ml
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6.5 Buffer for washing plates

0.15M phosphate buffer, pH 7.4 stock solution (10x)	1000 ml
Tween 80	5 ml
Physiological saline	9000 ml
Sodium azide	1 g