OPTIMIZATION, VALIDATION AND APPLICATION OF RADIOIMMUNOASSAYS FOR PLANT GROWTH SUBSTANCES IN AVOCADO (*PERSEA AMERICANA* MILL.) FRUITS

JONATHAN GARTH MELVILLE CUTTING.

B.Sc. Agric. (Natal)

Submitted in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in the

Department of Horticultural Science
Faculty of Agriculture
University of Natal,
Pietermaritzburg

June, 1984

OPTIMIZATION, VALIDATION AND APPLICATION OF RADIOIMMUNOASSAYS FOR PLANT GROWTH SUBSTANCES IN AVOCADO (*PERSEA AMERICANA* MILL.) FRUITS

CUTTING, JONATHAN GARTH MELVILLE
Ph.D., 1984, 190pp.
Department of Horticultural Science, University of Natal,
Pietermaritzburg

ABSTRACT

The objective was to develop, optimize and validate radioimmunoassays (RIA) for several plant growth substances (PGS) and then apply the RIA's to determine PGS trends in 'Fuerte' avocado fruits from fruit set to fruit ripening.

Antibodies to the cytokinin isopentenyl adenosine (IPA) were obtained from rabbits inoculated with a periodate-derived IPA-BSA conjugate. The antiserum cross-reacted (25%) with only 2iP (isopentenyl adenine). The RIA measuring range was from 0,1 to 100 ng. Anti-IPA serum was used to develop a RIA for 2iP, with a measuring range from 0,5 to 100 ng. Using Dowex 50W-X8 and cellulose TLC purified avocado fruit extract, 20 samples per day could be processed.

The RIA for abscisic acid (ABA) was developed from rabbit antibodies from an inoculated carbodiimide-derived (±)ABA-BSA conjugate. The free active ABA componer was isolated prior to quantitation by RIA by solvent partitioning and silica gel TLC.

The indole-acetic-acid (IAA) RIA was established from sheep-produced antibodies to a formaldehye-derived IAA-BSA conjugate, after repeated inoculations. For both the ABA and IAA RIA's, contaminants in the avocado tissue were removed by solvent partitioning.

Developing avocado fruits, and especially young fruits were rich sources of IAA and 2iP in particular with seed concentrations exceeding those of the fruit flesh. The concentration of ABA rose throughout fruit development, reaching 100

ng g^{-1} in the flesh at fruit maturity. Just prior to seed and fruit maturity, relatively high levels of IAA, 2iP and IPA were associated with the thick, fleshy testa, rapidly declining to zero as the testa dried. The avocado fruit is physiologically dependent on the seed right up to this stage, and testa maturity correlates well with minimum "legal" maturity of 80% moisture content of flesh.

In ripening avocado fruit, the concentration of free ABA rose as softening progressed. Total ABA concentrations fell initially, and then rose after a firmometer reading between 50 and 60 (100 coincides with eating ripeness). Later harvested fruit had double the ABA concentration of early harvested fruit. The ripening stimulus appeared to be related to moisture stress in the fruit.

This study has confirmed the prime advantages of sensitivity, specificity and rapidity of RIA, as well as its usefulness in multi-PGS studies and batch-type analysis.

DECLARATION

I hereby certify that the research work reported in this thesis is the result of my own investigation, except where acknowledged.

SIGNED .....

(J.G. CUTTING)

ACKNOWLEDGEMENTS

Many thanks are extended to my two supervisors, Prof B.N. Wolstenholme, Dept of Horticultural Science, University of Natal,

and

Prof A.W. Lishman, Dept of Animal Science, University of Natal, for helpful advice and discussion during the period of study as well as the preparation of the manuscript. Special appreciation to Prof Lishman for the free use of his Laboratory where much of this work was undertaken.

The constructive criticism and discussion of Dr Peter Hofman, and the help of Dr Andre van der Hoven in the instruction in antibody production, is much appreciated.

Thanks are extended to Mr Mike Lyne, Dept of Agricultural Economics, for help with the statistical analysis of the radioimmunoassay data.

Mr J.K. Train is thanked for the donation of avocado material used in this study.

The South African Avocado Growers Association, the Dept of Agriculture, the Nuclear Corporation, the Council for Scientific and Industrial Research, the University of Natal Research Fund and Westfalia Estate contributed financial support for this project.

Personal assistance in the form of a graduate assistantship and the John Travers Bursary from the University of Natal is gratefully acknowledged.

The Citrus and Subtropical Fruit Research Institute, Nelspruit, is thanked for the gift of Phaseic and Dihydrophaseic acid.

LIST OF ABBREVIATIONS

RIA	Radioimmunoassay
EIA	Enzymeimmunoassay
PGS	Plant growth substance
BSA	Bovine serum albumin
ABA	Abscisic acid
IAA	Indole-3-acetic acid
IPA	Isopentenyl adenosine
2iP	Isopentenyl adenine
PBS	Phosphate buffered saline
NSB	Non specific binding
TLC	Thin layer chromatography
Ta	Total counts in the system
cpm	Counts per minute
min	Minute
UV	Ultra violet
PPO	2,5 diphenyloxazol
Popop	Dimethyl popop
OCT	October
NOV	November
DEC	December
JAN	January
FEB	February
MAR	March
SE	Standard error

CONTENTS

CHAPTER PAGE

INTRODUCTION

The avocado (*Persea americana* Mill.) is an established fruit crop in many parts of the world (Knight, 1980), but yields are often low and unreliable (Sedgley & Grant, 1982). There are over one million avocado trees in South Africa of which over half are still to come into bearing, and the industry is expected to earn R50 million from fresh fruit exports in 1985 (Bredell, 1983). The volume of fruit exported has shown a steady decrease since 1980. In 1983 only 55% of the national avocado crop was exported due primarily to poor fruit quality and drought, and post-harvest problems, such as chilling injury, which are compounded by the long sea voyage to the major export markets in Europe (Williams, 1984).

As a crop the avocado is noted for several physiological irregularities or disorders. For many years alternate bearing cycles (Hodgson & Cameron, 1936) have been a problem, and can lead to irregular and reduced cropping (Hodgson, 1947). Massive early fruit drop (November drop in South Africa) can result in less than 0,3% of flowers initially setting fruits (Adato & Gazit, 1977; Sedgley, 1977). Lahav & Kalmar (1983) have shown that water relations are a very important factor in fruit retention. This would tend to be mediated via plant growth substance (PGS) control, particularly reduced synthesis of promotive PGSs during this critical period. PGSs are small molecular mass compounds produced endogenously in the plant which control various physiological responses in the plant. The PGS usually exerts its effect at a different site to where it was synthesized (Hill, 1980).

Tomer & Gottreich (1978) observed that abscissed avocado fruits often had abnormal or degenerate embryos. Degeneration was observed by Sedgley (1980) to occur in both fertilized and unfertilized fruitlets. She concluded that

lack of ovule fertilization was not the main reason for fruit drop as sufficient flowers had been fertilized to give an adequate crop. This would tend to indicate PGS regulation in fruit retention and abscission.

Some studies on PGS trends during avocado fruit development have been carried out (Blumenfeld & Gazit, 1970; 1972; Gazit & Blumenfeld, 1970; 1972). However, these studies hinged on only four or five sampling points and made use of older methods for estimating the levels of the PGS studied.

Several post-harvest disorders occur in avocado fruit, when grown under sub-optimal conditions in South Africa (Swarts, 1978). These include premature fruit softening (Bower, van Lelyveld & Nel, 1982) and pulp spot (van Lelyveld, Nel & Dixon, 1983). Studies on post-harvest physiology have largely centered on ethylene (Baile, 1941; 1960; Adato & Gazit, 1974a; 1974b). In another study the relationship between ethylene and abscisic acid was investigated (Adato, Gazit & Blumenfeld, 1976). One objective of the present study was to determine PGS trends in various 'Fuerte' fruit components from flowering to harvest, and from harvest (maturity) through softening to ripeness using validated radioimmunoassays (RIAs). This would give an indication of which PGSs were possibly involved in fruit development and enable the construction of a PGS trend model from flowering to ripeness. This would aid the formulation of general proposals for further long-term and more detailed fruit development and maturity studies.

In the past PGS research has centered strongly around detection of a PGS in a test material, and the determination of the PGS level. A great many techniques are available for this type of research, ranging from methods suitable for single determinations to techniques geared toward high volume batch-type analysis. RIA, a relatively new technique in the field of PGS physiology but well es-

tablished in endocrinology (Chard, 1981), is one such batch-type technique which has recently been used for PGS analysis (Weiler, 1982a). Advantages of RIA include short assay time (usually less than three hours), the potential for large sample throughput, and sensitivity and reduced purification of the biological extract due to a largely theoretical specificity.

Using various biomedical techniques well documented in the recent literature, the establishment of RIAs should be relatively routine. However, two important aspects of such a technique, namely optimization and validation, appear to have been only partially implemented or largely neglected. Another objective of the research reported here was therefore to establish several RIAs for different PGSs, to critically validate them and to determine whether they were suitable for use with avocado material.

CHAPTER 1
REVIEW OF LITERATURE

1.1 Introduction

At present it would be inappropriate to identify one particular method of PGS analysis as best. A tremendous number of approaches have been used to isolate and quantitate the various PGSs. These include partitioning (Ciha, Brenner & Brun, 1977; Horgan, 1978), column chromatography (Armstrong, Burrows, Evans & Skoog, 1969; Thompson, Horgan & Heald, 1975; Vreman & Corse, 1975; van Staden, 1976), thin layer chromatography (Sagi, 1969, Horgan, 1978; Graebe & Ropers, 1978), high performance liquid chromatography (Pool & Powell, 1972), gas chromatography (Rivier & Pilet, 1980), bioassay (Reeve & Crozier, 1980), gas chromatography and mass spectrometry (MacMillan & Pryce, 1968; Ehmann & Bandurski, 1974; Morris, 1977), fluorimetric assay (Stoessl & Venis, 1970) and immunological assay (Pengelly & Meins, 1977).

There is no doubt that more approaches will be developed (Horgan, 1981). The requirements for these new methods include accuracy, precision, sensitivity, selectivity, speed and cost. Brenner (1981) stated that certain physicochemical and immunological procedures offer potential for improving analytical methods for PGS. Horgan (1981) goes so far as to say that in principle at least, immunological assays would appear to be the ideal method for the quantitative analysis of PGS. They combine all the requirements of the perfect quantitative assay, namely accuracy, sensitivity, high selectivity, minimum purification and speed of execution.

This review covers some of the more important aspects of general radioimmunoassay development and PGS immunoassay, a

field which has been very active recently. The PGS control of avocado fruit development is also briefly reviewed.

1.2 Radioimmunoassay

The first radioimmunoassay (RIA) was developed for insulin by Yalow & Berson (1960). Since then RIA and other saturation assay techniques have made an explosive impact upon endocrinology and other areas of medicine, in which the accurate measurement of small concentrations of biologically potent compounds is vital. These methods represent a common analytical approach of great sensitivity that has been applied to the measurement of a great many substances (Ekins, 1974).

1.2.1 Terminology

The technique involves the combination of two primary components, one of which is referred to as the binder or antibody, the other (the substance being measured) as the ligand or antigen. However, there are many more components used in RIA. The definitions as given by Landon & Moffat (1976) are used to describe the more common components encountered :

Antibody or binder : a gammaglobulin which will combine with a specific antigen.

Antigen or ligand : a substance that will combine with a specific antibody.

Immunogen : a substance that will provoke an immune response.

Hapten : an antigen that must be linked to a larger molecule in order to invoke an immune response.

Labelled antigen or ligand : an antigen that has been modified in order to enable its presence to be accurately

measured. This modification usually involves the introduction of either a gamma or beta emitting isotope, but other labels such as enzymes can be used.

1.2.2 Basic principle of RIA

The basic principle of all binding assays (of which RIA is only one) is the same (Ekins, 1974). A simple illustration of the mechanism of RIA is presented in Fig. 1.1. The effect of different masses of ligand in the presence of binder is shown. The distribution of the antigen between

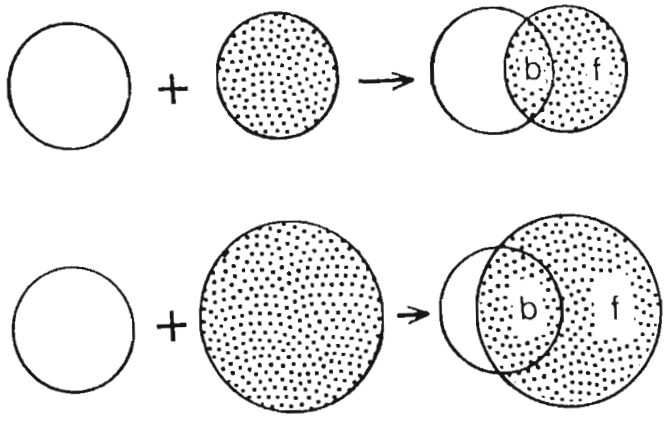


Fig. 1.1 The basic principle of a binding assay, using RIA as an example. If given amounts of antigen and antibody are allowed to react together an equilibrium antigen-antibody complex is formed (B) together with a portion of both antibody and free antigen (F). An increase in the amount of antigen (below) increases the amount of antigen-antibody complex (B). However, the increase in F is relatively greater and thus yields a lower bound to free ratio (Chard, 1981).

bound and free fractions is directly related to the total amount of ligand present, and thus provides a means for quantitating the latter (Kabat, 1980). In order to utilize this analytical principle, a means must be devised for separating the two components and consequently for quantitating the distribution of tracer ligand between them (Ekins, 1974).

A more productive approach to understanding the principle is through consideration of reversible reactions such as the reaction between an antigen and an antibody to form the antigen - antibody reaction complex :

$$Ag + Ab \underset{k2}{\overset{k1}{\rightleftharpoons}} AgAb$$

where Ag represents free antigen (the free fraction), AgAb the antigen present in the antibody bound form (the bound fraction), and k1 and k2 are the rates of forward and backward reaction respectively (Chard, 1981). The technique is based on determining the percentage of the total amount of Ag (free plus bound) that is present in the antibody bound fraction. This percentage is dependent upon three factors :

(a) It is directly related to the total amount of Ab present,

(b) It is directly related to the avidity with which the Ab binds the Ag and,

(c) It is inversely related to the total amount of Ag present.

In an immunoassay, the same concentration of the same Ab is present in each tube, i.e. factors (a) and (b) are kept constant, so that the only factor that influences the per-

centage of the total Ag in the bound fraction is the total amount of Ag present. In order to determine the percentage of the total Ag that has been bound, a constant amount of labelled antigen is added to each tube (to act as a tracer), and the bound and free fractions are separated by one of a number of techniques (Berson & Yalow, 1968). The percentage of the total counts present in the bound fraction can then be determined and will accurately reflect, and be inversely related to, the total amount of antigen present (Ekins, 1974). An unknown amount of the compound to be assayed can thus be quantitated by comparing the distribution of the tracer with the distributions produced by a number of standards (Landon & Moffat, 1976). Standards are a series of different concentrations of purified ligand against which the results of unknowns can be judged, and as such are no more than a technical convenience (Chard, 1981).

1.2.2.1 Binder dose-response curves

The binder dose-response curve involves the incubation of a fixed amount of tracer ligand with different concentrations of the binder (Odell, Abraham, Skowsky, Hescox & Fisher, 1971). This might, for example, consist of serial doubling dilutions of an antiserum. Following incubation, the distribution of the tracer in the bound or free fraction is ascertained. The general appearance of such a curve is shown in Fig. 1.2.

As a rule-of-thumb the concentration of binder chosen for use in a standard curve will be that which is sufficient to bind approximately 50% of the added tracer. At this concentration it is apparent that the addition of further ligand must lead to a substantially greater increase in the free fraction than the bound fraction. If a much higher binder concentration is chosen the amount of ligand required to produce a significant shift in the bound and free

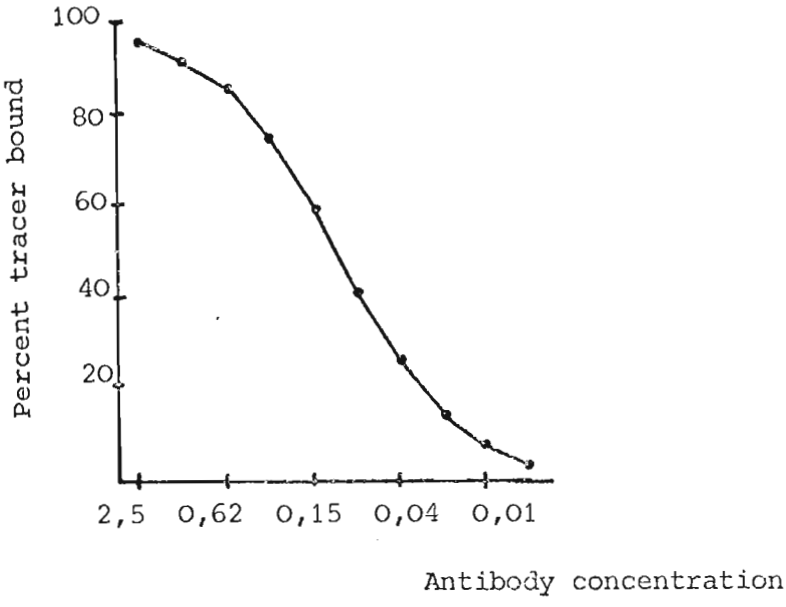


Fig. 1.2 Binder dilution curve. Serial dilutions of binder are incubated with fixed amount of tracer and the percentage of the latter plotted on the vertical axis (Chard, 1981).

fractions will be much greater, and the eventual assay less sensitive (Chard, 1981).

1.2.2.2 Standard dose-response curves

A standard dose-response curve involves the incubation of fixed amounts of tracer ligand and binder with different amounts of purified unlabelled ligand (Landon & Moffat, 1976). Plotted as the percentage of tracer bound against serial dilutions of the ligand on a logarithmic scale this gives a sigmoid curve (Fig. 1.3).

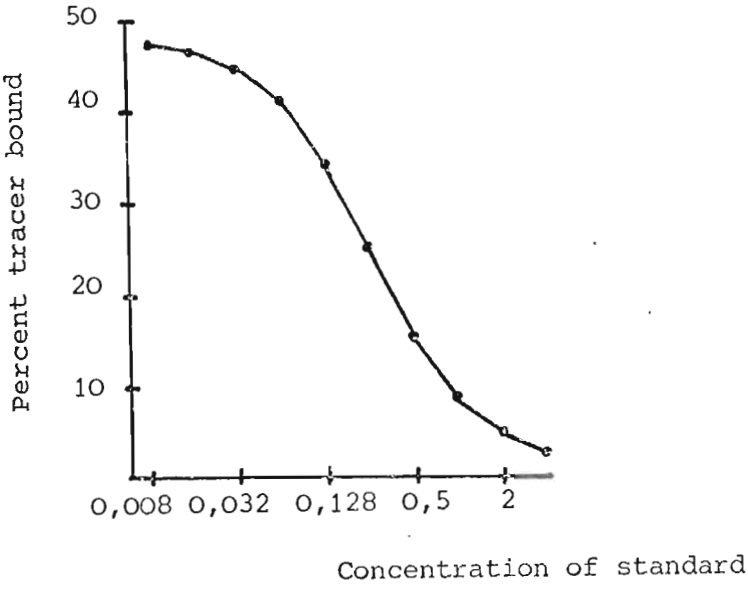


Fig. 1.3 A standard dose-response curve using fixed amounts of tracer and antibody (Chard, 1981).

In practical terms the steepest part of the slope represents the effective range of the assay. Thus in Fig. 1.3 the effective range would be from 0,032 to 2,0 (Chard, 1981). For an actual assay a biological sample replaces the standard and the result is read from the standard dose-response curve.

1.2.3 The binder or antibody

By-and-large the production of a suitable antiserum is the most important step in developing a RIA, as it is the antibody which determines the sensitivity and specificity of the material to be assayed by RIA (Hurn & Landon, 1971; Playfair, Hurn & Schulster, 1974; Landon & Moffat, 1976; Kabat, 1980).

1.2.3.1 Cellular events

The immune response can be divided into four phases (Parker, 1971) :

(a) An afferent phase in which the antigen is concentrated and processed by macrophages into an immunogenic complex.

(b) A recognition phase in which the processed antigen interacts with appropriate antigen-sensitive (antigen recognition) cells.

(c) A stimulatory phase in which antigen-sensitive cells are stimulated to differentiate and replicate to produce a large number of immunologically-committed plasma cells and small lymphocytes.

(d) An efferent phase in which antibodies and sensitized cells are generated and interact with the antigen to produce an inflammatory response.

The precise nature of the cellular events which lead to the production of large numbers of antibody-producing cells and sensitized lymphocytes is still not fully understood (Cunningham, 1978).

1.2.3.2 Chemistry of antibodies

The serum of normal vertebrates contains a large variety of proteins, including the five classes of immunoglobulins or antibodies. The basic structures and gross chemical properties of immunoglobulins are very similar, but their combining specificities vary widely, reflecting the

spectrum of antigens that the individual has encountered during its lifetime (Hood, Weissman & Wood, 1978).

Antibodies are made up of equal numbers of heavy and light polypeptide chains held together by non-covalent forces and interchain disulphide bridges (Hobart, 1976). The immunoglobulins comprise five classes, referred to as IgG, IgM, IgA, IgD, and IgE, all of which are based on a common structure (Fig. 1.4) (Alexander & Good, 1977; Bernier, 1978; van Regenmortel, 1982).

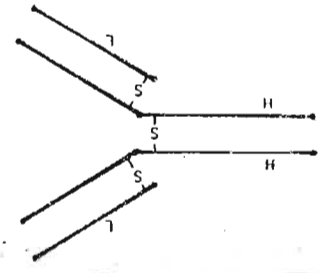


Fig. 1.4 The structure of the IgG molecule, consisting of two heavy chains (H) and two light chains (L) linked by disulphide bridges (S) (Chard, 1981).

While each class has a characteristic spectrum of activity, the nature of antibody populations is surprisingly variable. It can range from monoclonal responses to the widest possible heterogeneity involving all classes and subclasses. Many antibody responses share certain common features such as early IgM response (usually of low binding affinity) and a later, mainly IgG response (usually of a much higher binding affinity) (Turner, 1977). Due to the higher binding affinity of the IgG antibodies, this is the only class of any significance in RIA (Bernier, 1978; Chard, 1981).

There are two regions on both the heavy and light chains

known as the "variable" or V region and the "constant" or C region. The antigen combining sites lie in the variable region (Fig. 1.5). It is this variability, with the potential existence of many millions of different structures, which is responsible for the great specificity of antibodies (Hood, Weissman & Wood, 1978).

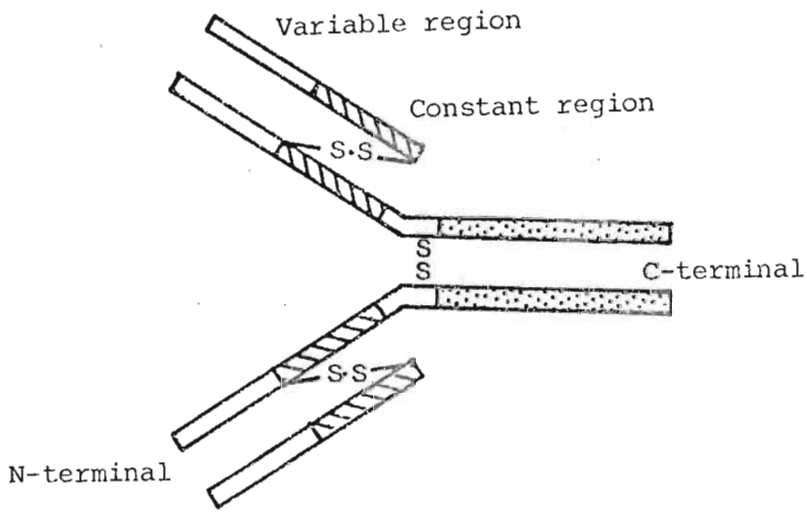


Fig. 1.5 Rabbit IgG molecule, showing the location of different domains (van Regenmortel, 1982).

1.2.3.3 The immune response

As stated earlier (Cunningham, 1978) the immune response at the cellular level is still poorly understood, although the complexity of the response is acknowledged.

When antigen is injected into a normal animal, there is a "lag" phase followed by the appearance of antibodies in the serum. If the animal has not been exposed to the antigen before, the "lag" phase of this response may be as long as 12 days. If the same antigen is re-injected, the "lag" phase is very much shorter (Hobart & McConnel, 1976). In

the instance of re-injection the predominant antibody produced is of the IgG class, which persists in the serum for weeks or even months (Alexander & Good, 1977).

The first antigenic experience primes the animal, so that it can make a secondary response which differs both quantitatively and qualitatively from the primary response (Hobart & McConnel, 1976). This is the phenomenon of immunological memory. The allergic response is mediated by a number of cell types, although the initial specific recognition of the antigen is done by lymphocytes which carry specific membrane receptors for the antigen.

Lymphocytes can be divided into T (for thymus) and B (for bone marrow) derived lymphocytes (Parker, 1971). T cells are involved with cell-mediated immune responses such as graft rejection or delayed hypersensitivity, whereas B cells are the direct precursors of mature antibody secreting cells. Both T and B type lymphocytes are required in the antibody response to most antigens. Although both are specifically involved with antigen recognition, only the B cells differentiate into antibody-secreting cells and this process is then controlled by the T lymphocytes. This phenomenon where T cells "help" B cells to respond to an antigen is known as cell co-operation (Hobart & McConnel, 1976).

Alexander & Good (1977) state that immunologic competence develops in an orderly manner in the very young, and that it gradually improves to reach a peak of responsiveness about the time of puberty. With increasing age however, the immunologic responsiveness begins to wane, indicated by the resultant increases in aged individuals to infections.

1.2.3.4 Immunogens

Immunogenicity is that property of a substance (immunogen) that endows it with the capacity to provoke a specific immune response. This consists of either the elaboration of antibody, the development of cell-mediated immunity, or both (Jackson, 1978).

The first and primary requirement for any molecule to qualify as an immunogen is that the substance be genetically foreign to the host (Landon & Moffat, 1976). In nature, an immune response will occur to a component that is not normally present in the body or normally exposed to the host's lymphoreticular system. However, not all foreign substances can induce an immune response, e.g. exposure to carbon in the form of coal dust will not induce an antibody response (Hood, Weissman & Wood, 1978). On occasion normal body constituents may be recognized as foreign and elicit an immune response (Jackson, 1978).

In general the immunogenicity of a material is directly related to its molecular mass. Materials with a molecular mass of 5 000 and greater are usually good immunogens (Garvey, Cremer & Sussdorf, 1977). Although some smaller molecules such as insulin do function as immunogens, the immune response is minimum in most hosts. It is also becoming increasingly clear that although immunogens are usually large substances, only restricted portions of the molecule may be actively involved with the antibody (Jackson, 1978). Materials of molecular mass less than 800 are not immunogenic (Chard, 1981).

There is no one molecular configuration that is immunogenic. Linear or branched polypeptides or carbohydrates, as well as globular proteins, are all capable of inducing an immune response. Nonetheless the antibodies that are formed in response to these different conformational

structures are highly specific and can readily discriminate these differences (Sela, 1966). When the conformation of an antigen has been changed, the antibody induced by the original form can no longer combine with it (Jackson, 1978).

Immunogenicity is not limited to a particular molecular charge; positive, negative and neutral substances can all be immunogenic. However, the net charge of the immunogen does not appear to influence the net charge of the resultant antibody. It has been shown that immunization with some positively charged immunogens resulted in the production of negatively charged antibodies (Jackson, 1978). The accessibility or spatial arrangement of the determinant groups on the immunogen will determine whether an immune response will occur (Sela, 1966).

1.2.3.5 Haptens

The term hapten was used to describe a substance with the capacity to react with an antibody but unable to induce an immune response. The term is now used to include artificial, as well as naturally occurring chemical agents. These substances are mainly of low molecular mass (less than 800). They are able to bind to antibody without inducing an immune response unless conjugated to a carrier protein (Turk & Parker, 1977).

When the hapten-carrier complex is used as a tool for the analysis of immunological specificity, it must be made certain that the observed biological effects are due to the attached hapten and not to side effects of the attaching procedure (Pohlit, Haas & van Boehmer, 1979). The density of the hapten on the carrier is important in the induction of an immune response (Parker, 1971). Generally five to eight moles antigen per mole of carrier is considered a sufficient dose (Turk & Parker, 1977). Some workers

however, use conjugates with a molar ratio of 15:1 and higher (Erlanger & Beiser, 1964; Ranadive & Sehon, 1967b). In theory, only one haptenic group per molecule of protein is required (Parker, 1971). It must be remembered however, that overloading does tend to reduce the antigenic effect (Turk & Parker, 1977).

The carriers most widely used for haptens are proteins such as albumins, 5-gammaglobulins and haemocyanins (Parker, 1971). A covalent link has to be formed between the two, usually a peptide bond between a carboxyl on the hapten and a free amino group on the protein molecule, principally on the side chain of lysine (Bauminger & Wilchek, 1980). If the hapten does not have a carboxyl then a suitable derivative must be formed with an active group which is present (Chard, 1981), or some other suitably reactive group used (Landon & Moffat, 1976).

There are many methods for forming the link between the hapten and the protein carrier (Erlanger, 1973). These include the periodate method of linking nucleotides to albumin (Erlanger & Beiser, 1964), and the carbodiimide method (Humayan & Jacob, 1973; Bauminger & Wilchek, 1980) for linking carboxyl groups to protein.

The site of linkage between the hapten and the protein is again emphasized. The aim should be to prepare an immunogen in which the principle functional groups of the hapten are remote from the linkage site and are thus presented to the immune system of the animal in an unaltered form. This is of critical importance for the specificity of the resulting antiserum (Chard, 1981).

1.2.3.6 Adjuvants

Adjuvants may be described as substances that when mixed with antigen prior to injection, enhance the antibody

response (Hurn & Landon, 1971). Garvey *et al.* (1977) suggest that adjuvants are particularly useful if only small amounts of antigen are available or if the material is of low immunogenicity. This has been shown by Vaitukaitis, Robbins, Nieschlag & Ross (1971) and Vaitukaitis (1981).

Waksal (1978) noted that although much remains to be understood, non-specific stimulators or adjuvants appear to induce their effects in a number of ways, including the following :

(a) Prolongation of the release of antigen - this is particularly enhanced in water-in-oil emulsions.

(b) Antigen denaturation increases the immunogenicity of serum proteins such as gamma-globulins.

(c) Recruitment of antigen-reactive cells due to the development of granulomas at the site of injection.

(d) Stimulation of cell-mediated immunity in addition to antibody formation due to an increase in delayed hypersensitivity to protein antigens, as with Freunds complete adjuvant.

Recent evidence now suggests that adjuvants may selectively expand T and B lymphocyte populations in addition to their more accepted classical roles (Jackson, 1978).

Freunds adjuvants induce powerful cell-mediated responses, humoral immunity, break tolerance and potentiate tumor rejection (Whitehouse, 1977). Freund (1951) devised two of

the most commonly used adjuvants. They are normally water-in-oil emulsions, composed of antigen in saline and a mixture of an emulsifier, Arlacel A, in mineral oil, with or without mycobacteria (Freunds complete or incomplete adjuvant respectively). Although both types of adjuvant enhance antibody formation, greater augmentation with certain antigens is achieved by the addition of killed mycobacteria (Freund, 1947). The presence of mycobacteria also enhances cutaneous hypersensitivity of the delayed type to the incorporated antigen (Garvey *et al.*, 1977).

Aluminium salts have also been used to raise the immunogenicity of toxoids in the development of anti-toxic antisera. Soluble aluminium salts precipitate many proteins or cause them to form large globular aggregates (Jolles & Paraf, 1973). The less soluble aluminium adjuvants probably have a two-fold action by providing a fairly large sorptive area to fairly soluble proteins, and limiting their bio-diffusion, thereby providing a particulate attraction to immunogen-processing cells *in vivo* by their low solubility (Whitehouse, 1977). Another important property of the aluminium salts is that they are not very toxic to cells mounting the immune response, unlike many other metal ions such as copper (Garvey *et al.*, 1977).

Silicon-containing materials can also be used as adjuvants. As these materials cannot readily pass into solution their activity depends primarily on their absorptive and macrophage stimulation properties (Whitehouse, 1977). Bentonite, a material related to montmorillonite, has been found to show marked adjuvant activity in stimulating cell-mediated and humoral responses in guinea pig (Chase, 1967).

1.2.3.7 Antibody production

Because variation between animals is so great and specific information so lacking, only general rules-of-thumb for

type and timing of injection are offered. Intravenous, intraperitoneal, subcutaneous or intradermal injection of materials are the most commonly employed procedures used with experimental animals (Garvey *et al.*, 1977).

Various immunization schedules have been formulated and successfully used (Hurn & Landon, 1971; Odell, Abraham, Skowsky, Hescox & Fisher, 1971; Vaitukaitis, Robbins, Nieschlag & Ross, 1971; Landon & Moffat, 1976; Garvey *et al.*, 1977; Chard, 1981; Vaitukaitis, 1981).

1.2.4 The ligand

Two types of ligand are required in RIA. A purified ligand is needed for immunization and standard dose-response curve establishment, and a tracer ligand to detect the levels in the assay determination.

1.2.4.1 Purified ligand

A supply of highly purified ligand is an essential pre-requisite to the development of any binding assay, and the application of the technique is limited to the substances for which this criterion can be met (Chard, 1981). The RIA depends on competitive binding of a substance in a test sample that is biological in origin. This makes it possible to define the system in absolute terms (Bangham & Cotes, 1974).

Generally, synthetically-prepared ligands present few problems, while ligands which have to be prepared from natural sources can present considerable problems.

Large protein hormones are the most common immunogens currently in use, especially for large animal hormones such as insulin (Buchanan & McCorroll, 1971). However, the problem of obtaining adequate supplies of pure hormone of

human or animal origin is no longer so critical, as synthetic alternatives are available for nearly all these hormones (Chard, 1971). As plant growth substances (PGS) do not have molecular masses in excess of 800 (Muir & Lantican, 1968; Lang, 1970; Milborrow, 1974; Leopold & Kriedemann, 1975) the problems associated with large protein molecules are of no consequence to the plant physiologist making use of RIA.

Most of the naturally-occurring PGS and metabolites can be prepared synthetically to a high degree of purity. However, commercial preparations must never be accepted as 100% pure (Bangham & Cotes, 1971) as in the course of preparation, "error" precursors are likely to be produced (Chard, 1981). Ideally, the ligand used in the assay (as tracer and standard) should be identical with the endogenous ligand which the assay is intended to measure (Bangham & Cotes, 1971). However, this is not always possible, and to highlight this fact abscisic acid (ABA) is considered. Naturally-occurring ABA (Milborrow, 1974) occurs as a plus isomer, whereas the synthetic preparations are a mixture of plus and minus isomers (Weiler, 1979). Any RIA developed for ABA will have to take this into account.

1.2.4.2 Tracer ligand

An essential element to any binding assay is a means for determining the distribution between the bound and free fractions (Landon & Moffat, 1976). For this purpose a small amount of highly purified labelled ligand (the tracer) is incorporated into the system (Ekins, 1974). The label may be any substance having the primary characteristic that it can be measured accurately by direct and simple methods, and that the sensitivity with which it can be detected is greater than that of direct methods for the measurement of the ligand itself. In

practise the label is almost invariably a radioactive isotope (Chard, 1978). Other labels such as enzymes can be used provided they meet the primary criteria (Landon & Moffat, 1976).

The choice of which radioactive isotope to use depends on the assay design and type of ligand. In the early 1970s iodination held sway (Hunter, 1971) in the belief that it was superior in all aspects when compared to tritium and ^{14}C. The popularity of tritium is growing (Peng, 1977) with the various commercial radiolabelling institutions offering tritium-labelling services (Anon., 1980).

Isotopes fall into two groups, each with its own advantages and disadvantages. The beta emitters ^{3}H and ^{14}C make up one group while the gamma emitters, particularly ^{131}I and ^{125}I comprise the other (Landon & Moffat, 1976). Chard (1981) outlines the following advantages of tritium :

(a) No marked steric changes to the molecule and therefore no marked influences on the antigenicity of the compound.

(b) Compound stability.

(c) Long half-life.

(d) Minimum health hazard.

Disadvantages for the use of tritium in RIA include low specific activity, cost and the apparatus required (Hunter, 1971).

Compounds labelled with gamma-emitting isotopes have a number of advantages when the number of samples to be assayed is large (Landon & Moffat, 1976). The samples can be counted directly in a gamma counter (Odell, Wilber &

Paul, 1965), without the need for liquid scintillant (Chard, Kitau & Landon, 1970), and much higher specific activities can be obtained (Greenwood, Hunter & Glover, 1963). ^{125}I has a long half-life and is not too great a health hazard (Landon & Moffat, 1976).

The purpose of the tracer is to provide a measure of the total ligand in the bound and free phases of the system. Its behaviour must be as nearly identical as possible with that of the unlabelled ligand (Anon., 1979). By definition the tracer must be slightly different from the pure ligand (Chard, 1978) due to the presence of the label on the molecule. However, provided its binding ability is not impaired this is of no significance.

1.2.5 Separation of bound and free ligand

All radioimmunoassays require a separation procedure because the bound fraction does not precipitate spontaneously at the low concentrations employed. This separation is necessary to determine the distribution between the free and bound forms. A wide variety of such procedures that exploit physicochemical or immunological differences between the two fractions are available (van Vanakis, 1980).

No single separation technique is ideal, and in establishing a new assay it is desirable to evaluate at least three to five different methods (Greenwood, 1971). The efficiency of a separation method can be defined as the completeness with which the bound and free phases are separated (Giese & Nielsen, 1971; Chard, 1980, 1981).

1.2.5.1 The ideal separation technique

Ratcliffe (1974) considers that an ideal separation should :

(a) Completely separate bound and free fractions with a wide margin for error in the conditions used for separation. Failure to meet this requirement will impair precision and sensitivity.

(b) Be practical i.e. be simple, quick and cheap and use reagents and equipment that are readily available.

(c) Not be affected by plasma or serum. Failure to meet this requirement causes difficulty in standardization.

A wide variety of separation techniques are currently employed in RIA with the original application being to peptide and protein hormones, but these techniques can be applied to a wider spectrum of antigens. All the popular separation techniques will be described as their application to PGS RIA is so novel.

1.2.5.2 Differential migration of bound and free fractions

These techniques rely on the differences in charge or the differences in relative molecular mass of the bound and free fractions.

1.2.5.2.1 Paper chromatoelectrophoresis

This method was employed by Yalow & Berson (1960) for insulin. It depends on the selection of a suitable paper that absorbs undamaged free hormone at its site of application. The bound fraction, "damaged" (hormone that has self-destructed due to the specific activity of the isotope) and free iodide move from the origin, under the influence of an electric current and buffer flow caused by

evaporation. The special advantage of chromatoelectrophoresis is that it separates damaged labelled peptide and free iodide, thus allowing a correction for the damaging effect of individual plasma samples. However, this technique is too complex, time-consuming and expensive to use routinely and cannot be automated (Ratcliffe, 1974).

Other electrophoretic techniques such as starch gel and cellulose acetate (Hunter & Greenwood, 1964) or wick (Orskov, 1967) chromatography may be used, but are limited by practical disadvantages (Chard, 1981).

1.2.5.2.2 Gel filtration

By definition the binder ligand complex must be larger than the ligand and can be clearly separated by using an appropriate grade of Sephadex or Biogel (Ratcliffe, 1974). Phase separation can be achieved by passing the incubate through a column under conditions such that the bound moiety is eluted, leaving the free moiety within the gel particles (Haber, Page & Richards, 1965). Giese & Nielsen (1971) used Sephadex G-25 in gel filtration whereas Genuth, Frohman & Lebovitz (1965) used G-75.

1.2.5.2.3 Gel equilibrium

In this method the gel is actually incorporated in the incubation medium. Low molecular mass material (free ligand) can then distribute freely both inside and outside the gel. The bound complex with its associated high molecular mass cannot enter the gel and is thus segregated in a small part of the system (Chard, 1981).

One of the advantages of this method is that a steady state is produced which, after a few minutes, is independent

of time. Gel equilibrium is probably the most satisfactory of the separation procedures currently available for competitive protein-binding assays (Ratcliffe, 1974).

1.2.5.3 Adsorption methods

Methods involving adsorption, usually of the free fraction, are widely employed in saturation analysis because of their simplicity, speed and large sample capacity (Chard, 1981). Adsorption (Ratcliffe, 1974) is determined by many factors such as surface area of absorbant, size and charge of antigen, temperature, ionic strength and pH.

1.2.5.3.1 Charcoal

Charcoal was first used for the RIA of insulin by Herbert, Lau, Gottlieb & Bleicher (1965). In general wood charcoals with a maximum particle size of less than 60μm have satisfactory adsorption characteristics. The major problem is that untreated charcoal has a high non-specific avidity for a wide range of substances, so that the bound as well as the free fraction may be adsorbed. However, despite the disadvantages both Hunter (1971) and Buchanan, McCarrol & Ardill (1971) recommend that charcoal be tried first in any new RIA.

Use of charcoal depends on recognizing that sufficient charcoal will bind antibody-ligand complexes as well as ligand, i.e. that dose-response relations exist for dose (or amount) of charcoal versus percentage of both antibody-bound and free ligand complexes. One must always, and for each ligand-antibody system of interest, study the full dose-response relations prior to selecting an amount of charcoal.

Variation of protein content in the medium bathing the

antibody complexes and ligand results in varying possibilities of competition for the charcoal surface. Thus, in general, with the amount of charcoal held constant, increasing protein content decreases the charcoal binding of both free ligand and antibody-ligand complexes (Odell, 1980).

Lastly, it is important to dispense with the concept of molecular sieving by dextran coating of charcoal. Gottlieb *et al.* (1965) suggested that by selecting the appropriate dextran (Sephadex) to coat the charcoal one could produce a reagent that selectively bound small molecules and failed to bind larger ones. It was hypothesized that a Sephadex molecular sieve had been produced. Binoux & Odell (1973) showed that Sephadex coating does not produce such a selective reagent. Sephadex does shift charcoal dose-response curves to the right. It also makes charcoal stickier, permitting easier centrifugation into a pellet, but it does not limit access to charcoal based on molecular size.

1.2.5.3.2 Silicates

Silicates have adsorptive properties which can be used for separation in RIA (Landon, 1971). Silicates with high silica content and large surface area can be added directly to incubation tubes in powder or tablet form. Adsorption occurs rapidly and separation is simple as the materials pack well on centrifugation. Rosselin, Assam, Yalow & Berson (1966) used talc powder to separate out bound and unbound peptide hormones.

1.2.5.4 Fractional precipitation

Fractional precipitation using neutral salts or organic solvents offers a simple method to achieve the separation of antibody-bound from free fractions in the radioimmuno-

assay (Chard, Martin & Landon, 1971). In this technique the immunoglobulin precipitates at a critical concentration of precipitant leaving the free fraction in solution (Ratcliffe, 1974). As the forces that determine this effect are largely electrostatic they can be influenced by many factors such as pH, temperature and protein concentration (Chard, Martin & Landon, 1971).

Ammonium sulphate (Goodfriend, 1968) has been successfully employed in assays of plasma and urine extracts for small peptide hormones. Chard, Kitau & Landon (1970), successfully used ammonium sulphate in their RIA for oxytocin. The use of ammonium sulphate and polyethylene glycol as reagents to separate antigen from antibody complexes has been thoroughly reviewed by Chard (1980).

Ethanol (Hedding, 1966) is perhaps more versatile, being suitable for a wide range of peptides. However, separation of certain antigens such as growth hormone varies considerably with time after the addition of ethanol, suggesting progressive disruption of the bound complex (Ratcliffe, 1974). Odell, Wilber & Paul (1965) advocated the use of ethanol and sodium chloride to achieve separation.

1.2.5.5 Double antibody method

This method employs a second antibody to precipitate soluble antigen-antibody complexes in the first antibody reaction. Utiger, Parker & Daughaday (1962) first applied this method for growth hormone. Precipitation reactions only occur at high concentrations of antigen and antibody, therefore separation by this technique requires relatively high concentrations of first and second antibodies (Chard, 1981). This method has been used successfully by Morgan & Lazarow (1963) and Hales & Randle (1963).

Den Hollander & Schuurs (1971) used a double antibody method where the precipitating serum was conjugated to an insoluble matrix such as cellulose. The practicability of the solid phase method is combined with the general applicability of the double antibody technique. The use of the double antibody method in RIA to separate antibody-bound from free ligand in RIA has been thoroughly reviewed by Midgley & Hepburn (1980).

1.2.5.6 Solid phase methods

In this method the binder is covalently linked to an insoluble support, enabling both it and the bound complex to be readily separated from the soluble free fraction. A wide variety of solid phase supports have been described including dextran and cellulose (Chard, 1981).

Two approaches have been used in the solid phase system. The binder may be attached to discs and tubes (Catt & Tregear, 1967), but as covalent bonds are not involved the links are unstable, and there is doubt about its general applicability and precision (Ratcliffe, 1974). In the second approach the binder is attached to a particulate solid phase (Wide & Parath, 1966). Here the gamma-globulins from an antiserum are attached to the particles by any one of a number of techniques designed to yield a covalent link between the protein and the particle. Chard (1981) states that for any assay with a large sample throughput, particle solid-phase systems are not technically convenient.

No separation method is universally satisfactory and each method has its weaknesses. Obvious factors to consider when selecting a method are practicality, simplicity, cost, general applicability, reagent availability and suitability for automation. At present costs militate against double

antibody systems whereas simplicity argues in favour of solid phase methods (Ratcliffe, 1974).

1.2.6 Statistical analysis of RIA data

The widespread use of RIA and related techniques has led to the development of methods for routine data analysis (Rodbard, Rayford, Cooper & Ross, 1968; Rodbard & Lewald, 1970; Rodbard, 1971). Unfortunately, many researchers still utilize graphical methods alone, or linear interpolation between adjacent points on the dose-response curve. These methods do not provide efficient utilization of the data, do not provide estimates of the precision of unknowns, are subject to erratic behaviour and subjective biases, and forfeit important information about the assay system (Rodbard & Frazier, 1975). The RIA dose-response curve presents two problems: non-linearity and non-uniformity of variance (Rodbard & Lewald, 1970).

Of the various methods of curve fitting, the log-logit transformation is preferable. It is the easiest to calculate, provides the simplest expressions for weighting and is theoretically justified (Rodbard & Frazier, 1975).

This is calculated as :

$$\text{logit } b = \log \frac{b}{(100-b)}$$

where b is the proportion of tracer bound expressed as a percentage of that in the zero standard.

Chard (1981) lists the following practical points for using the logit transformation :

(a) The assay blank must be subtracted from the percentage bound before the logit is cal-

culated. Failure to do so will result in a non-linear response.

(b) The upper and lower 10% of a standard curve are often non-linear in logit transform and should be eliminated when this is the case. Values recorded for unknowns above and below these limits should be rejected as inaccurate.

(c) The 'goodness of fit' of the straight line resulting from the logit transformation is judged by the correlation coefficient or r value of a linear regression.

(d) The validity of the logit transformation is very dependent on good estimation of the zero standard and the assay blank.

For several combinations of tracer and antibody concentrations, the log-logit method shows no departure from linearity (Feldman & Rodbard, 1971). By providing linearity, the log-logit method greatly facilitates dose interpolation over the entire dose range (Rodbard & Frazier, 1975).

For the monitoring of precision, Chard (1981) advocates the repeated determination of a quality control pool. The standard deviation expressed in absolute terms giving an absolute measure of dispersion (Rayner, 1967) is useful in the analysis of biological data.

Various quality control measures can also be applied to the standard dose-response curve statistics, such as zero standard and assay blank, as well as intercepts and slope of the standard dose-response curve (Lishman, 1971).

Chard (1981) advocates the placement of an arbitrary upper limit on the differences between replicates, and proposes the figure of 5% of the total counts in the system. Within-assay variation can be assessed as the coefficient of variation of duplicate samples :

$$\sqrt{\frac{\Sigma \left(\frac{d}{\bar{x}} \times 100\right)^2}{2n}}$$

where d = difference between duplicate estimates

$\bar{x}$ = mean of duplicate estimates

n = number of duplicate estimates.

Chard (1981) states that the estimation of confidence limits to the result of an unknown is unnecessary for most practical purposes, apart from quality control.

1.3 Immunological Assays for PGSs

Immunological assays and, in particular, RIA have been widely applied to animal hormones and drug assays (Landon & Moffat, 1976). Due to their high selectivity, minimal purification is required. Sensitivity is in the same range or better than the most sensitive physicochemical procedures (Weiler, 1982a). Horgan (1981) believes that in principle at least, immunological assays would appear to be the ideal methods for the quantitative analysis of plant hormones.

The prime requirement for any immunological assay is good quality antisera (Chard, 1981). PGS molecules are too small to be recognised by the animal immuno-system for the formation of specific antibodies (Turk & Parker, 1977). Therefore it is necessary to covalently link the PGS to a carrier molecule, usually a protein (Kabat, 1980). To

date immunological studies on PGS have concentrated largely on using bovine (BSA) or human serum albumin (HSA) as carrier molecules (Brenner, 1981).

Initial PGS immunological studies were carried out by Fuchs and co-workers (Fuchs & Fuchs, 1969; Fuchs, Haimovich & Fuchs, 1971; Fuchs, Mayak & Fuchs, 1972; Fuchs & Gertman, 1974), mostly on auxin and gibberellic acid. These studies were not done using RIA and the sensitivity of the method was low. Also revealed at this stage were the potential problems of selectivity and cross-reactivity inherent in immunological studies of this type. More recently the trend has been toward RIA and more selective linkage of the PGS to the carrier molecule (Brenner, 1981). Pengelly & Meins (1977) developed a specific RIA for auxin, for use with methanolic extracts. This technique was validated by a parallel comparison with GC-MS (Pengelly, Bandurski & Schultz, 1981). Weiler (1981) developed a similar RIA for indole-3-acetic acid and compared ^{131}I- and ^{3}H-tracer derivatives. In all aspects ^{3}H-tracer outperformed the ^{131}I tracer. More recently Weiler, Jourdan & Conrad (1981) described a very sensitive enzyme immunoassay for IAA. The problems associated with isotope instability could therefore be circumvented.

Initial work using gibberellins seemed to indicate that these compounds were unsuitable for immunological work (Fuchs & Fuchs, 1969; Fuchs, Haimovich & Fuchs, 1970; Fuchs & Gertman, 1974). Weiler & Wieczorek (1981) developed a RIA for GA_3, which could also be used to quantitate GA_7. The assay was very sensitive and stable. More recently Atzorn & Weiler (1983a; 1983b; 1983c) developed and applied various immunoassays for a wide range of gibberellins.

A sensitive antibody to isopentenyl adenosine was described

by Hacker, van Vunakis & Levine (1970). Khan, Humayun & Jacob (1977) described a RIA for isopentenyl adenosine. Weiler (1980a) developed a very sensitive RIA for zeatin riboside and mentioned the possibility of using the antisera to detect zeatin levels. RIAs to determine abscisic acid (ABA) levels in plant extracts were developed by Weiler (1979) and Walton, Dashek & Galston (1979). These two assays are essentially similar but do not differentiate between free and bound forms of abscisic acid. Unfortunately the antiserum cross-reacts as well or better with both abscisic acid glucosyl ester and abscisic acid methyl ester as it does with ABA. At present a very sensitive and specific enzyme immunoassay is available for ABA (Weiler, 1982b).

1.4 Avocado Fruit Development and Senescence

While numerous studies on avocado maturity have been made over the last 50 years (Barmore, 1977), studies on the physiological development of the avocado fruit appear to have been largely neglected. The anatomical development of the avocado fruit has however, been thoroughly investigated (Schroeder, 1953).

The growth of the avocado fruit follows the single sigmoid curve (Valmayor, 1964; Robertson, 1971). The early stages of fruit growth, regardless of whether it is an early or a late maturing cultivar are characterized by very rapid cell division (Barmore, 1977). Differences in fruit size of cultivars maturing at approximately the same time resulted primarily from differences in the rate of cell division during the first six months of development (Valmayor, 1964).

The avocado fruit is unusual in that cell division of the flesh is not restricted to the initial period of growth,

but also continues during cell enlargement and even in mature fruit attached to the tree. In some cases, cell enlargement stops when the fruit reaches 50% of its size at full maturity, while cell division accounts for continued growth (Cummings & Schroeder, 1942). Additional variation in fruit size can exist in the same cultivar due to cultivation practises, yield, water relations and climatic conditions (Barmore, 1977).

The relationship between size and development or maturity can be used as a determinant of maturity only if the above-mentioned factors which affect the size are understood. Studies have shown that, in general, larger fruit have higher flavour ratings than small fruit when tested early in the season at the time of minimum acceptability (Soule & Harding, 1955; Hatton & Reeder, 1969). However, as the season progressed differences between large and small fruit became less pronounced (Soule & Harding, 1955). In order to manipulate fruit growth in avocados it is necessary to develop an understanding of some of the endogenous controlling systems in the fruit. One such system is the regulation of fruit growth by endogenous plant growth substances (PGS).

1.4.1 PGS regulation of fruit growth

A detailed review of PGS regulation of fleshy fruit growth is not attempted here. Emphasis is upon work which used avocado as the test material.

The role of seeds upon fruit growth varies with fruit species - some are dependent upon their seeds for virtually the entire period of fruit growth, e.g. the strawberry (Nitsch, 1950); others are vegetatively parthenocarpic e.g. the banana (Luckwill, 1981); while in between we have the full range of fruit types dependent upon their seeds

for varying lengths of time (Leopold & Kriedemann, 1975). The avocado appears to be dependent on its seed (for normal growth) for virtually its full development period (Blumenfeld & Gazit, 1974).

The role of auxin in fruit growth and development appears uncertain at present (Leopold & Kriedemann, 1975). The richest source of auxin activity in avocado fruit is in the seed (Gazit & Blumenfeld, 1972). In most fruits there is little or no correlation between endogenous auxin concentration and fruit or seed growth (Goodwin, 1978). In avocado, Gazit & Blumenfeld (1972) found auxin activity in young fruit. At all stages of fruit development the seed and testa contained higher levels of auxin than the surrounding meso- and endocarp (flesh). They concluded that auxin increased the sink strength of the fruit and regulated seed tissue development.

Exogenous gibberellin treatments have been shown to stimulate fruit growth in certain species but not in others (Leopold & Kriedemann, 1975). A correlation between fruit growth and extractable gibberellin activity has been found in *Phaseolus vulgaris* (Skene & Carr, 1961), *Citrus sinensis* (Wiltbank & Krezdorn, 1969) and *Prunus* (Martin & Campbell, 1976). However, for avocado no such correlation could be established by Blumenfeld & Gazit (1972). Crane (1964) concluded that, in general, gibberellin levels did not correlate well with fruit growth rates. Blumenfeld & Gazit (1972) found high levels of gibberellin activity in the seed and testa of developing avocado fruit. The level in the testa decreased with fruit growth. No measurable gibberellin-like activity was detected in the meso- and endocarp (flesh) or embryo. They concluded that the testa was the site of gibberellin-like substance production in the avocado fruit.

The presence of cytokinins in developing fruits is fairly well established, especially during the early cell division period of fruit development (Leopold & Kriedemann, 1975). Seeds appeared to be the central source of cytokinins in apple fruit (Letham & Williams, 1969). Whether the seed is the site of cytokinin synthesis, or whether the fruit and seed are supplied with cytokinins from the sap (Kende, 1965; Letham, 1969) or roots (van Staden & Davey, 1979) does not appear to have been elucidated at this stage. In grape, exogenous cytokinin application brought about increases in fruit growth (Weaver & van Overbeek, 1963).

In avocado, Blumenfeld & Gazit (1970) found high levels of cytokinins in both the seed and testa, the levels of which decreased with development. At the time of testa shrivel and withdrawal, cytokinin activity was not detected. They concluded that the high levels of cytokinin activity in the young seed served to increase the "sink" strength of the fruit for nutrients and other metabolites. In another study, Gazit & Blumenfeld (1970) found that cytokinin activity in the mesocarp (flesh) was very low and decreased further with development. However, whether the reduction in growth was due to increased inhibitor levels or decreased cytokinin levels was not determined.

At present the role of ABA in fruit development remains speculative although certain evidence has been presented that ABA may play a role, particularly in seed development. A role for ABA was first proposed by Thompson (1961) working on strawberries. ABA levels rise and fall during the development of many types of seeds (King, 1976; McGlasson & Adato, 1976; Hsu, 1979). Water stress appeared to influence ABA levels in barley grains (Goldbach & Goldbach, 1977). A role for ABA in inhibition of seed germination in developing fruit has been advanced by several in-

vestigators (King, 1976; Morris, 1978).

Gazit & Blumenfeld (1970) detected an inhibitor in avocado mesocarp which they concluded was not ABA. The level of this inhibitor increased as growth slowed. In a further study (Gazit & Blumenfeld, 1972), three inhibitors were detected in avocado fruit material, one of which had chromatographic properties similar to ABA and the level of which remained nearly constant throughout development. Inhibitor levels in other fruit components were not presented.

1.4.2 PGS regulation of senescence

The senescence of fruits starts with the ripening of the mature fruit. The PGS regulation of ripening is very similar to other senescence phenomena such as abscission (Bruinsma, 1981).

Studies on the involvement of ethylene have, in the past, dominated the study of PGS control of fruit ripening. Externally applied ethylene can induce ripening in fruit, and potentially stimulatory concentrations of the gas are present in fruit at the inception and during ripening. Rhodes (1980) therefore concluded that the gas plays some fairly direct role in ripening. The precise nature of this role still has to be elucidated. As the development of a RIA for ethylene is impossible, due to its structure, this topic will not be further reviewed.

The control of maturation and the initiation of ripening is thought to be due to the interaction and balance between opposing promotory and inhibiting factors (Rhodes, 1980). ABA could be one of these promotory factors, as it accumulates in maturing fruits and its exogenous application may advance the onset of ripening in grapes

(Hale & Coombe, 1974). ABA has been shown to stimulate ethylene production in pre-climacteric apples (Lieberman, Baker & Sloger, 1977). In avocado, the concentration of ABA is constant during maturation but rises during ripening (Adato, Gazit & Blumenfeld, 1976). ABA levels increased during the ripening of some types of climacteric fruit and ABA increase tended to precede the increased ethylene production involved with ripening (Looney, McGlasson & Coombe, 1974). External treatments that delayed or advanced ripening such as cold treatment also delayed or advanced changes in ABA in pear (Wang, Wang & Mellethin, 1972). McGlasson & Adato (1976) found an increase in ABA levels during the major period of growth of the tomato fruit. In avocado the increase in ABA during ripening appears to be the result of synthesis, rather than release from the bound form (Adato *et al.*, 1976). ABA synthesis, like ethylene, appears in some cases to be stimulated by the initiation of the ripening process (Rhodes, 1980).

CHAPTER 2
RADIOIMMUNOASSAY FOR ISOPENTENYL-TYPE CYTOKININS

2.1 Materials and Methods

2.1.1 Conjugation of IPA to BSA

A modification of the method described by Erlanger & Beiser (1964) for coupling nucleosides to proteins was used. One hundred mg isopentenyl adenosine (IPA) was dissolved in 2ml of methanol. A total of 5ml 0,01M $NaIO_4$ was added to the solution over a 10 min period, followed by the addition of 0,3ml 0,1M ethylene glycol to neutralize excess periodate. The solution was allowed to stand for 5 min. The reaction mixture was added dropwise to a stirred solution of 100mg of BSA dissolved in 5ml distilled water, with the pH adjusted to 9,3 with 5% K_2CO_3. During the addition of the IPA mixture the pH was kept between 9,2 and 9,4 with further additions of 5% K_2CO_3.

The reaction mixture was allowed to stand for 60 min. Thereafter 5mg of solid $NaBH_4$ was added and after 40 min this step was repeated. The pH of the solution was adjusted to 6,5 using 1M acetic acid and the solution stirred for 2 hours. The conjugate was purified by dialysis against distilled water for 72 hours, freeze-dried and stored at -5°C.

The linking ratio of IPA to BSA was determined using the ultra violet (UV) spectrum of the conjugate. It was assumed that the mass of IPA attached per mole of protein is negligible. Twelve mg samples of both BSA and the IPA-BSA conjugate were separately dissolved in 50ml distilled water and their UV spectra between 200 and 300nm determined. Using a Σ_{max} = 15 000 for nucleotides (Erlanger & Beiser, 1964) and the data derived from the UV spectra, the linking ratio was determined (refer to 2.2.1). The answer was

checked using a UV standard curve constructed from serial dilutions of IPA at 260nm from an IPA standard dissolved in methanol.

2.1.2 Production of antisera

2.1.2.1 Immunization

The immunization method selected was the small dose multiple intradermal injection technique, which has the ability to generate specific antibody with both high titer and affinity (Vaitukaitis, 1981).

The water-in-oil emulsion for injection was prepared as follows. The freeze-dried IPA-BSA conjugate was dissolved in sterilized saline (0,9% m/v, pH 4,6) so that 1ml of saline contained 1mg of immunogen. This was transformed into a stable water-in-oil emulsion with the addition of 3ml Freunds complete adjuvant per 1ml saline, using a syringe transfer method. After loading, the syringes were set aside for 1 hour at 4°C to ascertain the stability of the emulsion.

Five healthy male rabbits approximately six months old were selected for immunization. The animals were fed a commercial pelleted ration. A patch of fur about 60mm x 100mm was removed from the animal's lower back, taking care not to break the skin or clip the fur too short. The area was sterilized with 70% alcohol. Using 20 or 21 gauge needles, between thirty and fourty 100µl intradermal inoculations were given in this area. The initial immunization was in August, 1981.

After a six month rest the rabbits were boosted using the above method. A second boost was given in August 1982, but here Freunds incomplete adjuvant was substituted for the complete adjuvant to reduce the hypersensitive reaction

that developed after the February, 1982 boost. Approximately 20ml of blood was collected (in 40ml glass centrifuge tubes), nine days after each boost from the marginal ear vein of each rabbit.

2.1.2.2 Purification and storage

The purification and storage of serum was a modification of the method described by Garvey, Cremer & Sussdorf (1977). The freshly drawn blood was allowed to stand for one to two hours in a water bath at 37°C for clot formation. The clot was carefully separated from the wall of the tube using a sterilized metal spatula. The blood was then stored at 4°C for 24 hours to permit clot contraction.

The serum was decanted into 4,5ml sample tubes and centrifuged at 2 000g for 20 min. The cleared supernatant was removed using a Pasteur pipette with minimum disturbance to the sediment. Aliquots of 2 to 3ml of the serum were placed in 4,5ml plastic sample tubes and snap frozen using a 99% methanol and dry ice mixture. The frozen sera were stored at -20°C. Antisera were not pooled.

2.1.2.3 Titer determination

Titer determination was done by RIA. Anti-IPA serum was diluted in 0,1% gelatine (®Knox unflavoured) in phosphate buffered saline (PBS) of pH 7,0. Dilutions which ranged from 1:50 to 1:500 were prepared and the percentage binding relative to the total activity added determined. The procedure was as for a standard RIA for IPA (refer to 2.1.3.2), the only alteration being the absence of standard or sample. Anti-IPA serum titer curves were established and all titers given are relative to 30% binding of the total activity added.

2.1.3 Radioimmunoassay

Before any RIA is accepted, various tests have to be performed to select the most suitable procedure or adjust the assay conditions (Chard, 1978). Thus the RIA is optimized for the determination of the biological compound of interest.

2.1.3.1 Optimization

2.1.3.1.1 Effect of separation technique

Four different separation methods were tested. Aspects under consideration were the efficiency of the separation procedure, its precision, and the effect upon the standard dose-response curve.

A dextran-coated charcoal suspension containing 250mg of activated charcoal (Norit A), 250mg of Dextran T-70 and 100ml of distilled water was prepared using a magnetic stirrer. Before use the suspension was cooled to 4°C. After stabilization of the primary reaction mixture (refer to 2.1.3.2), an 800µl aliquot of the charcoal suspension was added to each assay tube. After mixing on a Heidolph vortexer the solution was allowed to stand at 4°C for 10 min prior to centrifugation at 2 000g for 10 min at 4°C. The supernatant was carefully decanted into a counting vial to which 12,5ml of scintillation cocktail had been added. The radioactivity in the bound fraction was determined.

The second separation procedure evaluated was ammonium sulphate precipitation. A 90% saturated solution of ammonium sulphate was prepared and stored at 4°C. After incubation and stabilization of the primary reaction mixture (refer to 2.1.3.2), 300µl of the ammonium sulphate solution was added to each assay tube. After vortexing,

the tubes were allowed to stand at room temperature to allow the antibody to precipitate. After centrifugation at 2 000g for 30 min at 4°C, the supernatant was carefully decanted, and the radioactivity in the free fraction determined. The precipitate or bound fraction was dissolved in 400µl distilled water and the radioactivity in this fraction determined. Standard dose-response curves were drawn using data from either the free or the bound fraction.

The third separation technique assessed was polyethylene glycol precipitation. A 20% (m/v) solution of polyethylene glycol 6 000 was prepared in PBS (pH 7). The procedure followed was similar to that outlined for ammonium sulphate (refer to 2.1.3.1.1), the difference being that 600µl of polyethylene glycol solution was added in place of ammonium sulphate. Precipitation was instantaneous and irreversible, thus only data from the free fraction were used to draw up a standard dose-response curve.

Anti-rabbit globulin raised in sheep was used for a second antibody separation procedure. The anti-rabbit serum was made up in 0,1% gelatine in PBS (pH 7,0) using a 1:4 titer dilution, and contained 1% normal rabbit serum to act as a carrier.

Following the addition of protein coating buffer and antibody (refer to 2.1.3.2) the assay was allowed to stand at 4°C for 30 min. After the addition of radiotracer, the reaction mixture stood for a further two hours at 4°C. A 200µl aliquot of the precipitating serum was added to each assay tube and the assay incubated at 4°C for 18 hours. The resultant precipitate was pelleted by centrifugation at 2 000g at 4°C for 30 min. The supernatant was decanted into counting vials filled with 12,5ml of scintillation cocktail. The free fraction was then

counted for activity.

2.1.3.1.2 Effect of charcoal concentration

The effect of various concentrations of dextran-coated charcoal on the percentage binding were tested. The object was to remove as much of the free fraction as possible without a reduction in the percentage binding. The addition of dextran to the charcoal slurry was to make it stickier, permitting easier centrifugation into a pellet, and not to limit access to charcoal based on molecular size (Odell, 1980).

The charcoal concentrations tested ranged from 1 to 20mg per tube. After incubation and stabilization of the primary reaction mixture (refer to 2.1.3.2), the different concentrations of dextran-coated charcoal were added to the relevant assay tubes. The addition of the charcoal solution and subsequent procedure was as described in 2.1.3.1.1.

2.1.3.1.3 Determination of incubation time

Lengthy incubations have no advantages apart from the achievement of equilibrium, as various components of the assay, particularly tracer, are subject to damage by other components of the assay (Chard, 1978). Therefore as soon as equilibrium has been achieved the reaction should be stabilized and the separation procedure instituted.

After addition of the primary assay reagents (refer to 2.1.3.2) various incubation times from 3 to 30 min were tested in a water bath at 37°C. After stabilization in ice for 40 min the procedure was as described in 2.1.3.1.1. Results were calculated as a percentage of the highest binding value. The assumption was zero binding at zero time.

2.1.3.1.4 Effect of pH

The effect of pH on the percentage binding was tested. This enabled the selection of a buffer in which the standard used is most soluble.

Phosphate buffered saline with pHs of 6,5, 7,0 and 7,4, and borate buffer with pHs of 8,0 and 8,5 were tested. The procedure followed was as in section 2.1.3.2. The only difference was the addition of 100µl of the various buffers to their respective assay tubes at the onset of the experiment. Results were calculated as the percentage binding of the highest binding value.

2.1.3.1.5 Tracer preparation

Tritiated IPA was supplied by Amersham (United Kingdom) as a custom preparation with only the labile tritium removed. Due to radiochemical decomposition, use of the crude tritiated compound as supplied resulted in non-specific binding (NSB) values in excess of 20%. Therefore some form of further purification was necessary prior to use with RIA.

A Sephadex LH-20 column (16 x 900mm) in 35% ethanol was prepared. A flow rate of 20ml hr^{-1} was set using a peristaltic pump. Fifty mg of IPA standard in 300µl 80% ethanol was placed on the column. After 6 hours a fraction was collected every 30 min. The elution pattern for IPA was determined using UV spectrometry (280nm). The elution volume to bed volume ratio was then calculated ($\frac{Ve}{Vb}$ = constant for any given cytokinin standard), (Chen, 1983). This procedure was repeated for isopentenyl adenine (2iP). The derived data were used for the purification of tritiated IPA.

A 200 to 300µl aliquot of crude tritiated IPA was placed on

the column. The collection procedure was as outlined for standard IPA. A 100µl subsample was taken from each collected fraction and placed in scintillation vials filled with 12,5ml of cocktail (refer to 2.1.3.2). After the addition of 1ml distilled water to each vial, they were capped and gently shaken until a single phase was obtained. The vials were then assessed for activity and the tritiated IPA peak determined. The peak was diluted to 11 000 cpm per 100µl in 0,1% gelatine in PBS for the IPA RIA. A dilution of 15 000 cpm was used in the 2iP RIA.

2.1.3.2 Standard procedure

The completion of the various parameter tests ensured that an optimized RIA could be designed which utilized the combination of the best possible conditions. The assay procedure and equipment is standard unless otherwise stated.

The RIA was conducted in 10 x 75mm rimless soda glass test tubes. Dispensing of reagents was done using a ®Microlab-P programmable microprocessor controlled hand-held pipette fitted with a 2,5ml barrel unless otherwise stated. Centrifugation was in a ®Sorval RC-3B swing-out bucket centrifuge. Mixing was done on a ®Heidolph vortexer. All assay parameters were duplicated.

Standard or biological samples suspended in methanol were dispensed into the relevant assay tubes using a 100µl fixed volume hand-held pipette with disposable tips. Standard concentrations used in the assay ranged from 0,1 to 100ng per 100µl. A typical assay format is presented in Table 2.1. The standard or sample was reduced to dryness under air at 37°C. Assay tubes designated for the determination of NSB were coated with 0,1% gelatine dissolved in PBS (pH 7,0). The coating buffer used in all other tubes contained 1% BSA in PBS (pH 7,0). After vortexing the tubes were allowed to stand for 15 min to

dissolve the standard or sample.

Table 2.1 Typical assay format used as standard throughout the study unless otherwise stated

Tube No.		STD or unknown	
1	2	Ta	Total counts in the system
3	4	NSB	Non-specific binding
5	6	0	Binding in presence of zero standard
7	8	Me 0	As for tubes 5 & 6 but with removed methanol
9	10	0,1	
11	12	0,25	
13	14	0,5	
15	16	1,0	
17	18	2,5	
19	20	5,0	Std dose-response curve
21	22	10,0	
23	24	25,0	
25	26	50,0	
27	28	100,0	
29	30	Sample 1	
51	52	Sample x	
53	54	Recovery	
55	56	Blank extraction	
57	58	Ta	
59	60	NSB	

Suitably diluted anti-IPA serum (100µl) was then dispensed into all tubes except those designated for total counts

(Ta) and NSB. The dilution used was dependent upon the isopentenyl-type RIA conducted. The assay for IPA was designed around a binding value of 30%. The 2iP assay was designed around a 45% binding value due to the reduced specificity of the antibody for this cytokinin. This was followed by the addition of 100µl of radio-tracer to all assay tubes (refer to 2.1.3.1.5 for the relevant dilutions). The reaction mixture was then vortexed, and incubated in a water bath at 37°C for 10 min. Assay tubes were then again vortexed.

Stabilization of the reaction mixture was achieved in an ice bath after 40 min. Thereafter 800µl of dextran-coated charcoal (125mg charcoal and 250mg dextran in 50ml distilled water) at 4°C was added to all tubes except those designated for Ta. Assay tubes were again vortexed and allowed to stand for 10 min at 4°C.

The charcoal with the adsorbed free fraction was pelleted by centrifugation at 2 000g for 10 min at 4°C. The supernatant containing the bound fraction was carefully decanted into standard scintillation vials filled with 12,5ml of scintillation cocktail. The vials were gently shaken until a single phase was obtained. The scintillant was prepared as follows :

12g of PPO and 0,3g Popop were dissolved in a small volume of toluene with stirring. After dissolution the volume was made up to 3l with further addition of toluene. Thereafter 1,5l of Triton X-100 was added to the toluene solution and the mixture vigorously shaken.

After standing for 10 min the vials were placed in a ®Beckman LS 8100 liquid scintillation counter and the activity determined. The Sigma preset error was set at 2%. The calculation of results is presented in 2.1.3.3.

2.1.3.3 Statistical analysis and calculation of results

By appropriate manipulation using the logit transformation, the dose-response relationship in a binding assay can be linearised (Rodbard & Frazier, 1975). The logit was calculated as :

$$\text{logit } b = \log \frac{b}{100-b}$$

where b was the proportion of the tracer bound expressed as a percentage of that in the zero standard.

Plotted against log dose this yielded a straight line for most assays. The goodness of fit of a straight line is judged by the r value of a least squares regression (Rayner, 1967). This value was used as a measure of precision in the assay.

The use of a pool sample as recommended by Chard (1978) was impractical due to the limitations of sensitivity of the assay and the endogenous levels of the compound of interest (lower ng g^{-1} range). For biological samples a 5% limit was placed on the difference between replicates of the total counts in the system. Differences greater than 5% were discarded and the sample repeated. However, in practise, differences this great were never encountered in purified samples, probably due to the reduction in pipetting error by the use of automated dispensing equipment.

All standard dose-response curves were plotted on log-logit probability graph paper (Technical Aids for Engineering and Management, Tamworth, New Hampshire) unless otherwise stated.

2.1.4 Validation

For the measurement of endogenous samples it is important to verify that the compound of interest is the compound being measured by RIA. Validation requires the demonstration that endogenous compound and standard compound react with the antibody identically under the conditions of the assay. Other factors, should they be present in the biological extract, must not affect the determination (Yalow & Berson, 1971).

2.1.4.1 Extract dilution curves

To detect any potentially interfering compounds present in the biological extracts, extract dilution curves were made. A 40g sample of young actively expanding avocado (*Persea americana* Mill. cv 'Fuerte') leaf was harvested and extracted in 80% ethanol (refer to 2.1.5.1). Prior to Dowex cleanup the sample was split so that a crude extract dilution curve could be obtained. After Dowex treatment (refer to 2.1.5.2) the remaining sample was again divided in two so that a post-Dowex dilution curve could also be determined.

The remaining 10g fresh mass equivalent, reduced to 800µl in 99% methanol, was strip-loaded onto two 0,5mm thick cellulose microcrystalline preparative TLC plates with added indicator using a 100µl Hamilton syringe. Three-quarters of the plate width was utilized. Five µg reference spots of IPA and 2iP were spotted onto the plate in the unused section. Refer to 2.1.5.3 for development and elution of the cytokinins.

After separation and elution, the two isolated cytokinin samples and the two crude composites (one pre-, one post-Dowex) were treated similarly. Each sample was split 1:2 and treated as follows :

(a) The pre-Dowex crude composite 1/3 sample had 500ng of both IPA and 2iP added. Dilutions containing between 100% and 5% of the extract were prepared using 99% methanol (spectranol grade).

(b) The pre-Dowex 2/3 crude composite sample had no standard added and the same dilution format as in (a) was applied.

(c) The post-Dowex 1/3 crude composite sample was prepared as for (a).

(d) The post-Dowex 2/3 sample was prepared as for (b).

(e) The pooled IPA 1/3 sample after TLC had 50ng IPA standard added and dilutions were as in (a).

(f) The pooled IPA 2/3 sample after TLC had no added standard and dilutions were as in (a).

(g) The pooled 2iP 1/3 sample after TLC had 50ng 2iP standard added. Dilutions were as in (a).

(h) The pooled 2iP 2/3 sample after TLC had no standard added and was treated as for (f).

The standard RIA procedure (refer to 2.1.4.2) was used for the determination of the dilution curves, the only difference being that 200µl of the prepared extracts was added to each assay tube. Results were plotted on standard log-logit probability graph paper.

2.1.4.2 Antibody selectivity (cross-reaction studies)

Various compounds such as related cytokinins, precursors or structurally similar compounds were assessed for cross-reactivity. From these data it could be determined if non-specific or cross-reactivity factors were the reason for the non-parallelity of the dilution curves in the crude samples assayed (refer to 2.1.4.1). It could also be determined which components of the isopentenyl adenosine compound were involved in the immunochemical recognition response.

Ten mg standard preparations of isopentenyl adenosine, isopentenyl adenine, zeatin, zeatin riboside, dihydro-zeatin, adenine, adenosine, 6-furfurylaminopurine, 6-benzyl-aminopurine and 6-hexylaminopurine in 10ml 99% methanol (spectranol grade) were prepared.

The dilutions of IPA and 2iP used in the RIA have been described in section 2.1.3.2. Dilutions of the other standards ranged from 0,1 to 10 000ng per 100µl.

The standard RIA procedure was followed (refer to 2.1.3.2) and cross-reactivity curves were plotted on 6 cycle log-logit probability graph paper. The percentage cross-reactivity was calculated relative to 50% inhibition of binding of IPA.

2.1.5 Biological sample preparation

The procedure described hereafter was that used as standard for the preparation of biological samples for measurement of cytokinins by RIA unless otherwise stated.

2.1.5.1 Extraction

Cytokinin extraction was a modification of the method as described by van Staden (1980). Ten g of plant tissue was homogenized in 50ml of 80% ethanol using an Ultra Turrax. Extraction took place over 24 hours at 4°C in the dark. After extraction the homogenate was stirred, equally halved and placed in two glass 50ml centrifuge tubes. The liquid fraction was cleared by centrifugation at 1 000g for 15 min. There was no temperature control.

2.1.5.2 Purification

Cation exchange resins have been widely used for the purification of extracts (Vreman & Corse, 1975; van Staden, 1976, 1980). A slurry method (Whenham, 1983) was used due to the number of samples run.

The cleared liquid fraction (refer to 2.1.5.1) was decanted into a 100ml beaker and the pH adjusted to 2,5 with 1N HCl. The adjusted supernatant was placed in a 100ml beaker charged with 10g of Dowex 50W-X8 cation exchange resin (Sigma chemical Company H^+-form; 50-100 mesh) and slurried for 30 min at room temperature in a shaker. The slurry was allowed to stand overnight at 4°C. The liquid was decanted off and the mix again slurried using 50ml of 80% unadjusted ethanol. The liquid fraction was removed and discarded.

Cytokinins were released from the resin with 50ml of a 3,5N ethanol ammonium hydroxide solution. After shaking for 30 min the solution was decanted and reduced to dryness under vacuum at 35°C. The residue was resuspended in 5ml of 99% methanol.

2.1.5.3 Isolation

After both silica gel and cellulose microcrystalline thin layer chromatography (TLC) had been tested, cellulose TLC was selected. Only this technique is described as it was used routinely. Two g fresh mass equivalent samples suspended in 1ml methanol were streaked onto 0,4mm thick cellulose microcrystalline plates containing UV indicator (200 x 200mm). Plates were prepared as follows :

A slurry mix consisting of 28,5g cellulose microcrystalline, 0,3g fluorescing indicator and 73ml 10% ethanol was prepared using an Ultra Turrax homogeniser. After degassing there was enough mixture for five 200 x 200mm plates. Plates were allowed to dry for 24 hours before being used.

Two samples were applied per TLC plate using a 100µl Hamilton syringe with a shortened and bevelled 22 gauge needle. Reference standards of IPA and 2iP were applied using a 5µl spotter. All streaking and spotting was done under a heated air blower.

The plates were developed using distilled water as the solvent. The front was allowed to advance about 150mm after which the plate was removed from the tank and allowed to dry. Reference standards were detected and marked under UV light. The zones corresponding to authentic IPA and 2iP were individually removed and the cellulose placed in 25ml Ehrlenmeyer flasks. Ten ml of methanol (spectranol grade) was added to each flask. After shaking for 30 min the flasks were left at 4°C overnight.

After agitation the cellulose mixture was poured into 20ml glass centrifuge tubes and vigorously vortexed. After centrifugation at 1 000g for 30 min the cleared supernatant was reduced to dryness under vacuum at 35°C. The residue was resuspended in 1ml absolute methanol, split and used for

the determination of either IPA or 2iP by radioimmunoassay.

2.2 Results

2.2.1 IPA-BSA conjugate

The ultra violet spectra of BSA and IPA standards are given in Fig. 2.1. The IPA has a maximum absorbance at 266nm while BSA has a maximum at 220nm. However, as the Σ_{max} = 15 000 for nucleotides given by Erlanger & Beiser (1964) was at 260nm, the calculations have been based on data derived from this wavelength. BSA-IPA conjugate spectra are presented in Fig. 2.1 with the blank as either distilled water, or BSA in distilled water. Calculation of the linking ratio was as follows :

$$\frac{\dfrac{(\text{Absorbance (conjugate)} - \text{Absorbance (BSA)})}{\Sigma_{max}}}{\dfrac{\text{BSA (g l}^{-1})}{\text{molar mass of BSA}}}$$

$$\text{IPA} = \frac{0{,}700}{15\ 000}$$

$$= 4{,}66 \times 10^{-5} \text{ mol l}^{-1}$$

$$\text{BSA} = 0{,}012\text{g in } 50\text{ml}$$

$$= 3{,}4 \times 10^{-6} \text{ mol l}^{-1}$$

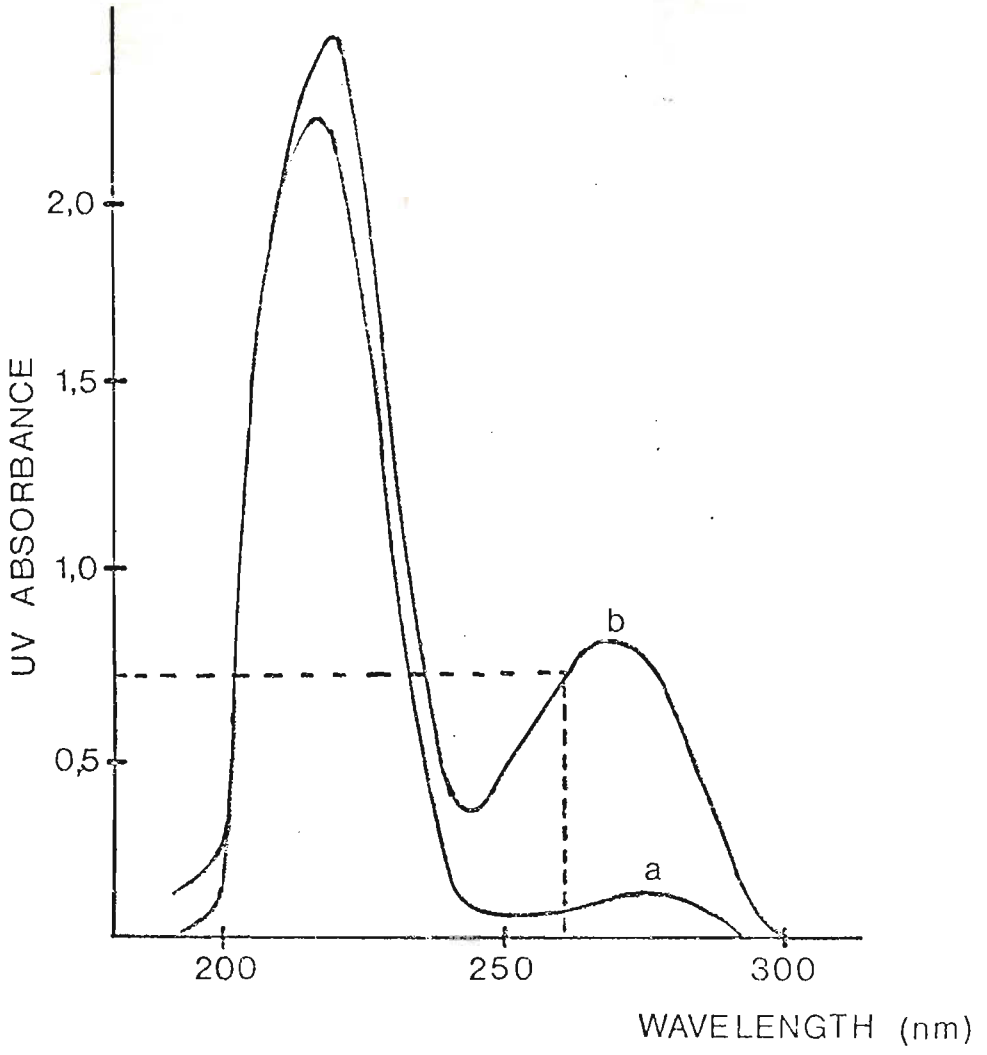
$$\text{Ratio IPA:BSA} = \frac{4{,}66 \times 10^{-5}}{3{,}4 \times 10^{-6}}$$

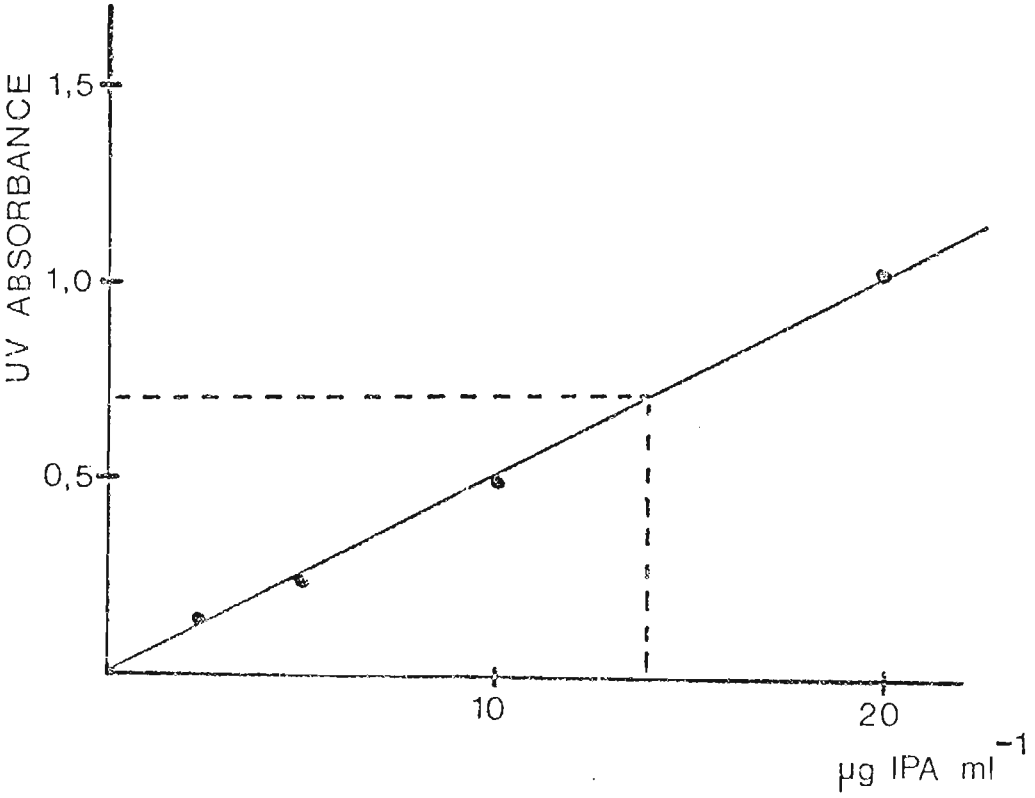
$$= 13{,}7:1$$

A standard curve for IPA at 260nm is given in Fig. 2.2. The amount of IPA in 0,012g of conjugate was obtained as a direct readoff from the standard curve. After conversion

Fig. 2.1 The UV spectra of BSA (a) and the IPA-BSA conjugate (b) between 200 and 300nm. The broken line (260nm) shows where the UV readings used in the calculation of the linking ratios were taken.

Fig. 2.2 The standard UV curve for IPA at 260nm. The broken line transformed the UV reading from Fig. 2.1 to an absolute IPA concentration of 13,8µg ml^{-1} which was used in the calculation of the IPA-BSA linking ratio.


UV ABSORBANCE
2,0
1,5
1,0
0,5
b
a
200
250
300
WAVELENGTH (nm)


UV ABSORBANCE
1,5
1,0
0,5
10
20
µg IPA ml⁻¹

to mol l^{-1}, the linking ratio was calculated.

IPA = 13,8µg ml^{-1}

= 4,11 x 10^{-5} mol l^{-1}

BSA = 3,4 x 10^{-6}

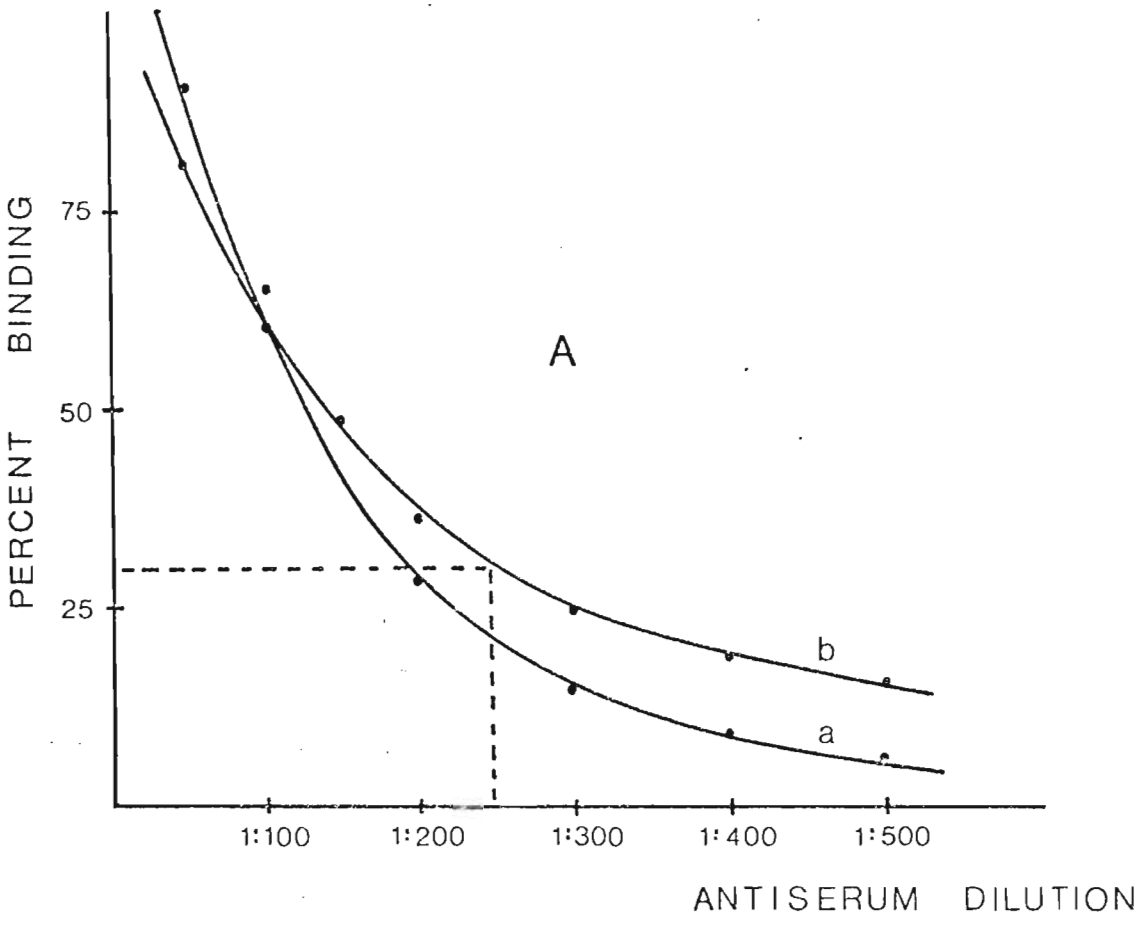
IPA:BSA = 12,1:1

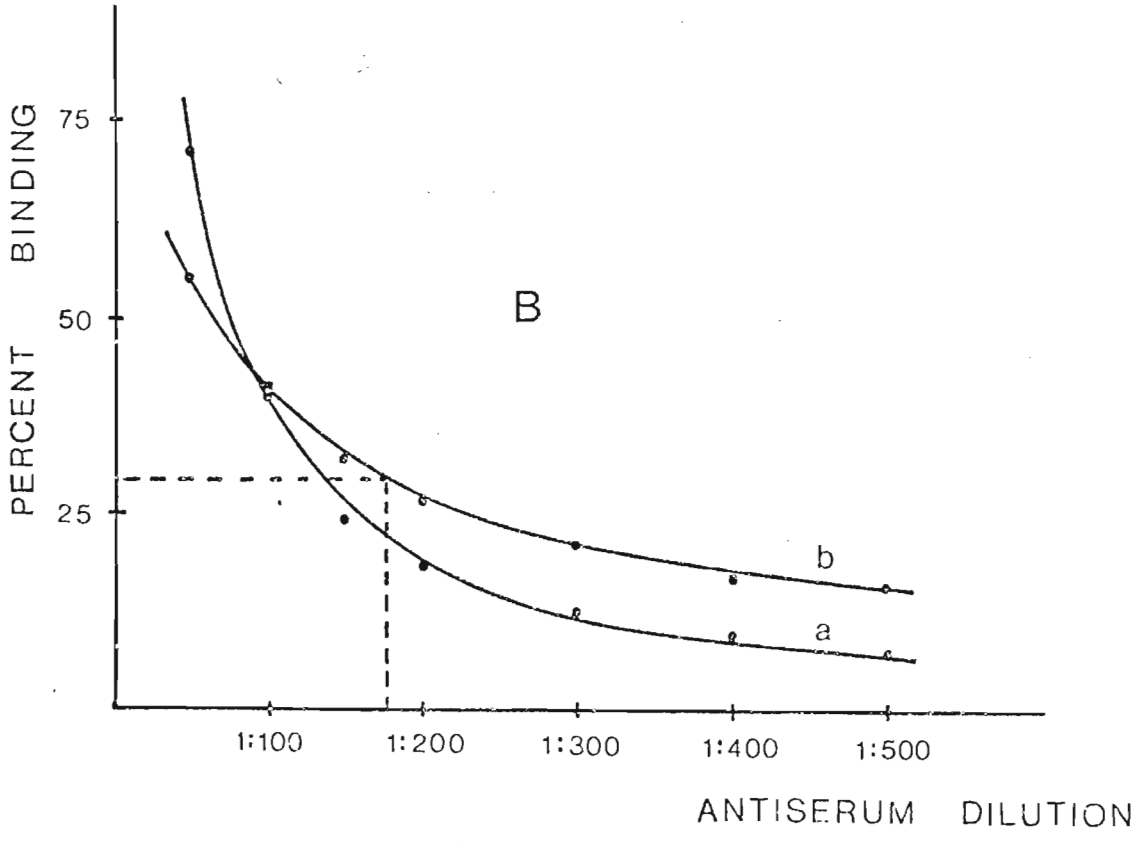
The two answers matched closely and after verification the extinction coefficient, Σ_{max} = 15 000, was thought suitable for future linking ratio determinations of other cytokinins to BSA.

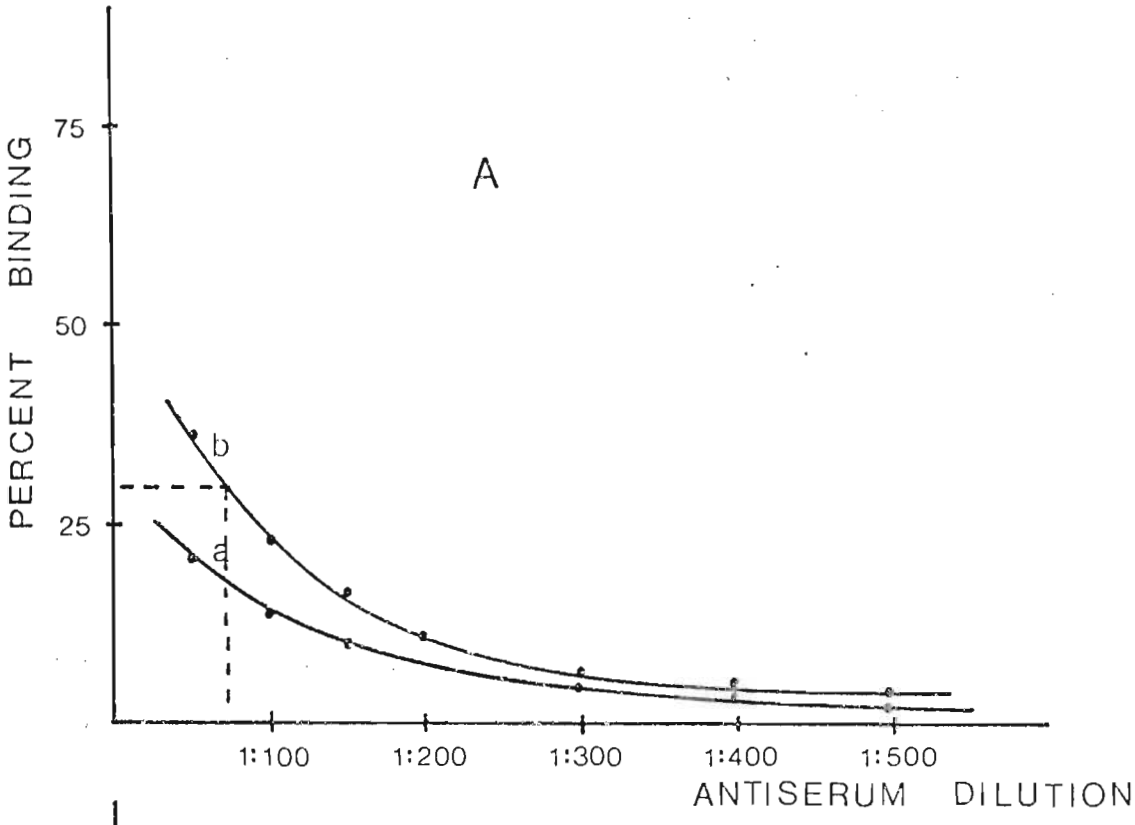
2.2.2 Response to immunization

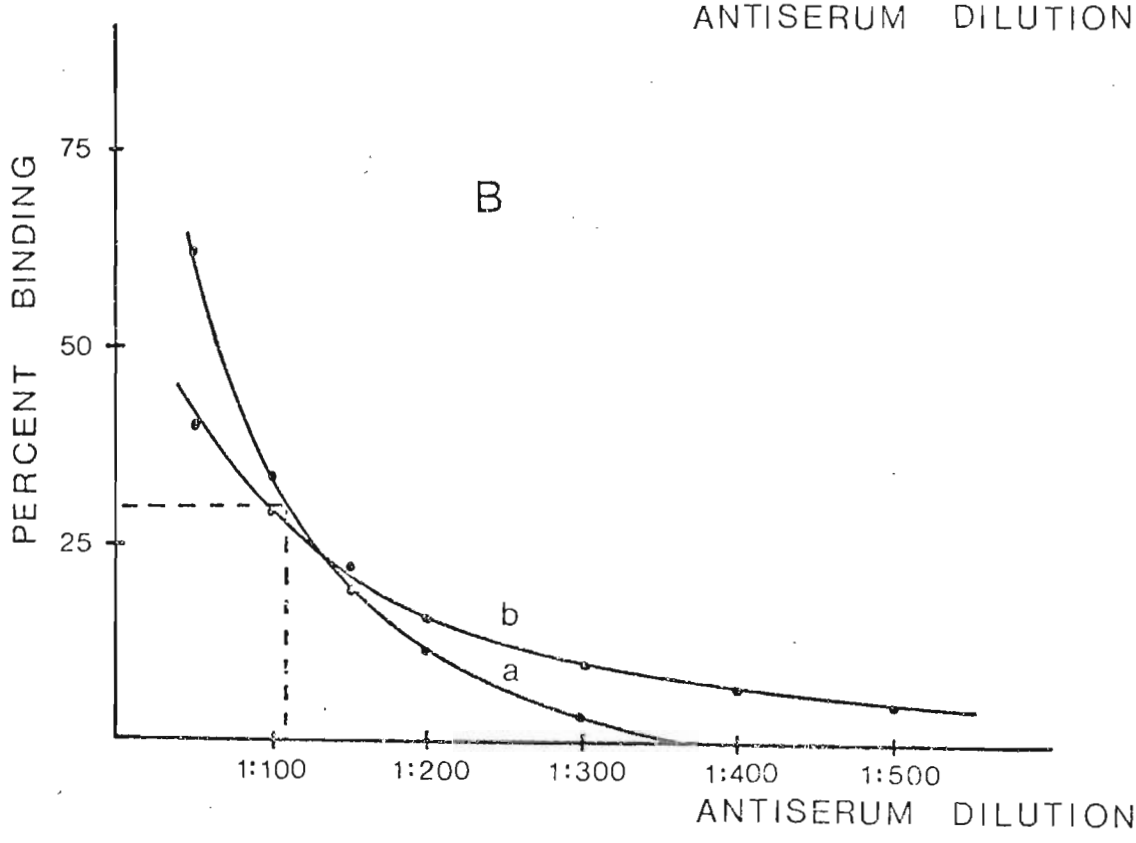
The immune response of rabbits inoculated with the IPA-BSA conjugate is shown (Fig. 2.3 & 2.4). One of the immunized animals died, therefore data from this animal were discarded and the titer curves of the remaining four rabbits considered for RIA. The antibody titer which bound 30% of the added ^{3}H-IPA tracer was in all cases higher in response to the second booster immunization. After consideration of titer, standard dose-response curves (Fig. 2.15), extract dilution response curves (Fig. 2.18 & 2.20) and cross-reactivity data, serum from rabbit IPA-1 was used in both isopentenyl-type RIAs.

The assay for IPA was run at a dilution of 1:250 and the serum bound 30% of the added tracer. At a dilution of 1:150 used in the 2iP assay the serum bound 45% of the added tracer.


PERCENT BINDING
75
50
25
A
b
a
1:100
1:200
1:300
1:400
1:500
ANTISERUM DILUTION


PERCENT BINDING
75
50
25
B
b
a
1:100
1:200
1:300
1:400
1:500
ANTISERUM DILUTION


A
PERCENT BINDING
75
50
25
b
a
1:100
1:200
1:300
1:400
1:500
ANTISERUM DILUTION


B
PERCENT BINDING
75
50
25
b
a
1:100
1:200
1:300
1:400
1:500
ANTISERUM DILUTION

2.2.3 Radioimmunoassay

2.2.3.1 Effect of separation technique

Dextran-coated charcoal gave good separation results. Regression analysis (r = 0,98) indicated good precision and the assay was shown to be suitable to linearization using the logit transformation (Fig. 2.5). NSB was very low and ranged from 1,2 to 2,5% (n = 12) of the total tracer added to the system.

However, the charcoal reduced the apparent titer of the antiserum in the presence of similar quantities of tracer when compared to ammonium sulphate, polyethylene glycol and second antibody as separation procedures (Table 2.2).

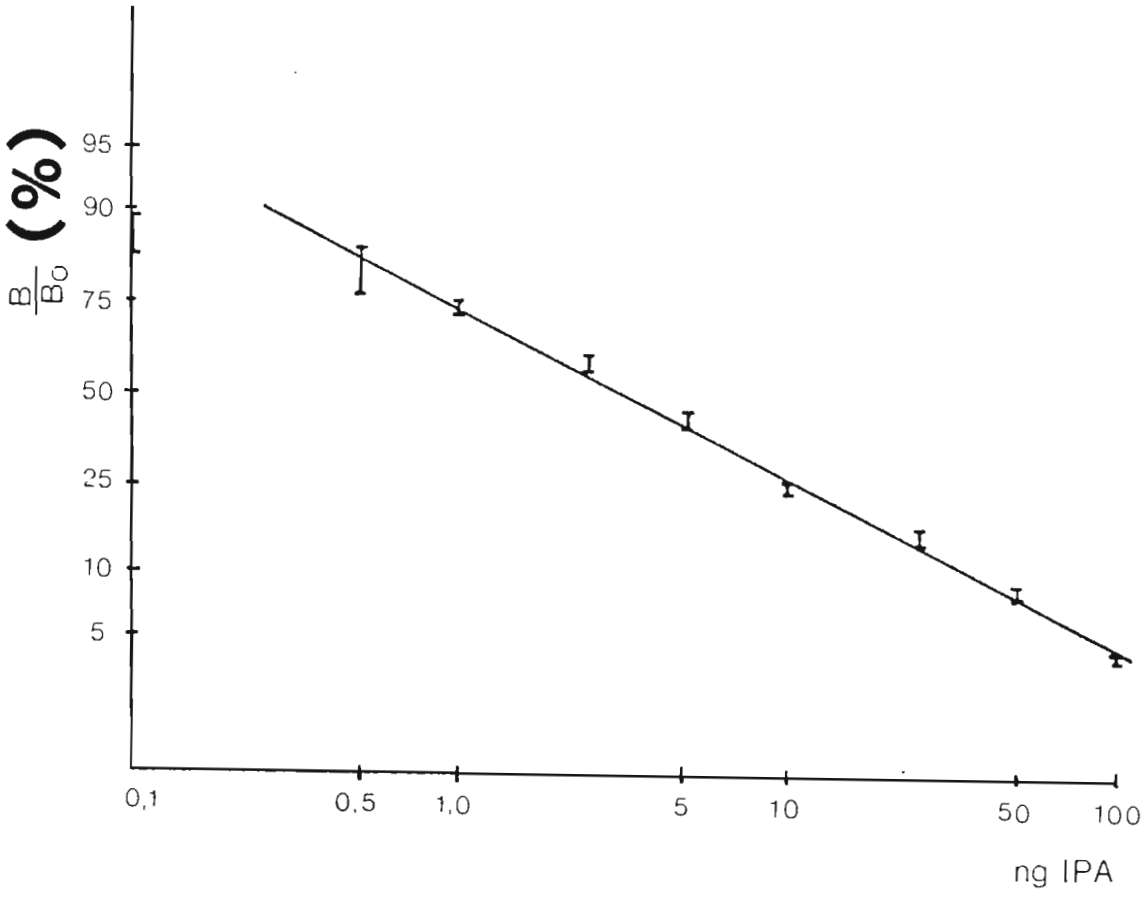
Table 2.2 Effect of separation technique on titer and standard concentration at 50% inhibition of binding

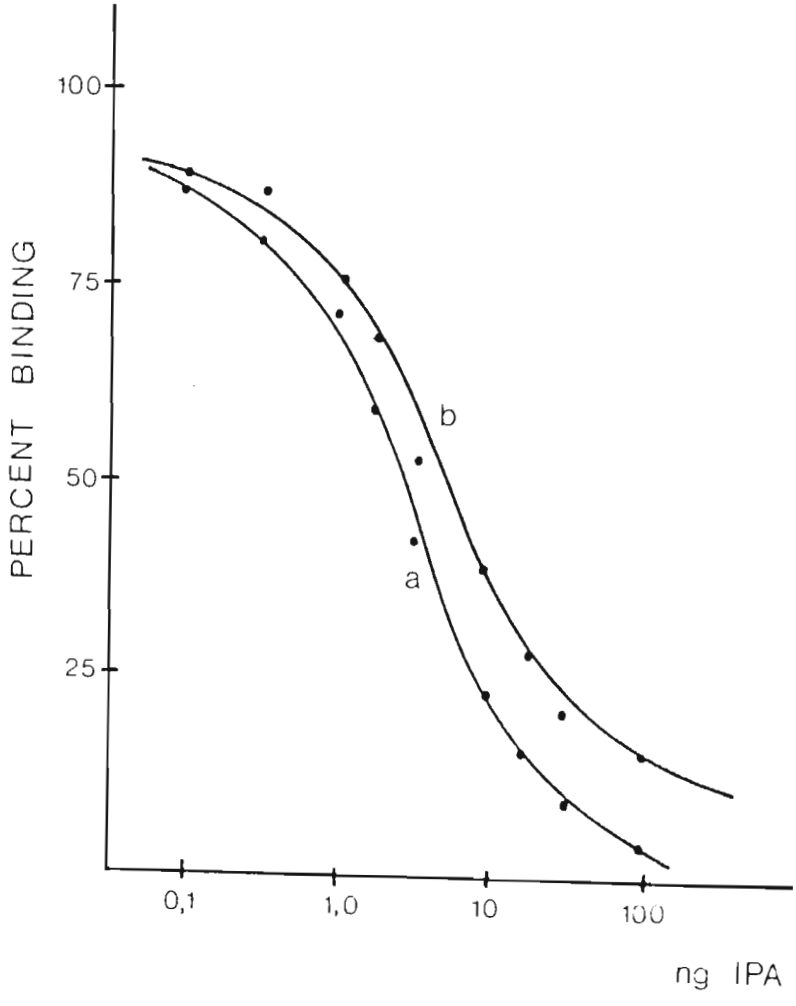
Separation procedure	Ta	Titer	Standard concentration at 50% inhibition of binding in ng
Charcoal	11 250	25%	3,1
Polyethylene glycol	10 900	42%	3,4
$(NH_4)_2SO_4$	13 100	52%	7,5
Second antibody	11 600	50%	7,5

The effect of charcoal as opposed to ammonium sulphate on the standard dose-response curve is shown in Fig. 2.6. The lowered titer is due to competition by the charcoal with the antibody for binding thus 'stripping' or removing a proportion of ligand from the bound complex. The overall result was a more sensitive assay.

Fig. 2.5 Standard dose-response curve for IPA using dextran-coated charcoal as a separation procedure.

Fig. 2.6 A comparison of dextran-coated charcoal (a) and ammonium sulphate (b) as separation procedures for the IPA and their effect upon the standard dose-response curve.


B/Bo (%)
95
90
75
50
25
10
5
0,1
0,5
1,0
5
10
50
100
ng IPA


PERCENT BINDING
100
75
50
25
b
a
0,1
1,0
10
100
ng IPA

Ammonium sulphate separation was not as good as with dextran-coated charcoal. NSB was in the range from 8,5 to 10% (n = 4) of the total tracer added to the system (Fig. 2.7). Precision as measured by regression (r = 0,95) was poor, particularly in the more sensitive regions of the standard dose-response curve. However, when averaged concentration points were subjected to regression analysis (Fig. 2.8), r = 0,98 indicated that the assay was indeed suitable for linearization. Due to the reduced sensitivity of the assay (refer to 2.2.3.1.1) combined with poor precision and high NSB values, this technique was not considered further.

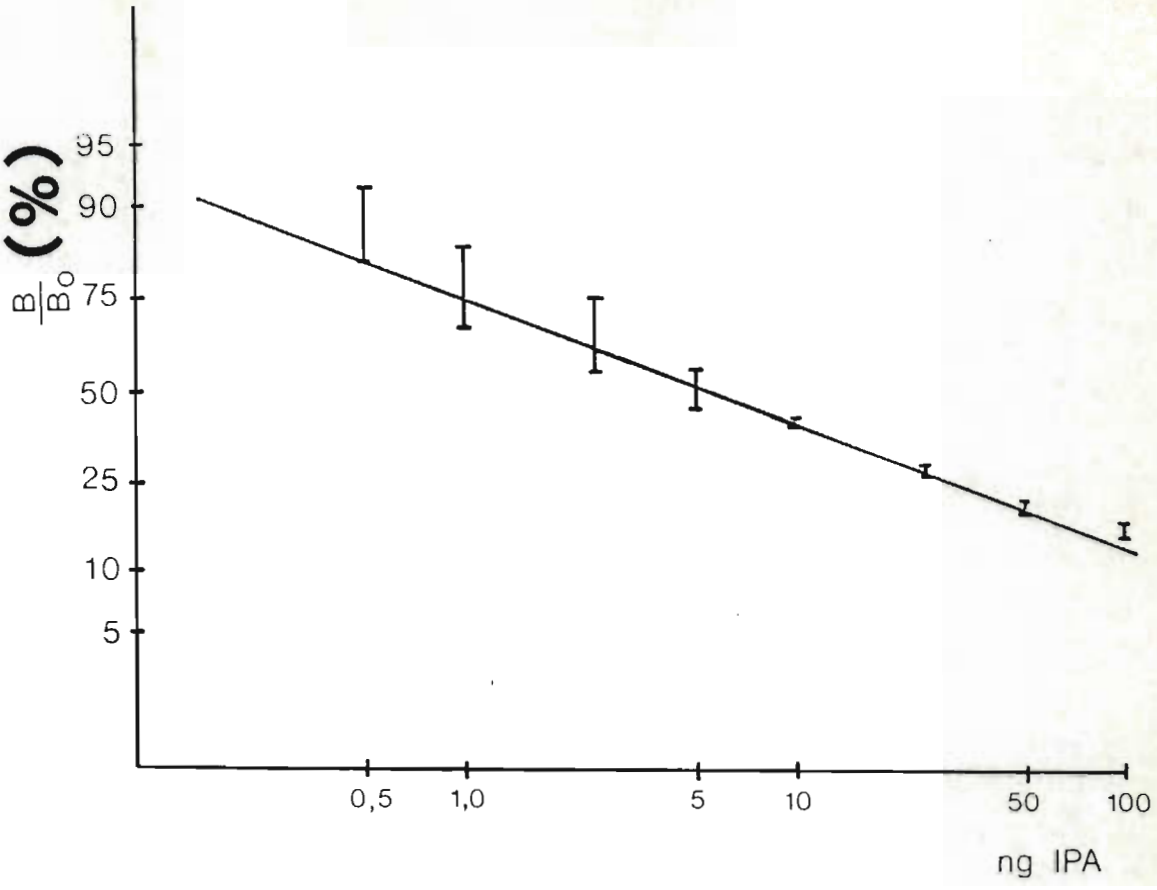
Polyethylene glycol separation of bound and free fractions was good and non-specific binding ranged from 3 to 4% (n = 4). Titer was slightly reduced when compared to ammonium sulphate, the reason not being clear. Precision, as measured by regression analysis (r = 0,94) was poor over the entire standard dose-response curve, particularly in the sensitive regions of the curve (Fig. 2.9). For similar reasons to ammonium sulphate this technique was not investigated further.

Results using anti-rabbit gammaglobulin for second antibody separation were very good as regards NSB (2,5 to 3,0, n = 4), and precision (r = 0,98). The regression analysis also indicated that linearization was suitable for this method of separation (Fig. 2.10).

Unfortunately several important practical disadvantages were encountered. Firstly the assay required an extra day to complete, effectively halving the number of assays that could be run, a most undesirable situation for assay developmental studies. Secondly due to the relatively high anti-rabbit globulin titer used, supplies of this serum were rapidly consumed which made the system expensive. For these reasons this system was not investigated further.

Fig. 2.7 Standard dose-response curve for IPA using ammonium sulphate precipitation as the separation procedure, showing poor precision.

Fig. 2.8 Standard dose-response curve for IPA using ammonium sulphate precipitation as the separation procedure. This curve was constructed using averaged data.


B/B0 (%)
95
90
75
50
25
10
5
0,5
1,0
5
10
50
100
ng IPA

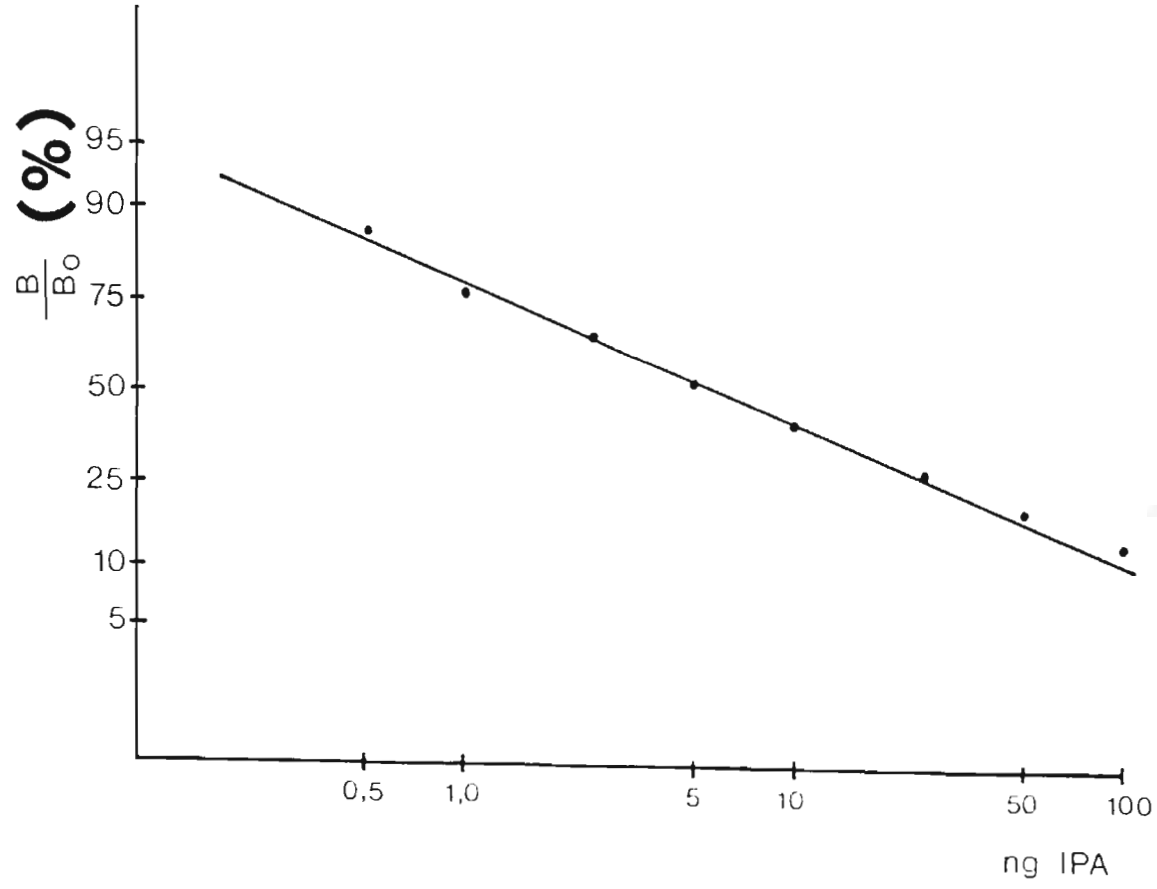
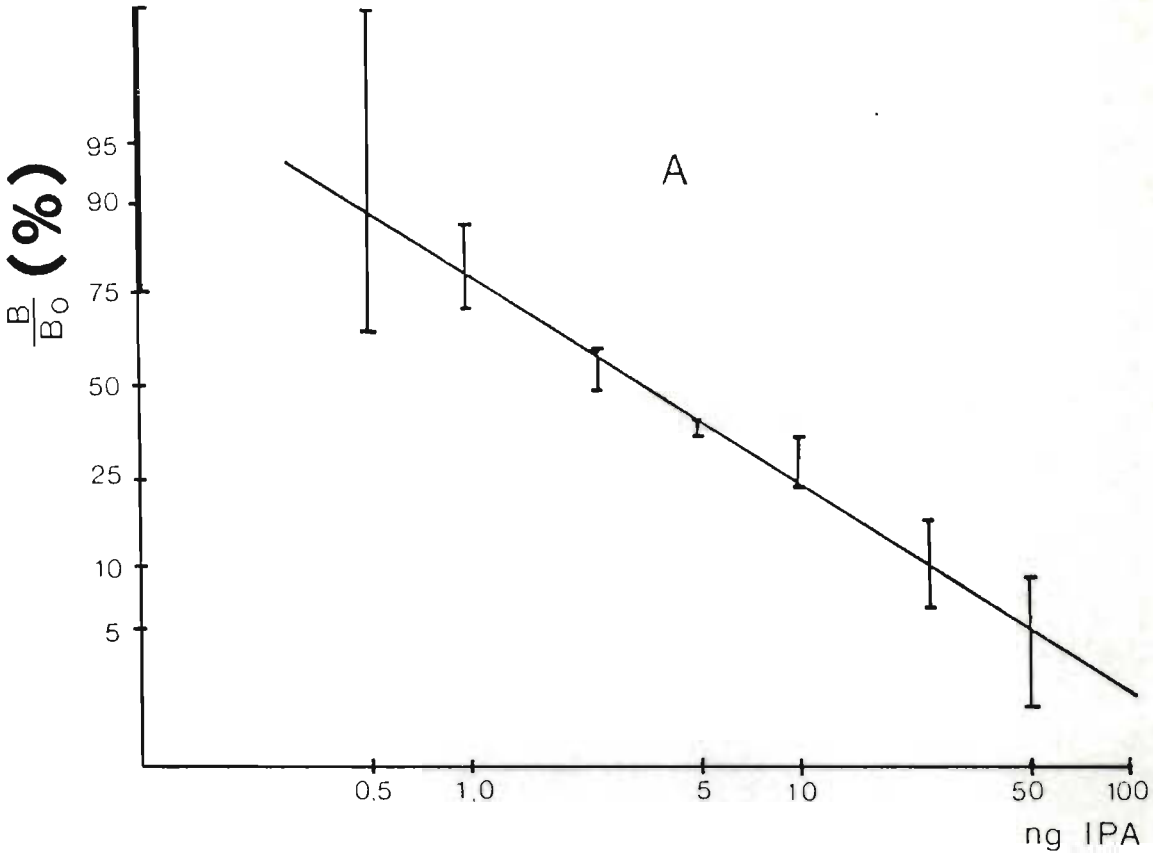
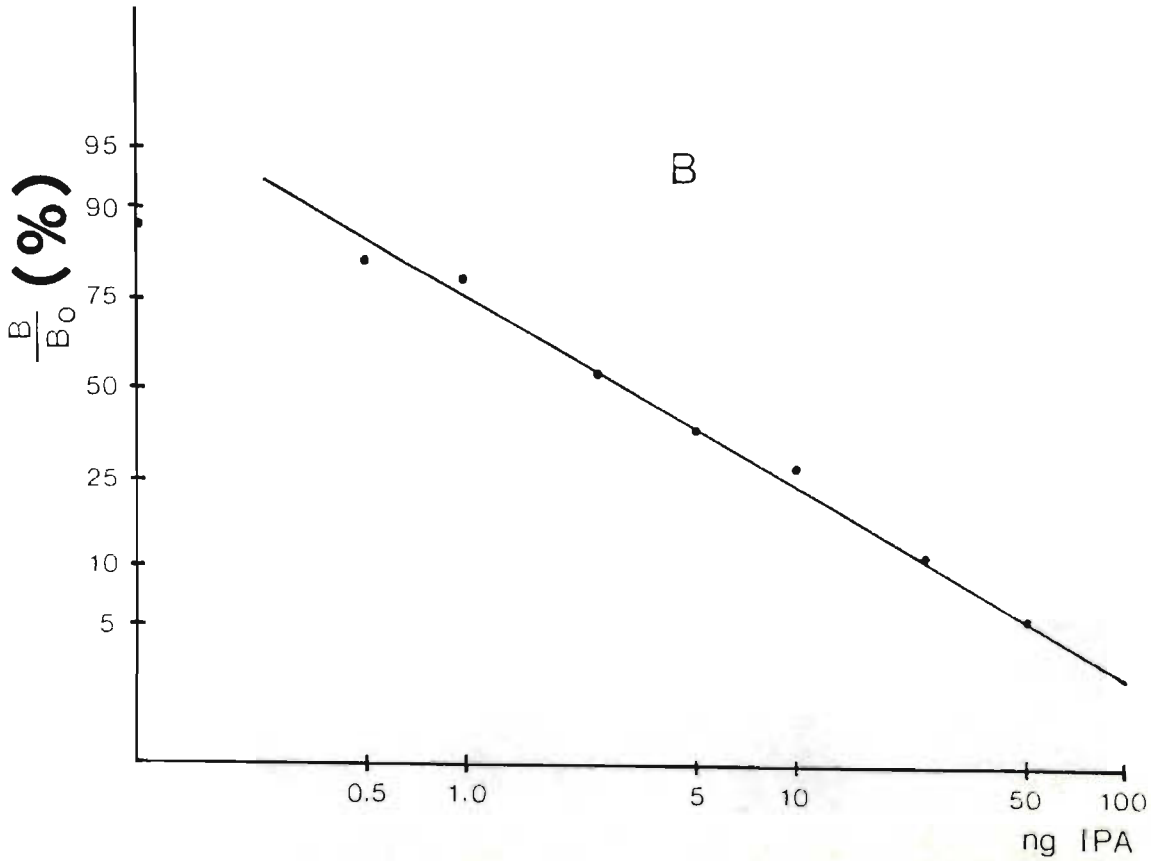

B/B0 (%)
95
90
75
50
25
10
5
0,5
1,0
5
10
50
100
ng IPA

Fig. 2.9A Standard dose-response curve for IPA using polyethylene glycol precipitation as the separation procedure, showing poor precision.

Fig. 2.9B Standard dose-response curve for IPA using polyethylene glycol precipitation as the separation procedure. This curve was constructed using averaged data.


A
$\frac{B}{B_0}$ (%)
95
90
75
50
25
10
5
0.5
1.0
5
10
50
100
ng IPA


B
$\frac{B}{B_0}$ (%)
95
90
75
50
25
10
5
0.5
1.0
5
10
50
100
ng IPA

However, due to the good precision and NSB of this system, it would be a good first choice as an alternative system, should any problems arise with the primary separation procedure.

2.2.3.2 Effect of charcoal concentration

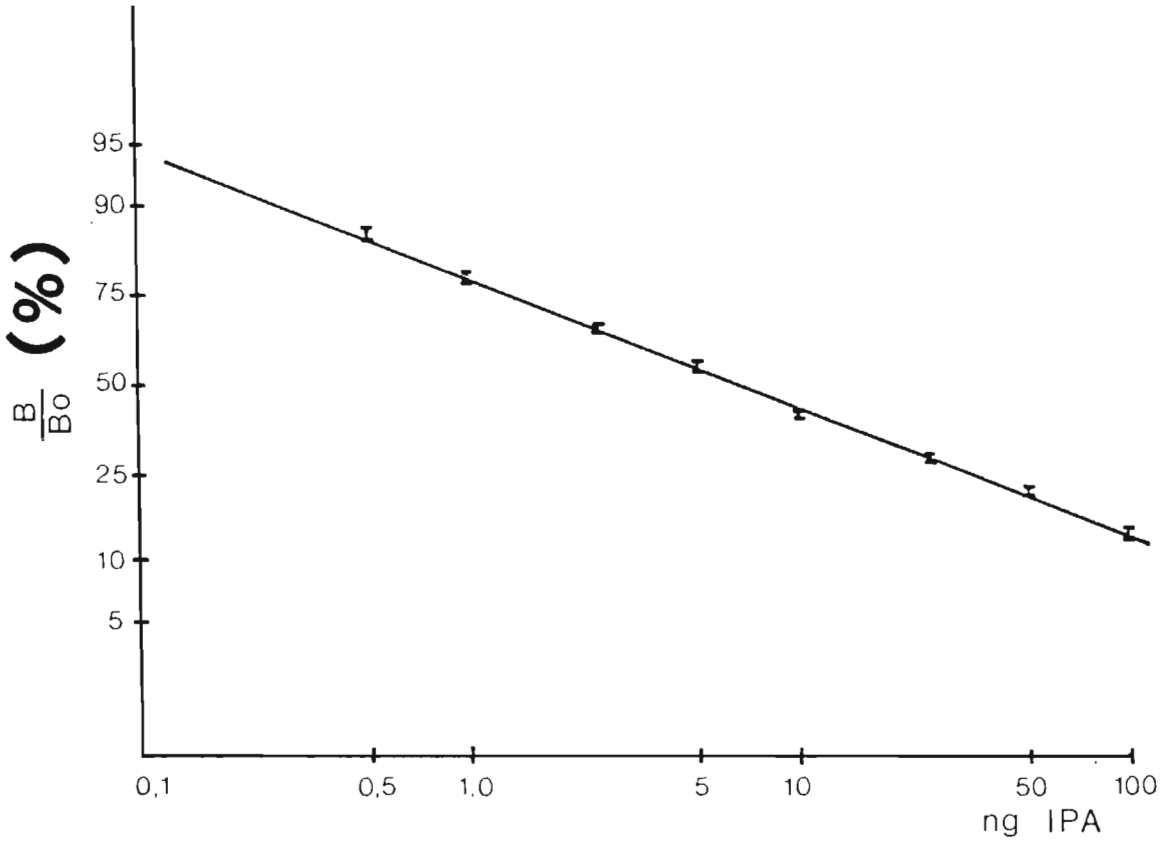
The charcoal dose-response curve is presented in Fig. 2.11. It shows that the amount of charcoal that could be added to each tube under the conditions stated (refer to 2.1.3.1.2) could range from 1mg to 3mg. An amount of 2ng per tube was selected (2.1.3.2). It was found that increasing charcoal concentration bound increasing amounts of free and bound ligand, the amount of free ligand rapidly reaching a maximum. Whether the charcoal actually held the bound ligand or stripped it from the antibody complex is unknown. It was also observed that during the decanting of the supernatant after centrifugation, it was necessary to touch the mouth of each tube onto the scintillant surface to remove as much of the supernatant as possible. This helped to keep precision high.

2.2.3.3 Effect of incubation time

The incubation time-response curve is presented in Fig. 2.12. Maximum binding in excess of tracer was achieved after 7 min. There was no measurable dissociation of the tracer ligand from the antibody complex for at least 40 min after maximum binding had been attained. A reasonable amount of flexibility was therefore possible in the selection of an incubation time, provided it was longer than 7 min. The short incubation time (10 min) selected tended to reduce the risk of tracer damage, resulting in low NSB for the assay.

Fig. 2.10 Standard dose-response curve for IPA using second antibody as the separation procedure. The method showed good precision.

Fig. 2.11 Charcoal dose-response curve showing the percent of ^{3}H-IPA removed from the incubation volume (A) and the percent ^{3}H-IPA 'stripped' from the bound complex (B). A charcoal concentration of between 1 and 3mg per tube (C) removed all the free tracer without stripping tracer from the bound complex.


B/Bo (%)
95
90
75
50
25
10
5
0,1
0,5
1,0
5
10
50
100
ng IPA

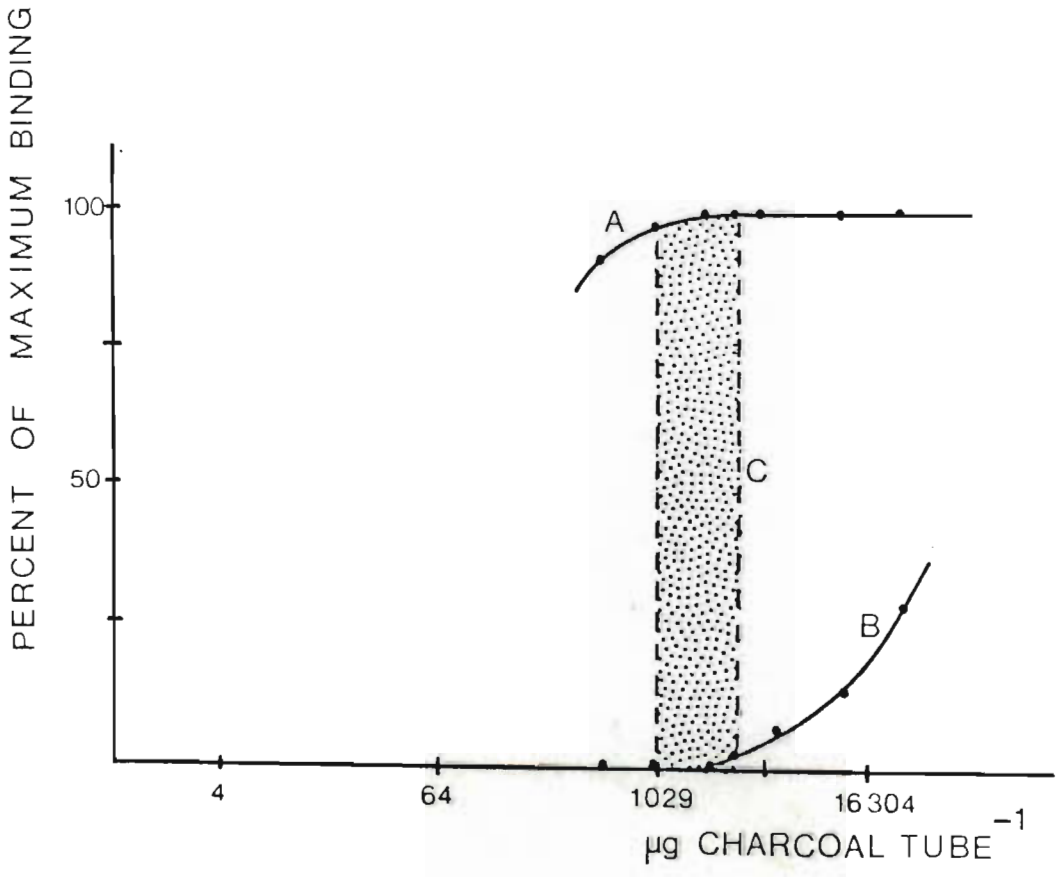
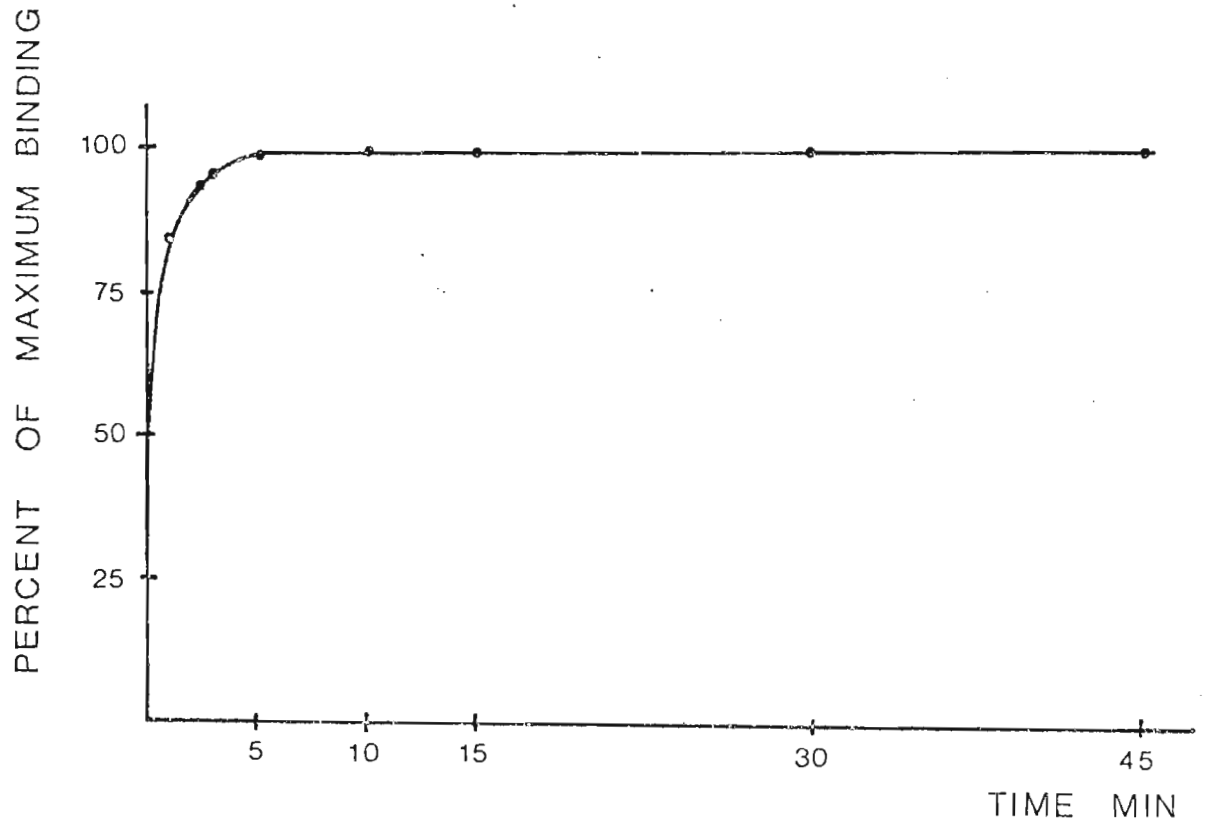
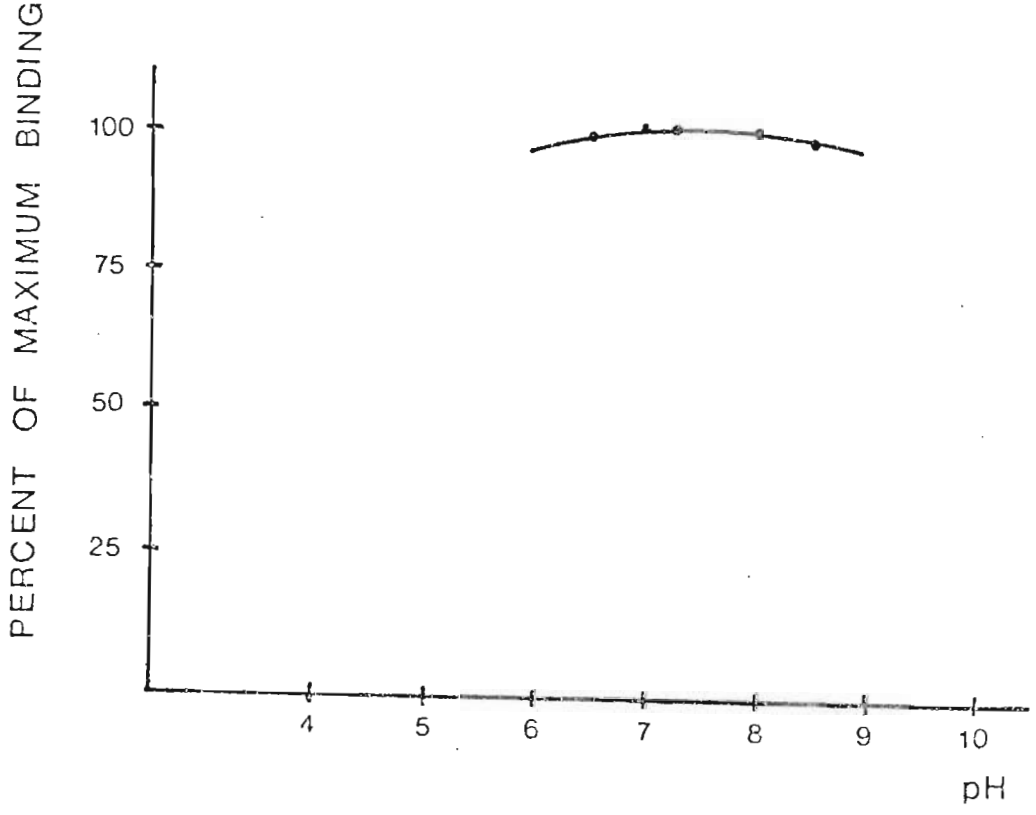

PERCENT OF MAXIMUM BINDING
100
50
A
B
C
4
64
1029
16304
µg CHARCOAL TUBE^{-1}

Fig. 2.12 The incubation time-response curve for serum from rabbit IPA-1 at an incubation temperature of 37°C.

Fig. 2.13 The effect of incubation pH on the percentage binding of serum from rabbit IPA-1.


PERCENT OF MAXIMUM BINDING
100
75
50
25
5
10
15
30
45
TIME MIN


PERCENT OF MAXIMUM BINDING
100
75
50
25
4
5
6
7
8
9
10
pH

2.2.3.4 Effect of pH

The effect of pH on percentage binding is presented in Fig. 2.13. There was no reduction in binding over the range tested except at pH 8,5, which reduced binding by 2%. Therefore, a buffer pH of 7 was selected for the preparation of all primary reagents used in the assay (refer to 2.1.3.2).

2.2.3.5 Tracer properties

The Ve/Bv ratios for IPA and 2iP were 1,6 and 1,9 respectively. The radioactivity in the various fractions from the Sephadex LH-20 column are presented in Fig. 2.14, with the UV absorption spectra of the two cytokinins superimposed. From this it can be seen that the major proportion of the radioactivity could be associated with IPA. The tracer appeared to self-destruct with time. When NSB rose above 3% the tracer was discarded and new tracer purified.

2.2.3.6 Standard dose-response curve

Two typical standard dose-response curves for isopentenyl adenosine are presented in Fig. 2.15. One was adjudged normal and one had increased sensitivity (and reduced precision, particularly below 0,5ng). Typically the assay has a measuring range from 100pg to 100ng.

The two typical standard dose-response curves presented for 2iP (Fig. 2.16) show the effect of reduced cross-reactivity on the curve. The effect is a more sensitive assay due to a more dilute antiserum and hence a decreased binding percentage. Therefore, the binding percentage was increased for this assay.

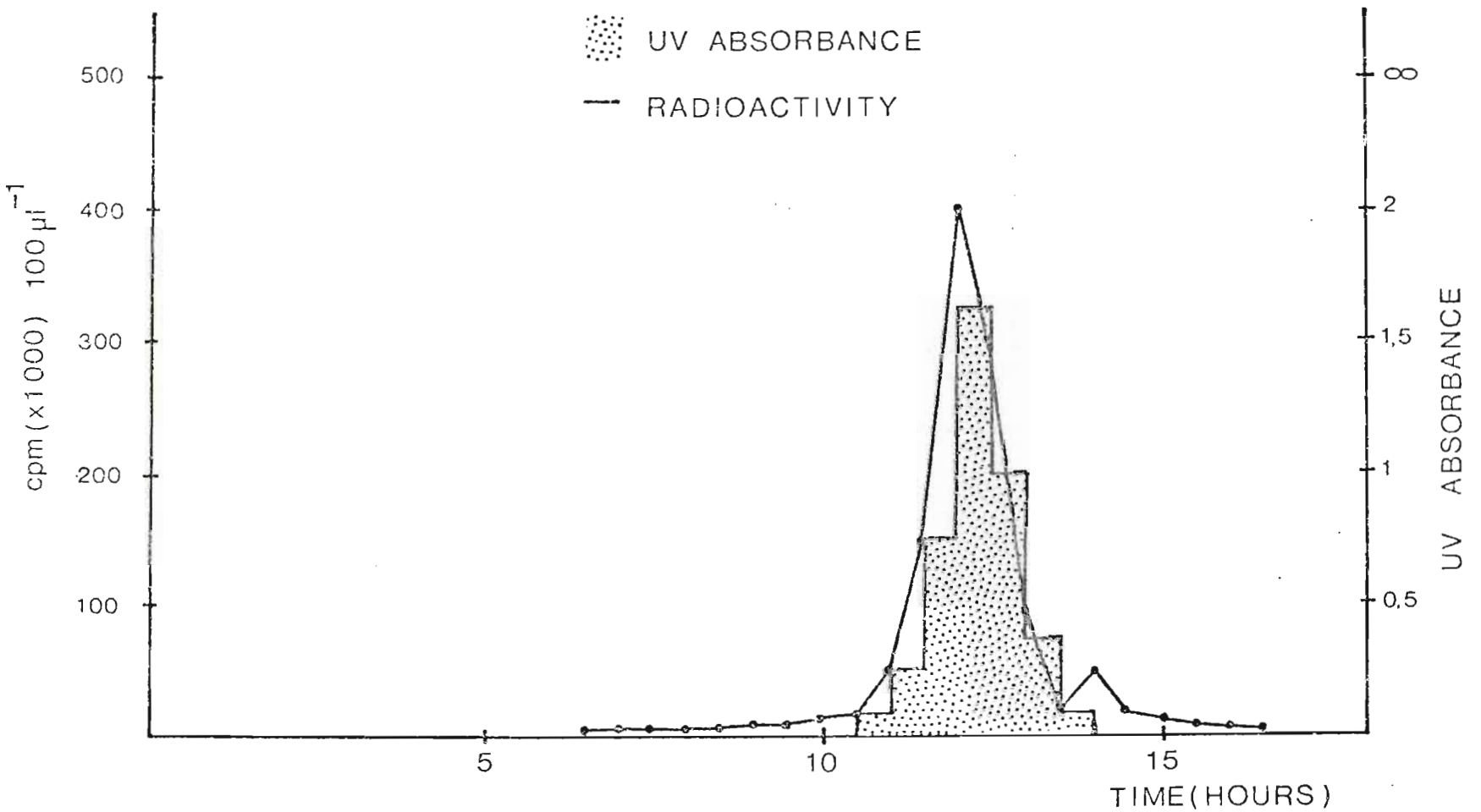
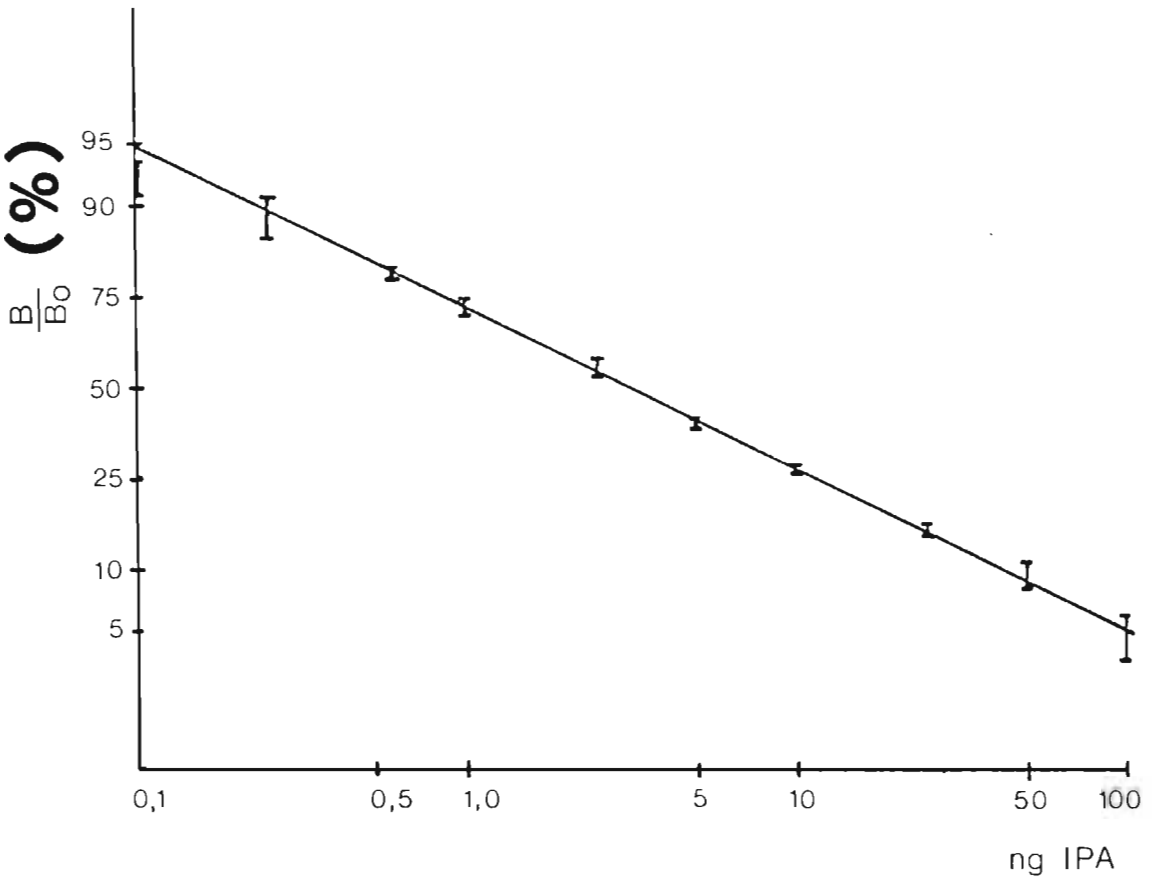


Fig. 2.14 The radioactivity trace and UV absorbance of ^{3}H-IPA and authentic IPA standard respectively; collected as 30 minute fractions from a Sephadex LH-20 column eluted with 35% ethanol.

Fig. 2.15 Two typical standard dose-response curves for IPA, one with increased sensitivity, the other with reduced sensitivity.


B/B0 (%)
95
90
75
50
25
10
5
0,1
0,5
1,0
5
10
50
100
ng IPA

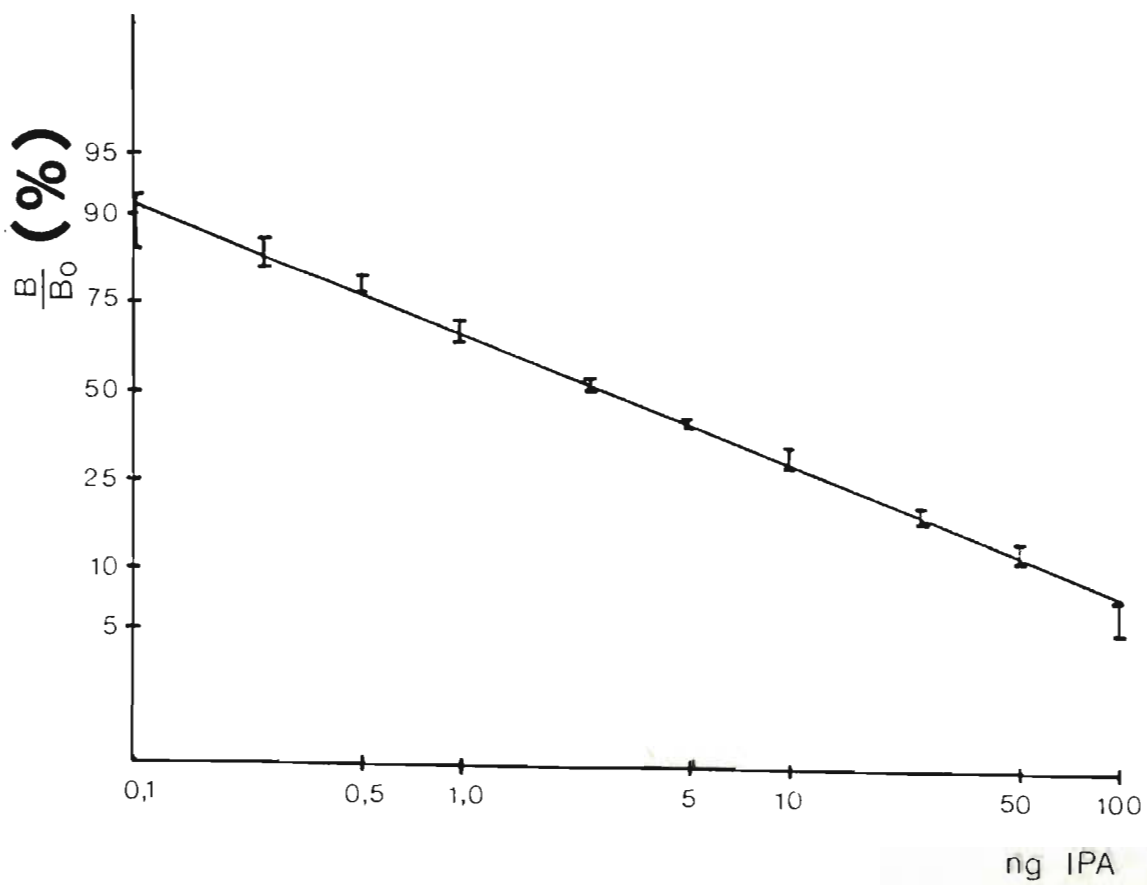
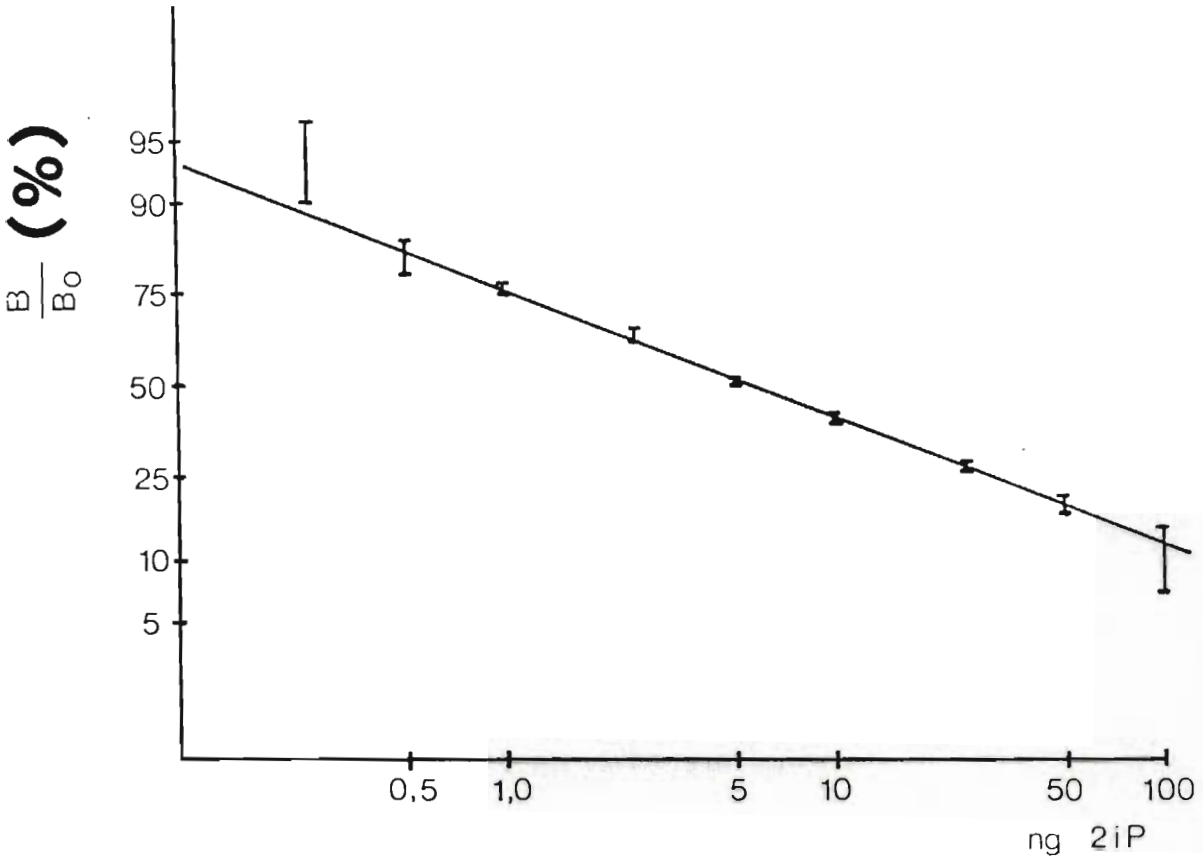
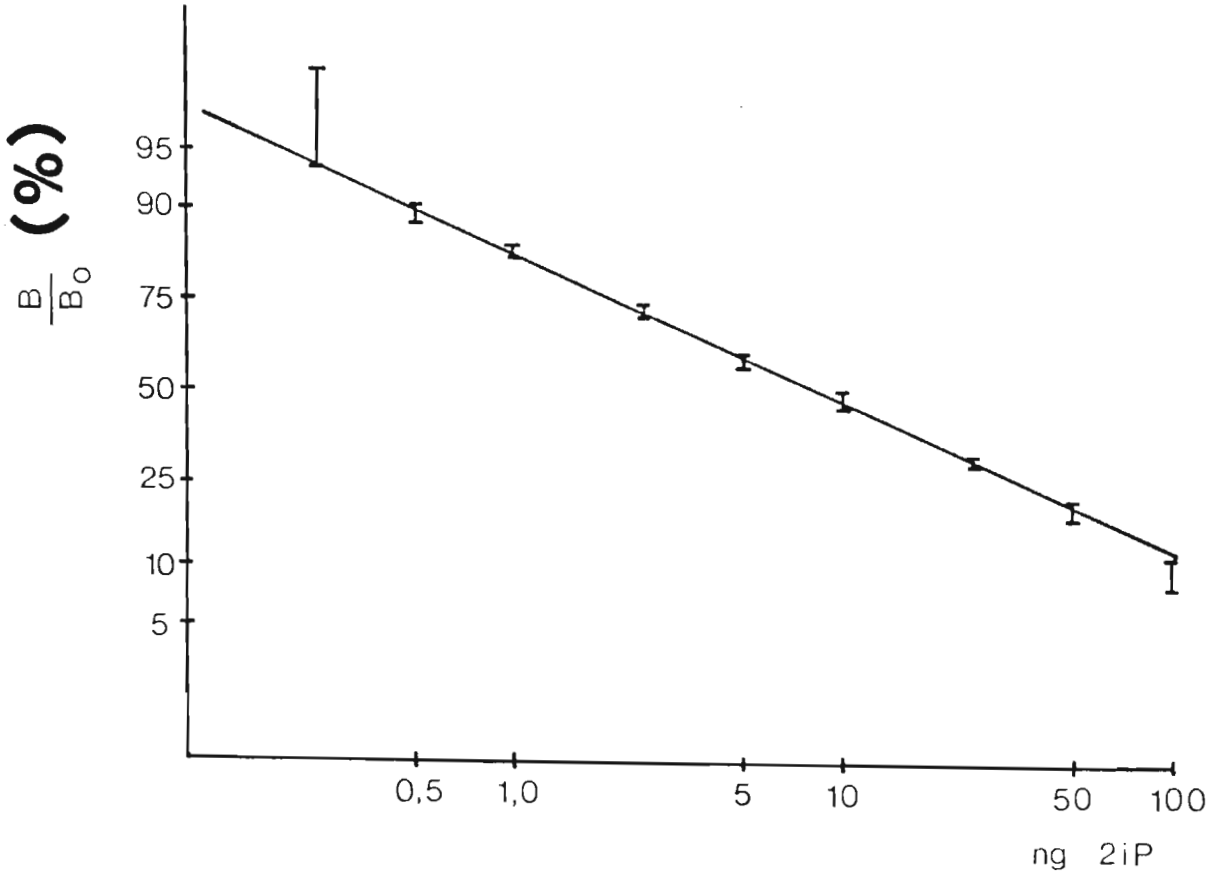

B/B0 (%)
95
90
75
50
25
10
5
0,1
0,5
1,0
5
10
50
100
ng IPA

Fig. 2.16 Two typical standard dose-response curves for 2iP, one with increased sensitivity and one with reduced sensitivity (b).


B/B0 (%)
95
90
75
50
25
10
5
0,5
1,0
5
10
50
100
ng 2iP


B/B0 (%)
95
90
75
50
25
10
5
0,5
1,0
5
10
50
100
ng 2iP

2.2.4 Validation

2.2.4.1 Extract dilution curves

The crude pre-Dowex treatment gave binding percentages in excess of the preset level (binding in the presence of zero standard). From this it appeared that there was some contaminant in the crude extract which served as a non-specific binder. This could have been oil which was visible in the extracts. Fig. 2.17 shows a slight slope in the two extract curves, probably due to some dilution of the contaminant. However, it did not parallel the standard dose-response curve. Therefore crude avocado extracts were unsuitable for analysis by RIA until this contaminant was either removed or inactivated.

The Dowex treatment appeared to remove most of the contaminant from the sample. However, the extract dilution slopes still did not parallel the standard dose-response curve (Fig. 2.18). This was thought to be due to the presence of a cross-reactive component 2iP in the extract, as the slope was slightly depressed.

After TLC the two dilution curves paralleled the standard dose-response curve (Fig. 2.19). To some extent this was expected as the cross-reactive contaminants were removed and virtually only the compound of interest was left in the extract. From the two slopes it appeared that the standard and biological ligand competed identically for sites on the antibody.

For 2iP the pre-Dowex crude extract gave results similar to IPA. Binding was in excess of the preset binding percentage. These data, being of similar nature to that for IPA, are not presented.

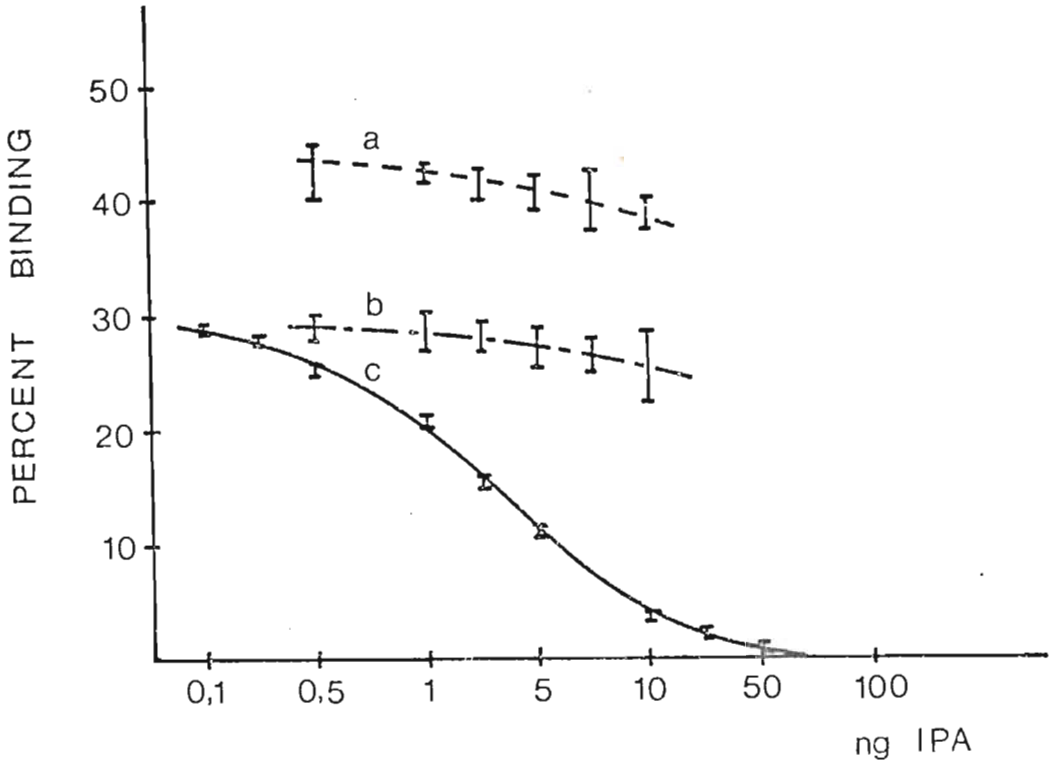
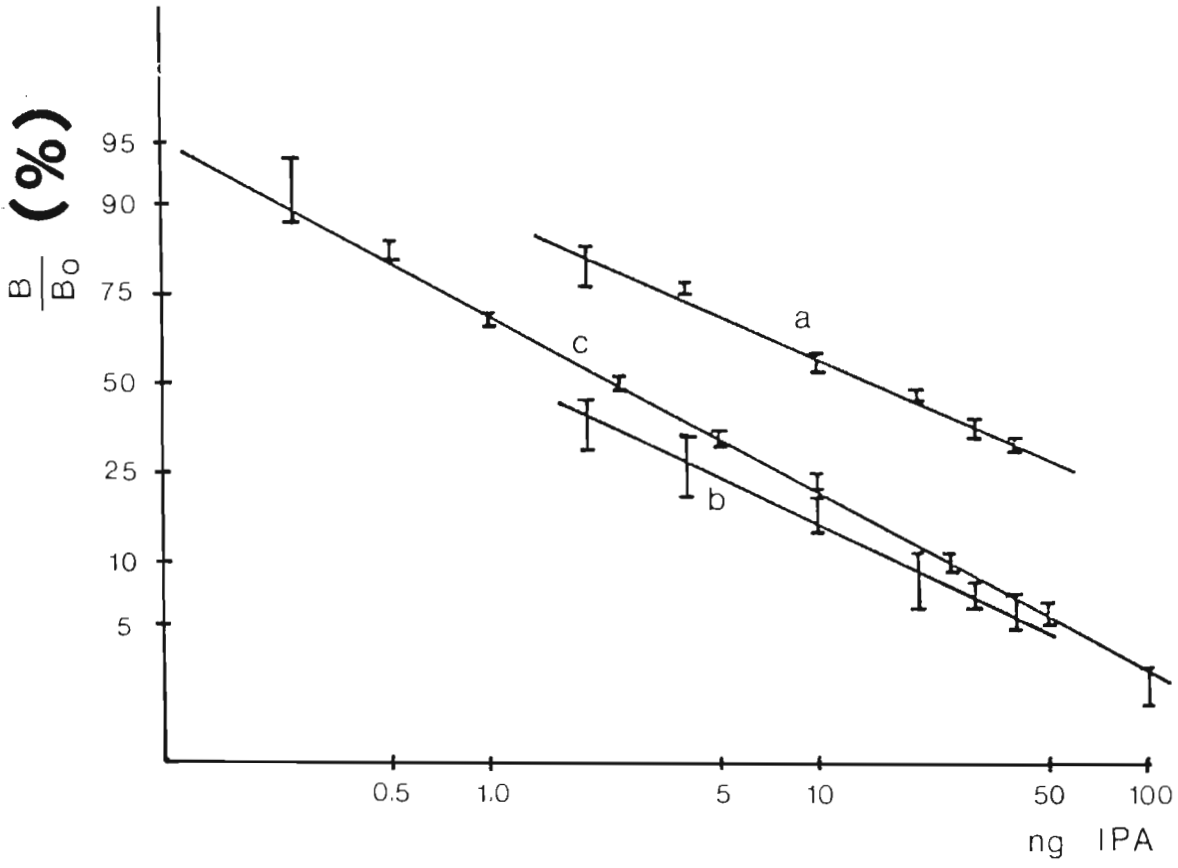


Fig. 2.17 Extract dilution curves for a crude extract prior to any purification, showing the effect of a contaminant (a). The curves for the extract plus authentic IPA standard (b) and the standard dose-response curve (c) are also shown.


B/B0 (%)
95
90
75
50
25
10
5
a
b
c
0.5
1.0
5
10
50
100
ng IPA

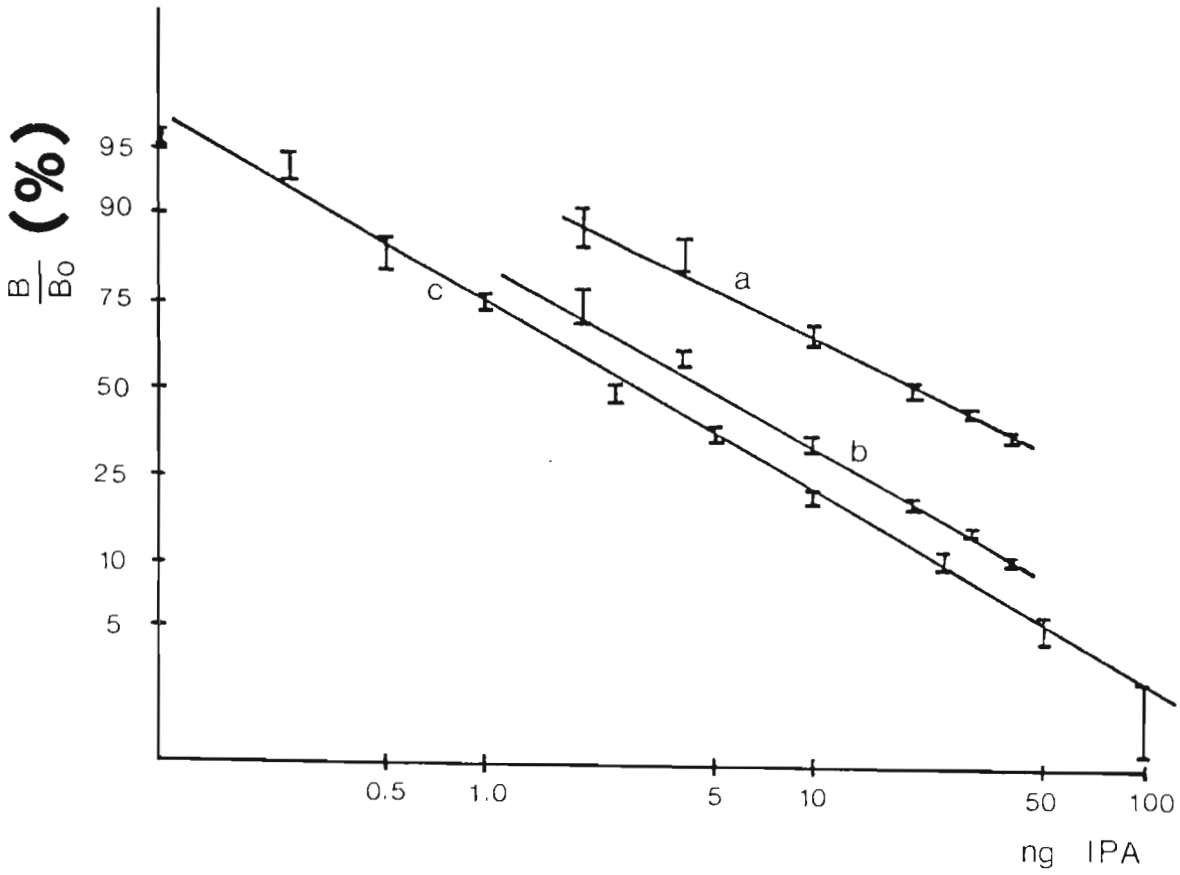
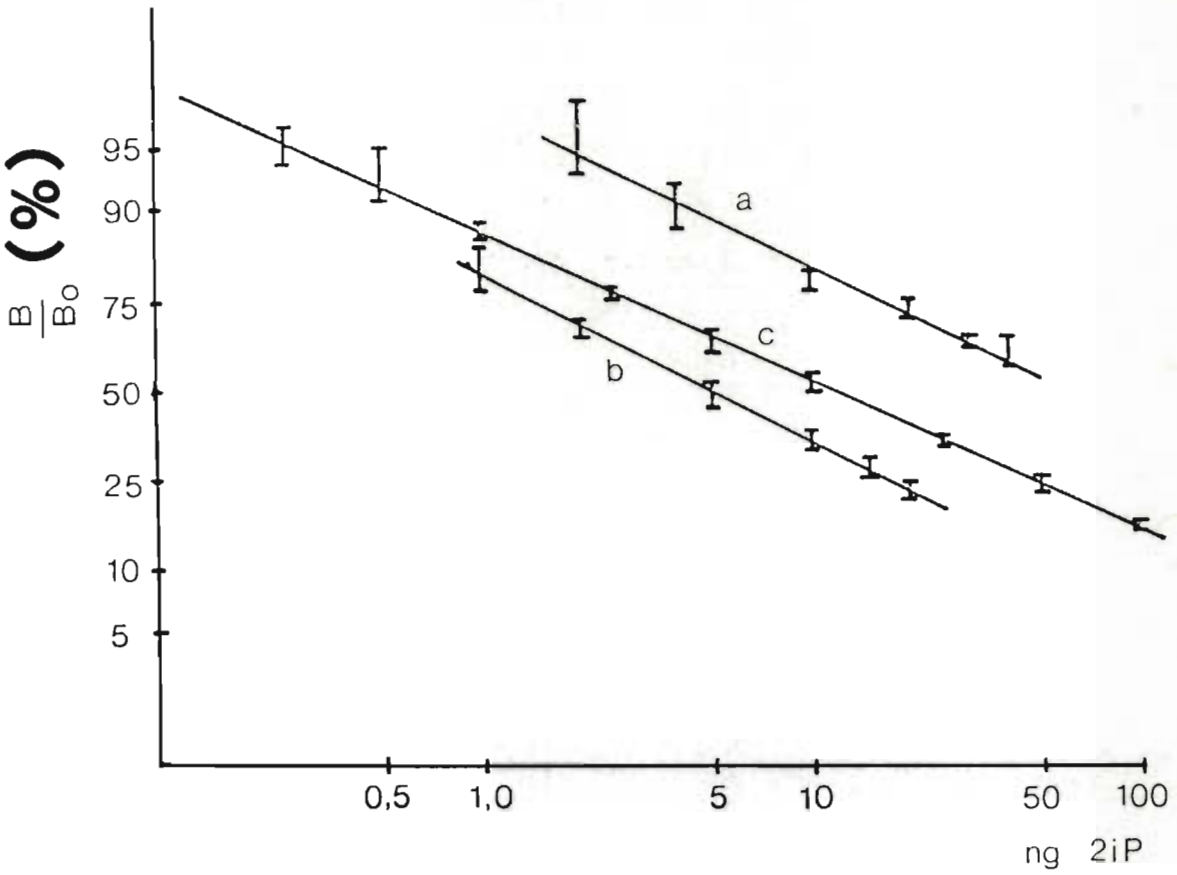
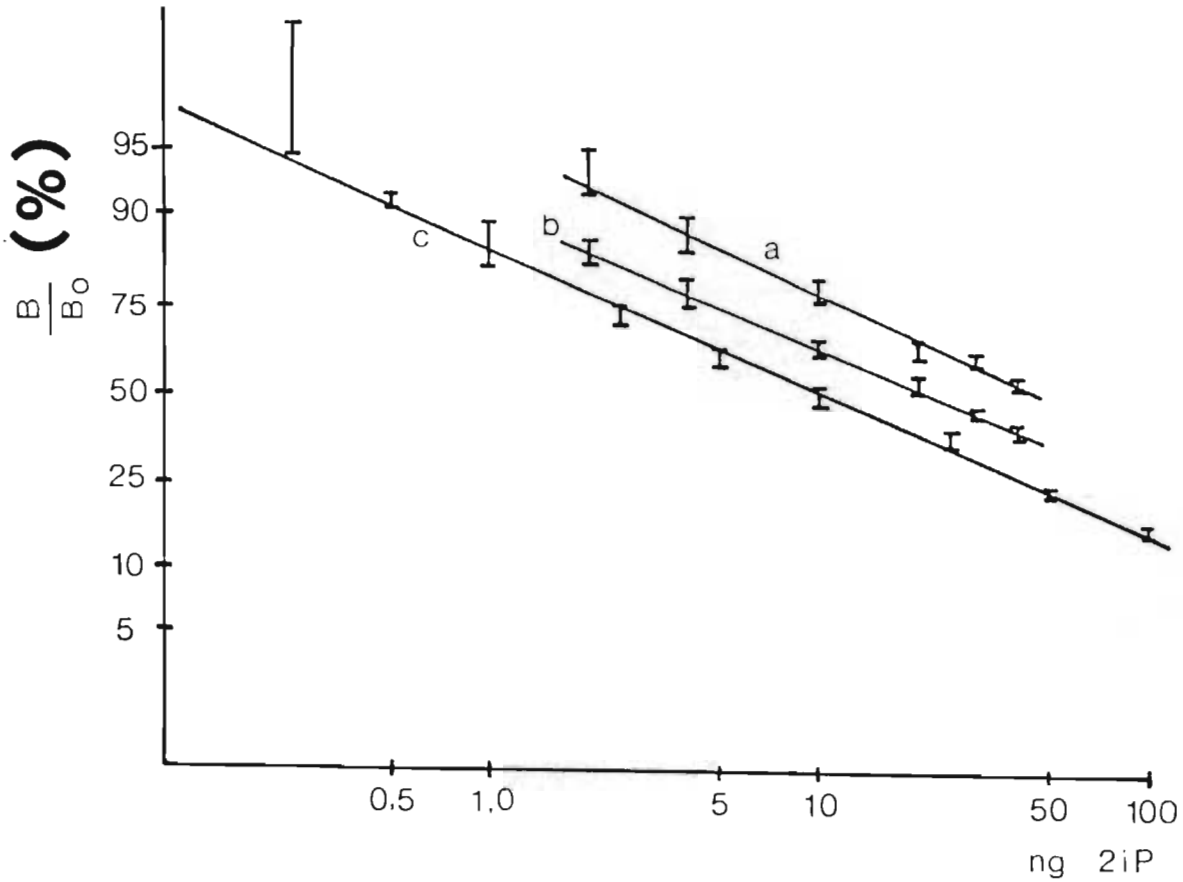

B/B0 (%)
95
90
75
50
25
10
5
a
b
c
0.5
1.0
5
10
50
100
ng IPA

Fig. 2.20 Extract dilution curves for 2iP in avocado extract after Dowex purification but prior to TLC isolation for extract only (a), extract plus authentic standard (b) and standard only (c).

Fig. 2.21 Extract dilution curve for 2iP in avocado extract after purification and isolation for extract only (a), extract plus authentic standard (b) and standard only (c).


B/B0 (%)
95
90
75
50
25
10
5
a
b
c
0,5
1,0
5
10
50
100
ng 2iP


B/B0 (%)
95
90
75
50
25
10
5
a
b
c
0,5
1,0
5
10
50
100
ng 2iP

Dowex treatment appeared to remove the non-specific contaminant in the extract. The slope of the extract dose-response curves (with and without added standard) was 2,1 while the slope of the 2iP dose-response curve was 1,8 (Fig. 2.20). This was thought to be due to the increased avidity of the antibody for the IPA fraction in the extracts.

TLC treatment effectively removed the IPA fraction from the extract. The slopes of the two extract dose-response curves paralleled the standard 2iP dose-response curve (Fig. 2.21). Under these conditions it appeared that the standard 2iP and biological ligand competed identically for the limited antibody sites. Therefore after TLC, biological extracts were suitable for quantitation of IPA and 2iP by RIA.

2.2.4.2 Cross-reactivities to Anti-IPA

From the results in Table 2.3 it was concluded that the antiserum is highly reactive with both IPA and its free base (2iP). The serum showed very low reactivity to other naturally occurring cytokinins (zeatin and dihydrozeatin types).

The fact that the purine bases showed no reactivity at all indicated that the side chain of the cytokinin is definitely involved in the binding to the antibody. The inclusion of a single hydroxyl (zeatin riboside) reduced reactivity by over 99%.

The cross-reactivity of the antiserum to 2iP (25%) did not interfere in the assay since IPA and 2iP were separated out using TLC and quantitated individually.

Table 2.3 Cross-reactivities of various cytokinin and cytokinin-like compounds to anti-isopentenyl adenosine

Compound	% Cross-reactivity
Isopentenyl adenosine	100
Isopentenyl adenine	25
Zeatin	0,48
Zeatin riboside	0,34
Dihydrozeatin	0,90
6-furfurylaminopurine	1,23
Adenine	0
Adenosine	0
6-benzylaminopurine	3,80
6-hexylaminopurine	1,72

2.3 Discussion

The RIAs for IPA and 2iP were not as sensitive as was hoped. This meant that instead of being able to use 100mg samples, it was necessary to extract samples of 5 to 10g. The similarity of the various sera (data not presented), suggested that the antibodies generated by the various rabbit immune systems were very similar, the only difference being titer. An increase in sensitivity could possibly be achieved by synthesis of a tracer ligand with a higher specific activity, or generation of an antibody with a higher avidity for the ligand. A tracer with a higher specific activity would appear to be an easier and faster solution. An alternative approach could perhaps be the development of an enzyme immunoassay.

The titers achieved were never very high when compared to

those obtained for animal steroid hormones (Odell et al., 1971). This was possibly due to inexperience in antibody generation. After booster inoculations, particularly when using Freunds complete adjuvant, tremendous localised reaction occurred, followed in most cases by secondary infection. It was thought that the animal's immune system was overtaxed leading to a reduction in titer against the compound of interest.

The system of using anti-IPA serum to detect levels of 2iP is not perfect. An improvement would be the generation of antibodies against 2iP. However, this would require the linking of 2iP to BSA in such a way that the N-group which links to the ribose unit in IPA, is left free. Another method of increasing titer and avidity to 2iP would be the insertion of a spacer chain between the BSA and the 2iP molecule. A further aspect that requires investigation with low immunogenic compounds is the time interval between the boost and serum collection.

In contrast to other researchers (Weiler, 1979, 1980a; Walton *et al.*, 1979) the use of dextran-coated charcoal as a separation procedure was selected. It was found that the simplicity, speed and effectiveness of the system was so good that it was the first choice in any other RIAs contemplated. In general the RIA as outlined in 2.1.3.2 was reliable and precise. Since the onset of this study three other research groups have succeeded in the development of RIAs for IPA (Weiler & Spanier, 1981; MacDonald, Akiyoshi & Morris, 1981; Ernst, Schafer & Oesterhilt, 1983). Ernst *et al.* (1983) showed that RIA and gas chromatography mass spectrometry analysis gave very similar results on crude extracts, but concluded that some form of isolation was necessary before a reliable answer was obtainable by RIA.

The preparation of the biological material was the most time consuming step, and it was found that considerable losses occurred during separation of IPA and 2iP. Recoveries after TLC on cellulose were found to be only 40% but this was consistent and reproducible. Experimentation with other solvents was not found to increase recoveries. It was thought that a proportion of the cytokinins became bound into the structure of the microcrystalline. In contrast to other workers (Weiler & Spanier, 1981; Ernst *et al.*, 1983) it was found that this RIA was seriously affected by contaminants in crude extracts. This was thought to be caused by the oil present in the avocado extracts acting as a non-specific binder and effectively out-competing the antibody. However, after removal of the contaminant the RIA performed both reliably and reproducibly, as the antibody was unable to distinguish between authentic standard ligand and biological ligand.

CHAPTER 3
RADIOIMMUNOASSAY FOR ABSCISIC ACID

3.1 Materials and Methods

In general the methods used in the development of the RIA for ABA were similar to those used for the development of the IPA and 2iP RIAs. In order to prevent excessive repetition of methodology, where methods were similar, reference will be made to the relevant section in Chapter 2. However, where obvious differences exist, such as preparation of immunogen and treatment of biological sample, these will be described in detail.

3.1.1 Conjugation of (±)ABA to BSA

A modification of the method of Fuchs & Fuchs (1969) for linking an active carboxyl group to BSA was used. One hundred mg (±)ABA was dissolved in 2ml water:dimethyl formamide (1:2), and then added dropwise to a stirred solution of 110mg BSA dissolved in distilled water with a starting pH of 8,5. The pH was then adjusted to 8 with 1M NaOH. Fifty mg quantities of N-ethyl-N-(3-dimethyl-aminopropyl)-carbodiimide-HCl were added every 30 min to the reaction mixture until a total of 200mg had been added, and the solution then stirred at room temperature for a further 24 hours. After dialyses against distilled water for 72 hours the solution was lyophilized and stored at -30°C.

The linking ratio of (±)ABA to BSA was determined from the ultra violet (UV) spectrum of the conjugate. The assumption was that the mass of the attached ABA per mole of protein was negligible. Twelve mg of BSA and 12mg of ABA-BSA conjugate were separately dissolved in 50ml of borate buffer (pH 8,5) and their UV spectra between 200 and 350nm determined. The UV spectrum for (±)ABA standard dissolved in

methanol was also determined, and a UV standard curve using serial dilutions of (±)ABA at 260nm constructed. Using the derived data from the UV spectra the linking ratio was determined (3.2.1).

3.1.2 Production of antisera

3.1.2.1 Immunization

The immunization method selected was the same as for IPA (2.1.2.1) with a few modifications, due primarily to the good results achieved with the IPA-BSA conjugate.

The (±)ABA-BSA conjugate was suspended in saline (pH 4,6), in which it was only partially soluble, so that 1ml of saline contained 2mg of conjugate. The preparation of the stable water-in-oil emulsion using Freunds adjuvant was as in 2.1.2.1.

Five rabbits, two male and three female, were selected for immunization and treated as in 2.1.2.1. The initial immunization was in October, 1982 and the booster injection in March, 1983.

Due to a poor response the rabbits were re-inoculated in July, 1983, with some minor changes to immunogen preparation. The conjugate was found to be fully soluble in borate buffer (pH 8,5). Therefore this buffer was used in all further ABA immunogen preparation, there were no other changes to the immunization and blood collection sequence (refer to 2.1.2.1). Rabbits were bled nine days after the July, 1983 boost and then every 30 days thereafter.

3.1.2.2 Purification and storage

Blood purification and storage were as described in 2.1.2.2.

The only modification was that clot contraction took place over 48 hours at 4°C.

3.1.2.3 Titer determination

As with anti-IPA (refer to 2.1.2.3) titer determination was conducted by RIA. Anti-ABA serum was diluted in 0,1% gelatine in PBS (pH 7,0). Dilutions ranged from 1:10 to 1:200. Calculation of results was as in 2.1.2.3. Once the titer curves had been obtained all anti-ABA titers were expressed relative to 30% binding of the total activity added.

3.1.3 Radioimmunoassay

As with the IPA-RIA, various tests were performed to select the most suitable procedure for the ABA RIA.

3.1.3.1 Optimization

3.1.3.1.1 Effect of separation technique

Three different separation techniques were tested. A second antibody method was not attempted due to the lengthy incubation times required for this procedure. As with the IPA RIA, aspects under consideration when selecting a possible separation procedure were efficiency of separation, its precision and its effect upon the standard dose-response curve. General methodology was the same as in 2.1.3.1.1.

3.1.3.1.2 Determination of incubation time

After addition of the primary reagents (refer to 3.1.3.2), various incubation times, in a water bath at 37°C, from 3 to 90 min were tested. After stabilization in ice for 120 min the procedure and calculation of results was as in 2.1.3.1.3.

3.1.3.1.3 Effect of charcoal concentration

Initially the methodology used for the ABA RIA was very similar to that for IPA. However, precision in the ABA assay was very poor. Therefore, the effect of charcoal concentration on assay precision was investigated. The charcoal concentrations per tube ranged from 1 to 40mg. For procedure and calculation of results refer to 2.1.3.1.2.

3.1.3.1.4 Effect of pH

Three different pHs were tested, viz. two PBS buffers of 6,5 and 7, and one borate buffer at 8,5. The procedure was as in 3.1.3.2. Results were calculated as the percentage binding of the highest binding value.

3.1.3.2 Standard procedure

After completion of the various parameter tests an optimized RIA procedure for ABA was formulated. Standard equipment was the same as for IPA (refer to 2.1.3.2).

All assay parameters were duplicated. Standard concentrations of (±)ABA ranging from 10pg to 10ng per 100µl, and biological sample, suspended in methanol were dispensed into the relevant assay tubes. The typical assay format used was similar to that presented in Table 2.1. Protein coating of the assay tubes and dispensing of the primary assay reagents was as in 2.1.3.2. After vortexing, assay tubes were incubated in a waterbath at 37°C for 15 min and the tubes were then again vortexed.

Stabilization of the reaction mixture was achieved in an ice bath after 120 min. Thereafter 800µl of dextran-coated charcoal (250mg charcoal and 500mg dextran slurried in 50ml distilled water) at 4°C was added to all assay tubes except

those designated for Ta which had 800µl distilled water added. Centrifugation and preparation of the bound fraction for the determination of activity (refer to 2.1.3.2), statistical analysis and calculation of results were as for IPA and 2iP (refer to 2.1.3.3).

3.1.4 Validation

3.1.4.1 Extract dilution curves

To determine the presence of any potentially interfering compounds present in the biological extracts, extract dilution curves were made. A 50g sample of water-stressed avocado leaf material was harvested and extracted (refer to 3.1.5.1) in 90% methanol acidified with 1% acetic acid. Dilution curves were established for crude extracts and for extracts that had undergone solvent partitioning and TLC on silica gel (refer to 3.1.5.2 for solvent partitioning and TLC).

After extraction the sample was centrifuged at 1 000g for 10 min at 4°C and the supernatant split into three fractions and treated as follows :

(a) A crude fraction was suitably diluted and assayed directly to establish a crude extract dilution curve.

(b) The same as for (a) but with the addition of 50ng (±)ABA standard.

(c) Fifty µl of crude extract was placed on a silica gel (Merck 5554) TLC plate, developed and had the relevant zone removed and eluted with absolute methanol. Suitable dilutions of the eluent were made and then subjected to RIA.

(d) The same as for (c) but with the addition of 50ng (±)ABA standard prior to TLC.

(e) A sample that had undergone solvent partitioning and TLC was suitably diluted and subjected to RIA.

(f) The same as for (e) but with the addition of 10ng (±)ABA prior to solvent partitioning.

The standard RIA procedure for ABA was used (refer to 3.1.2.3) for the determination of dilution curves. Results were plotted on standard log-logit probability paper.

3.1.4.2 Antibody selectivity (cross-reaction studies)

Various ABA-related compounds and precursors were assessed for cross-reactivity. From these data it was hoped that the cause of the non-parallelity of the dilution curves could be ascertained. It was also hoped to determine which components of the ABA molecule were involved in the recognition response. Ten mg standard preparations of the various compounds were individually suspended in 10ml of absolute methanol. Dilutions used in the RIA ranged from 10pg to 5 000ng per 100µl.

The standard RIA procedure was followed (refer to 3.1.3.2) and cross-reactivity curves plotted on 6 cycle log-logit probability graph paper. The percentage cross-reactivity was calculated relative to 50% inhibition of binding of ABA on a mass basis.

3.1.5 Biological sample preparation

The procedure described below was used as standard for the preparation of biological samples for measurement of ABA by

RIA unless otherwise stated.

3.1.5.1 Extraction

Organic solvents have been used to extract ABA from biological material (Weaver, 1972). A modification of the method described by Weiler (1979) was used.

Samples of 10g of plant material were homogenized in 50ml 90% methanol acidified with 1% acetic acid. After extraction at 4°C for 48 hours in the dark, the material was centrifuged at 1 000g for 10 min. The supernatant was reduced to dryness under vacuum at 30°C and taken up in 5ml absolute methanol so that 1ml methanol contained the equivalent of 2g fresh material. Samples were again centrifuged (1 000g for 10 min) and stored at -20°C prior to purification.

3.1.5.2 Purification

A modification of the solvent partitioning method of Tillberg & Pinfield (1982) was used. One ml of extract (2g equivalent) was added to 10ml potassium phosphate buffer (0,5M; pH 8,0), mixed, reduced to the aqueous phase under vacuum at 30°C, placed in a 100ml separation funnel, and extracted with 40ml diethyl ether (2 x 20ml). The lower phase (aqueous buffer) was retained and acidified to pH 3,0 with phosphoric acid. After further extraction with 40ml diethyl ether (2 x 20ml) the upper phase (ether phase) was retained and reduced to dryness under vacuum at 30°C. The residue was taken up in 2ml diethyl ether and stored at -20°C prior to TLC.

3.1.5.3 Isolation

To separate out the free ABA from the composite, 80µl of the

solvent-partitioned extract was placed on a silica gel TLC plate (Merck 5554). Reference standards were placed alongside the biological samples. After drying the plate was developed with toluene : ethyl acetate : acetic acid (50:30:4). Using UV, the R_f zone corresponding to authentic ABA was isolated and removed. Elution took place overnight at 4°C in the dark using 20ml absolute methanol. After reduction to 1ml the eluate was split and assayed in duplicate.

3.2 Results

3.2.1 ABA-BSA conjugate

The UV spectra of (±)ABA standard, BSA and the ABA-BSA conjugate are given in Fig. 3.1. The BSA had an absorbance maximum at 220nm. The conjugate had a maximum absorbance at 215 and a lesser peak at 260nm. The peak at 215 coincided very closely with BSA, and therefore deemed unsuitable for linking ratio determination. An ABA standard was found to have a high absorbance over the range from 310 to 200nm. A UV standard curve for (±)ABA at 260nm is presented in Fig. 3.2.

The linking ratio was calculated as follows :

$$\text{ABA} = \frac{26,6}{1\,000 \times 264,3}$$

$$= 0,0001000 \text{ mol } l^{-1}$$

$$= 1,0 \times 10^{-4} \text{ mol } l^{-1}$$

$$\text{BSA} = 0,012\text{g in } 50\text{ml}$$

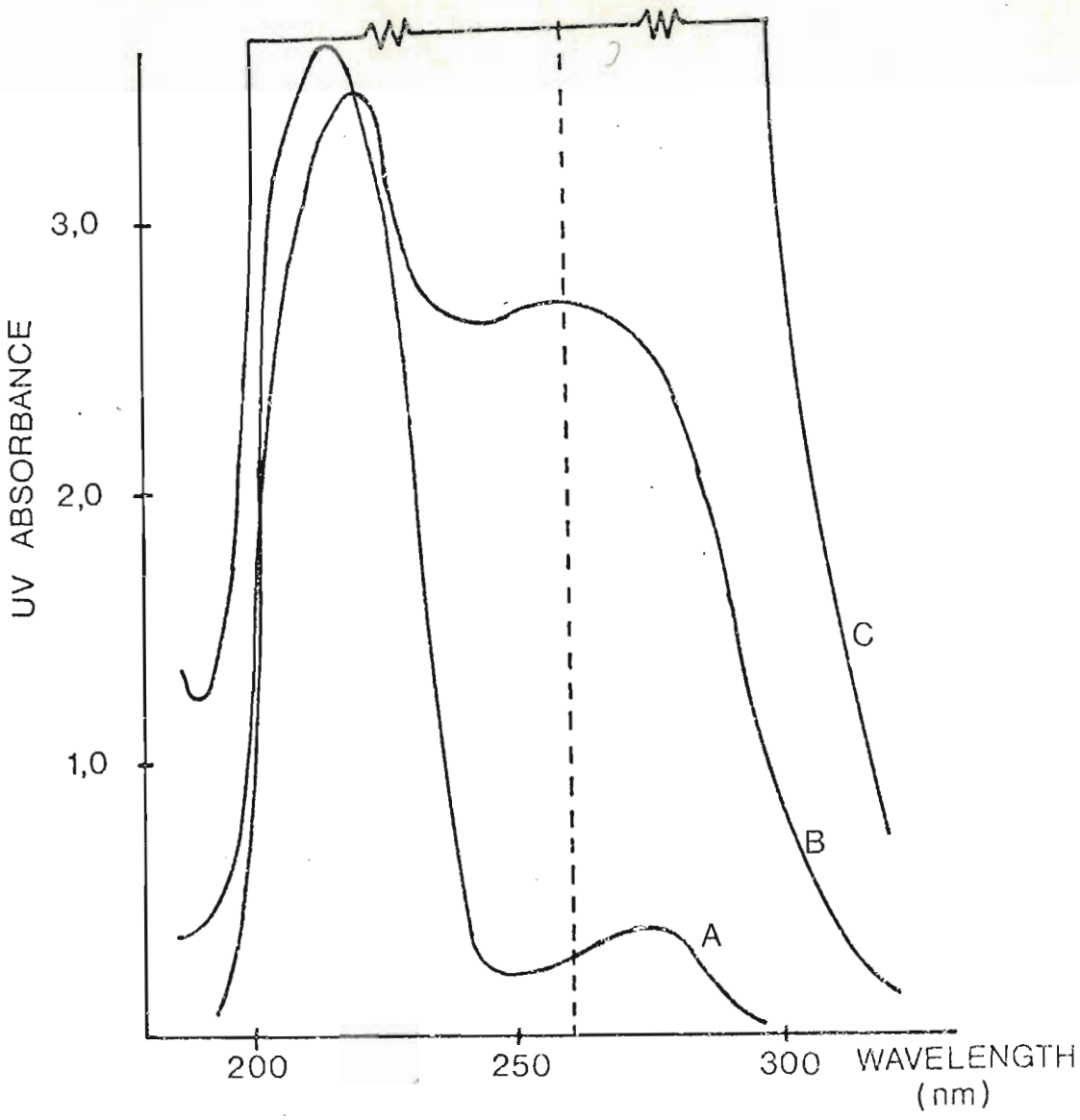
$$= 0,240\text{g } l^{-1}$$

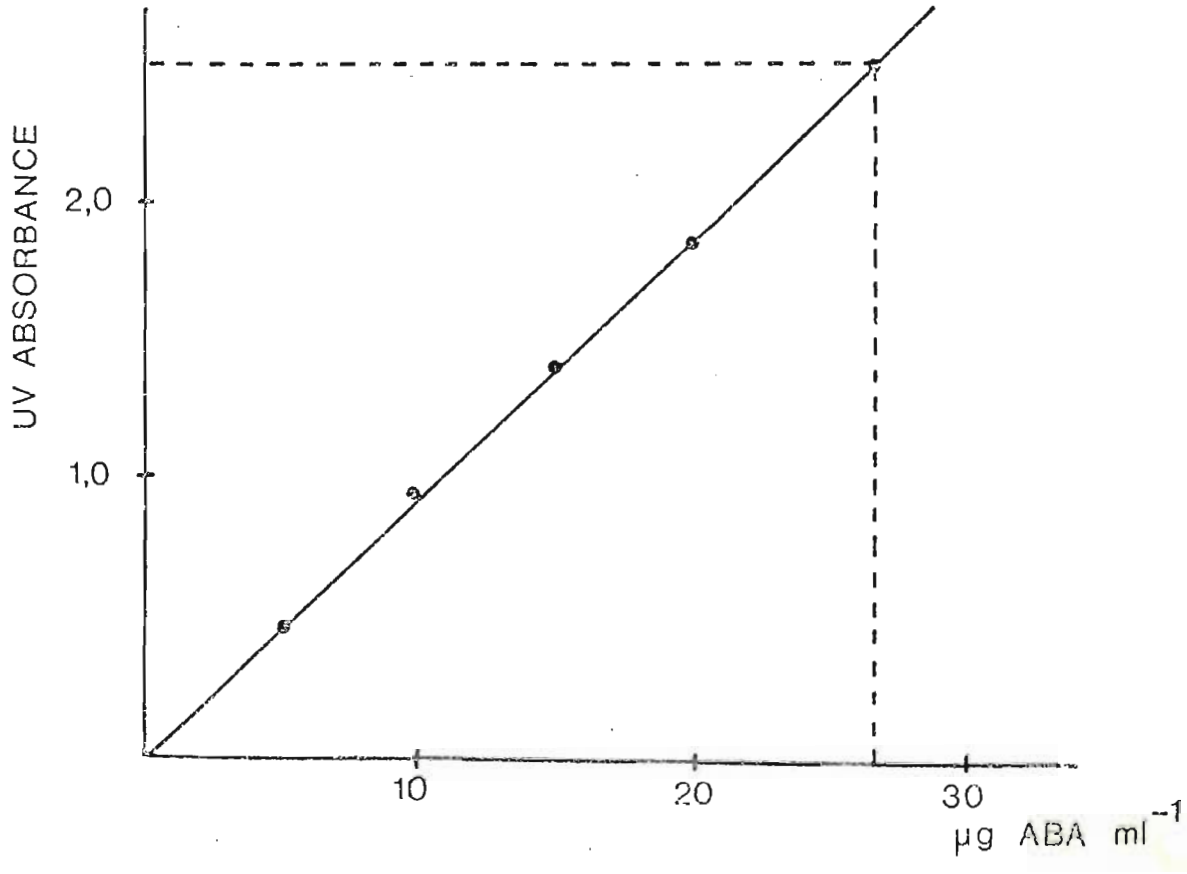
$$= \frac{0,24}{70\,000}$$

$$= 3,4 \times 10^{-6} \text{ mol } l^{-1}$$

Fig. 3.1 The UV spectra of BSA (A), (±)ABA (C) and the (±)ABA-BSA conjugate between 200 and 300nm. The broken line (260nm) shows where the UV readings used in the calculation of the linking ratio were taken.

Fig. 3.2 The standard UV curve for (±)ABA at 260nm. The broken line transformed the UV reading from Fig. 3.1 to an absolute (±)ABA concentration of 26,6µg ABA ml^{-1} which was used in the calculation of the linking ratio of (±)ABA to BSA.


UV ABSORBANCE
3,0
2,0
1,0
200
250
300
WAVELENGTH
(nm)
A
B
C


UV ABSORBANCE
2,0
1,0
10
20
30
µg ABA ml⁻¹

$$\text{Ratio ABA:BSA} = \frac{100 \times 10^{-6}}{3,4 \times 10^{-6}}$$

$$= 29,4:1$$

3.2.2 Response to immunization

The responses of the rabbits to inoculation with the (±)ABA-BSA conjugate is presented in Figs. 3.3 and 3.4. Two rabbits, ABA-1 and ABA-4, produced serum with a high antibody titer. For the balance of this study serum from rabbit ABA-1 was used. The antibody titer which bound 30% of the added (±)^{3}H-ABA tracer was superior in response after the second booster immunization in every case. The RIA for ABA was run at a serum dilution of 1:175 which bound 20% of the total added tracer.

3.2.3 Radioimmunoassay

3.2.3.1 Effect of separation technique

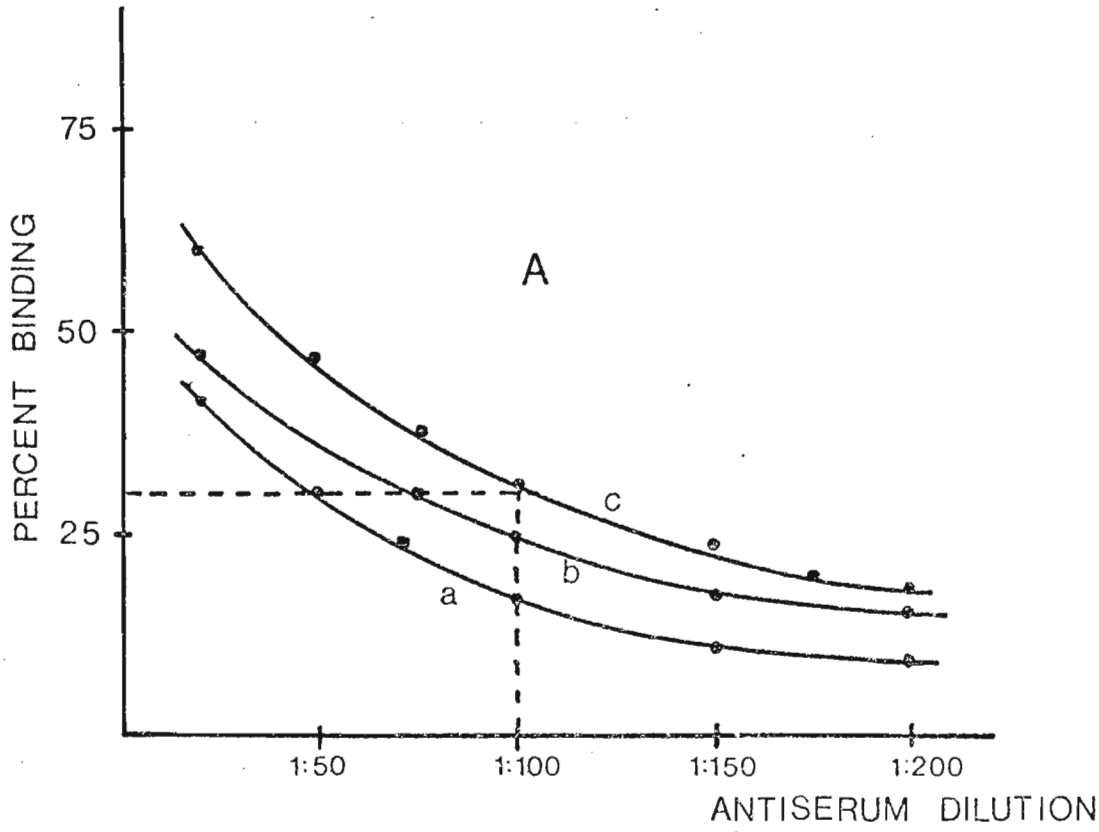
Dextran-coated charcoal gave separation adjudged good. Regression analysis (r = 0,986) indicated good precision and the assay was shown to be suitable to linearization using the logit transformation (Fig. 3.5). NSB was low and ranged from 1,0 to 3,5 (n = 30) of the total tracer added to the system.

As with the IPA-RIA, the charcoal reduced the apparent titer of the antiserum in the presence of similar quantities of tracer when compared to ammonium sulphate and polyethylene glycol (Table 3.1). For charcoal the overall result was a more sensitive assay.

Separation of bound and free fractions using ammonium sulphate was poor when compared to charcoal. NSB for ammonium sulphate was high and ranged from 10 to 15% (n = 4) of the

Fig. 3.3A Antiserum dilution curves from rabbit ABA-1 bled on the following days; 1983:03:17 (a), 1983:07:22 (b) and 1983:08:21 (c).

Fig. 3.3B Antiserum dilution curves from rabbit ABA-2 bled on the following days; 1983:03:17 (a), 1983:07:22 (b) and 1983:08:21 (c).


75
50
25
PERCENT BINDING
A
c
b
a
1:50
1:100
1:150
1:200
ANTISERUM DILUTION

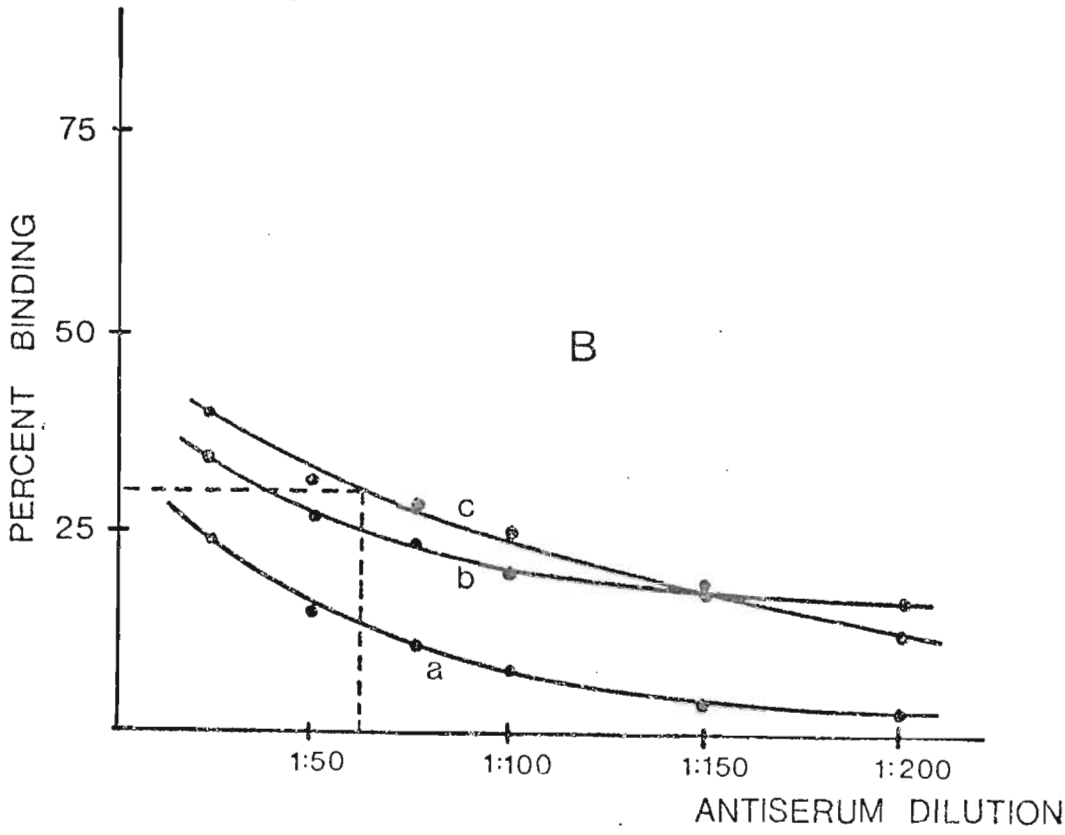
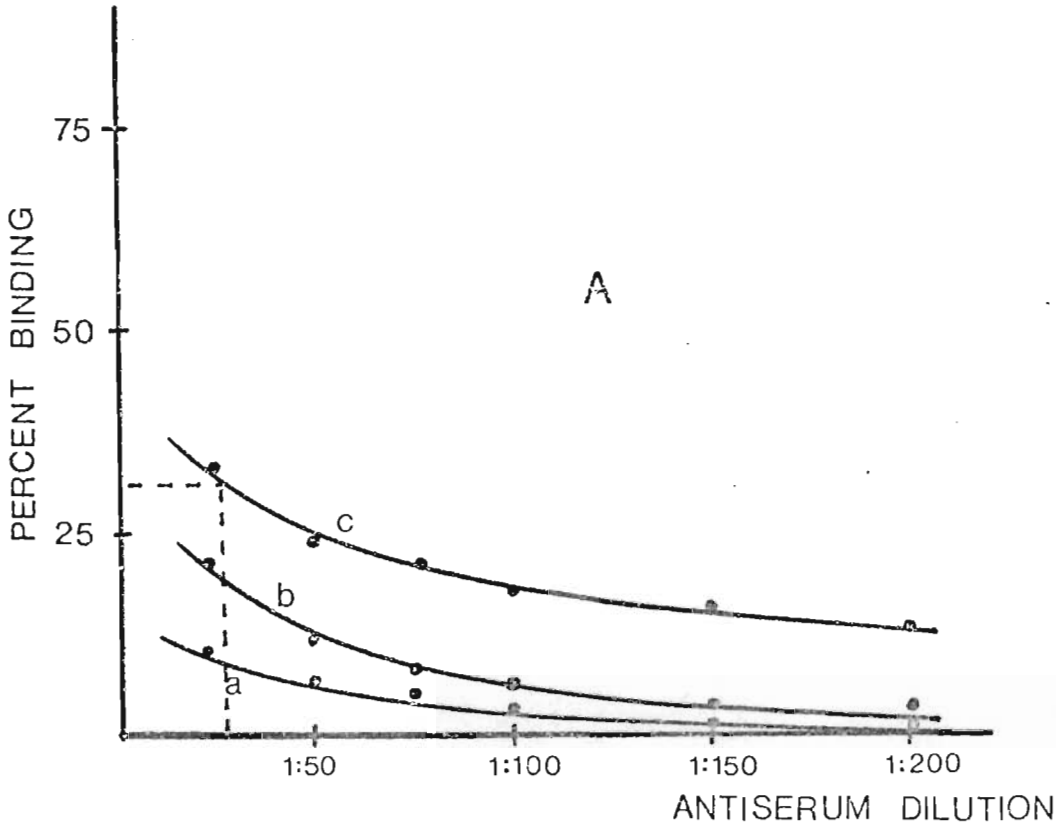
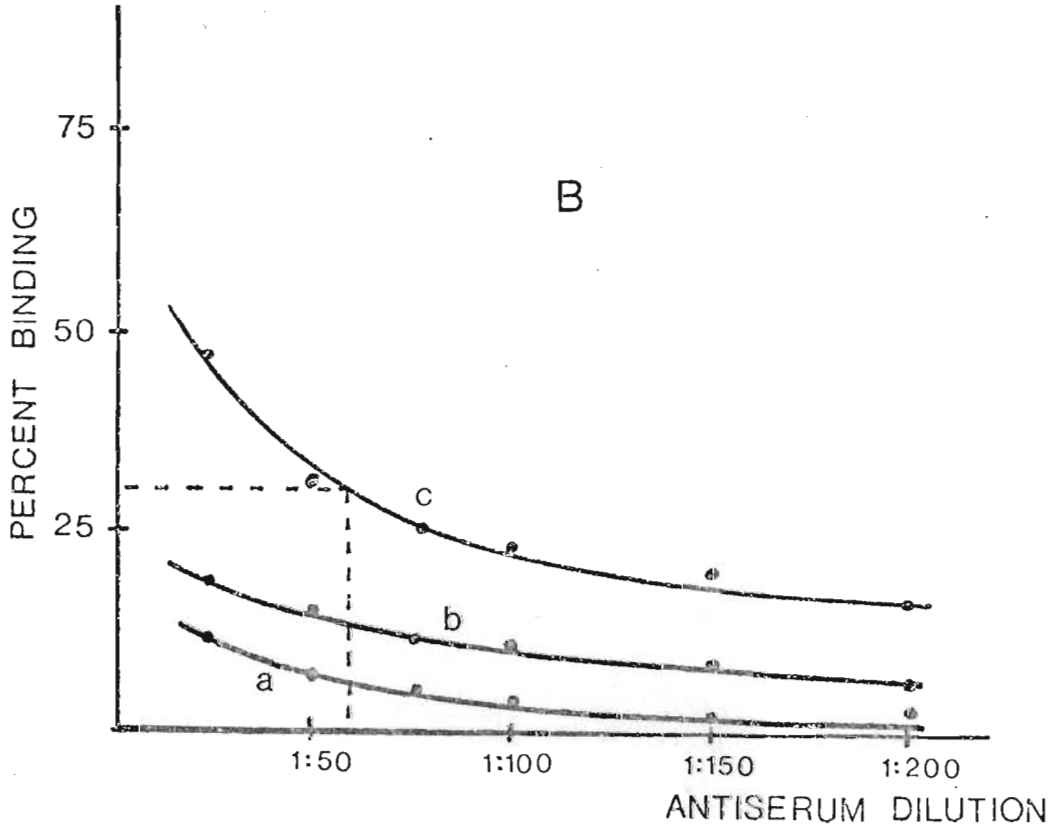

75
50
25
PERCENT BINDING
B
c
b
a
1:50
1:100
1:150
1:200
ANTISERUM DILUTION

Fig. 3.4A Antiserum dilution curves from rabbit ABA-3 bled on the following days; 1983:03:17 (a), 1983:07:22 (b) and 1983:08:21 (c).

Fig. 3.4B Antiserum dilution curves from rabbit ABA-4 bled on the following days; 1983:03:17 (a), 1983:07:22 (b) and 1983:08:21 (c).


A
75
50
25
PERCENT BINDING
c
b
a
1:50
1:100
1:150
1:200
ANTISERUM DILUTION


B
75
50
25
PERCENT BINDING
c
b
a
1:50
1:100
1:150
1:200
ANTISERUM DILUTION

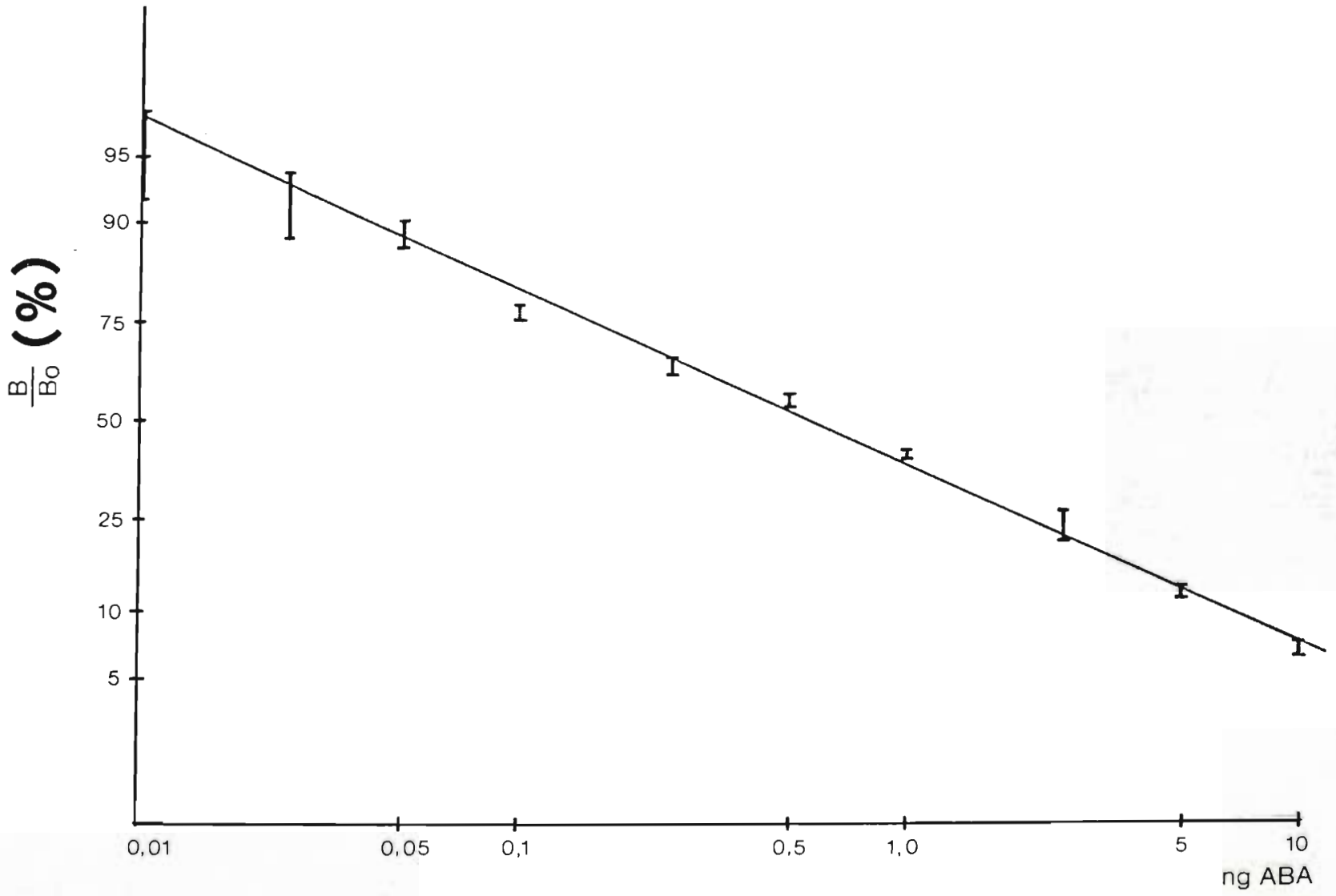


Fig. 3.5 Standard dose-response curve for ABA using dextran-coated charcoal as a separation procedure.

total tracer added to the system. Precision as measured by regression analysis (r = 0,94) was unacceptable, particularly in the more sensitive half of the curve (Fig. 3.6). As with the IPA-RIA, averaged values when subjected to regression analysis indicated that this method gave an assay that was suitable to linearization by logit analysis.

Table 3.1 Effect of separation procedure on the standard dose-response curve

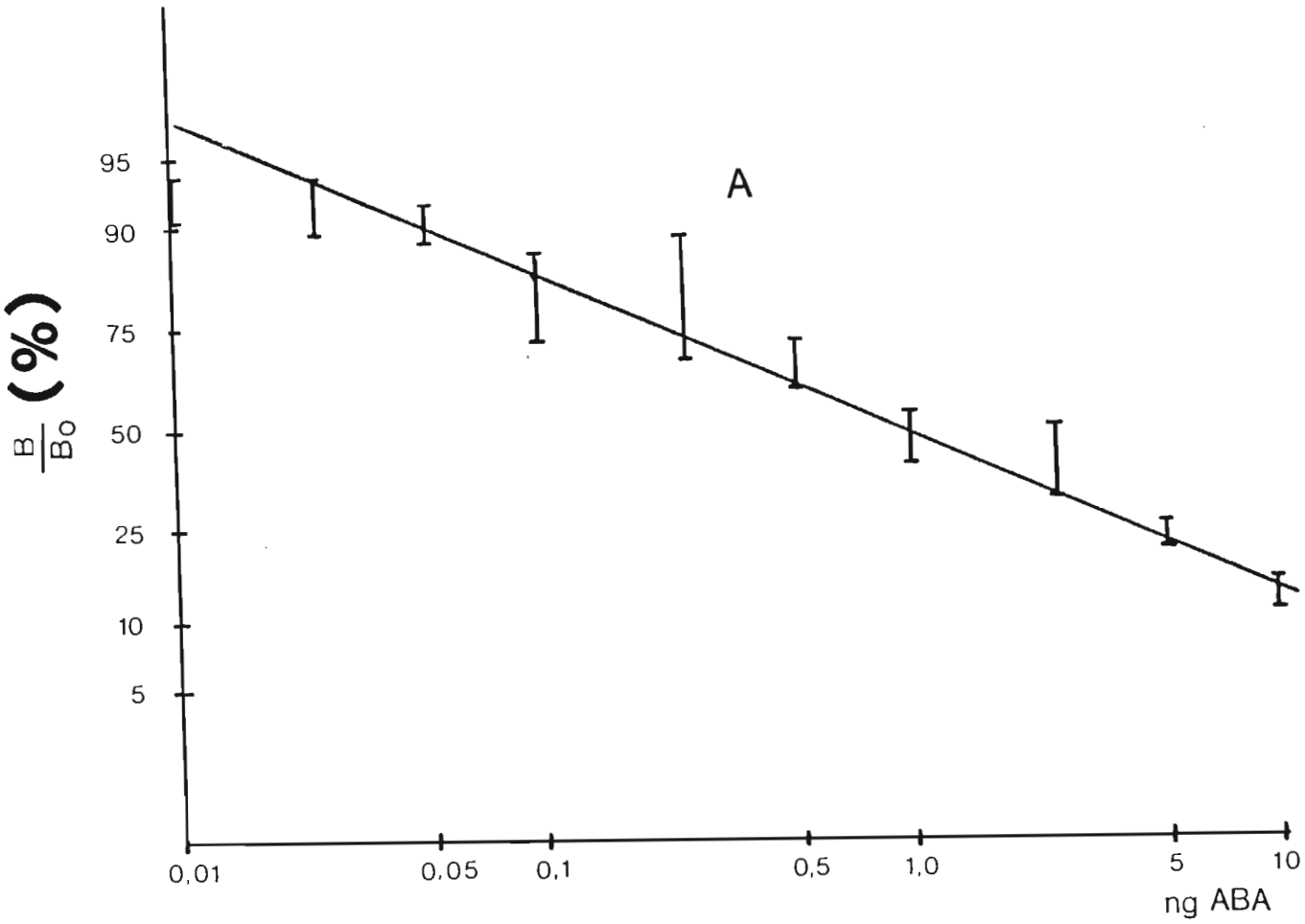
Separation procedure		Standard concentration at 50% inhibition of binding in ng
Dextran-coated charcoal	12 000	0,54
NH_3SO_4	12 000	0,90
Polyethylene glycol	12 000	0,78

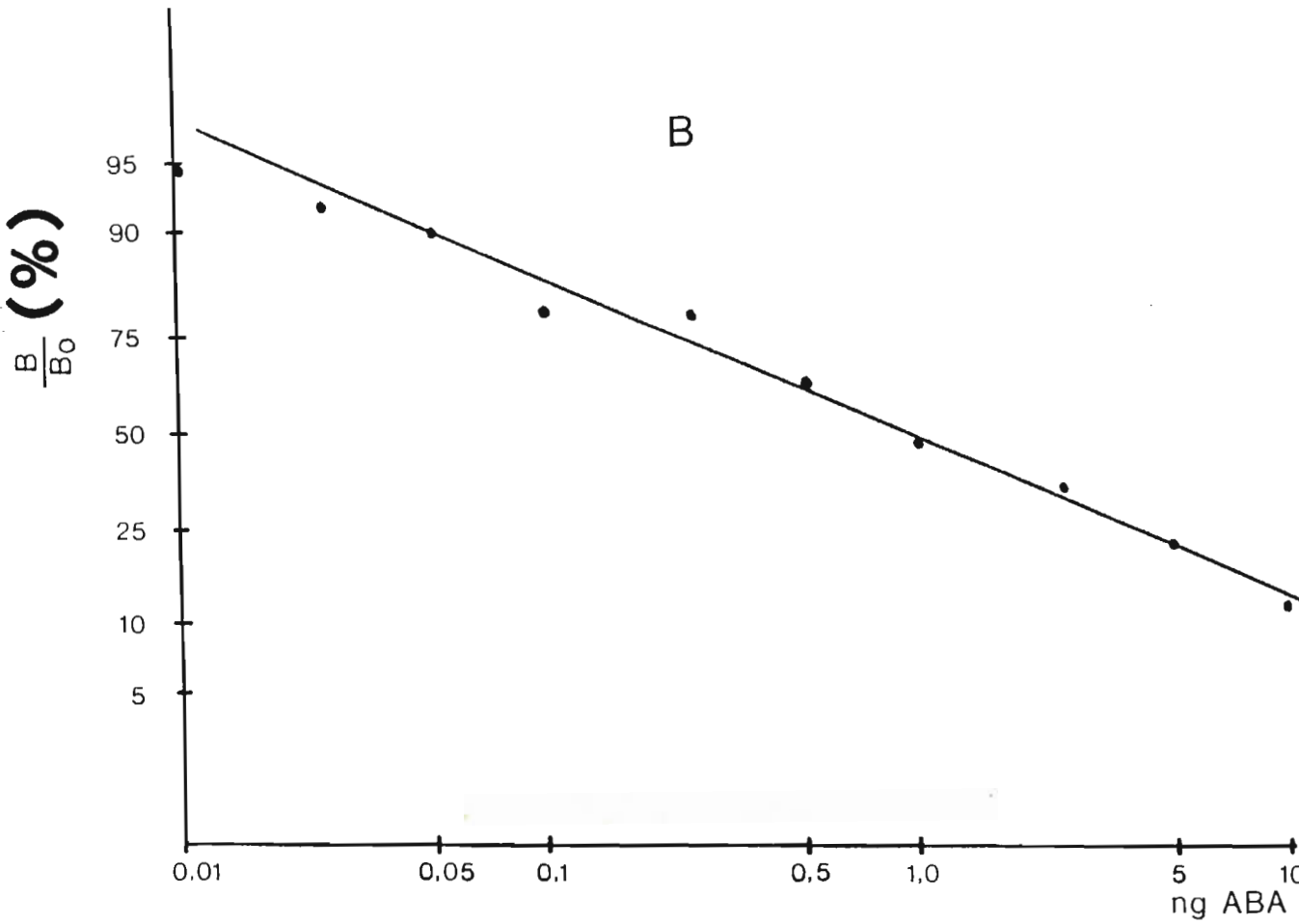
High NSB values and poor precision militated against the further consideration of the ammonium sulphate technique. In Fig. 3.7 a comparison of charcoal and ammonium sulphate as separation techniques and their effect upon the standard dose-response curve is presented.

Polyethylene glycol separation was good, with low NSB ranging from 3 to 6% (n = 4) of the total added tracer. However, regression analysis showed poor precision (r = 0,94), and this technique was not investigated further.

Fig. 3.6A Standard dose-response curve for ABA using ammonium sulphate precipitation as a separation technique. Precision for this separation technique was poor.

Fig. 3.6B Standard dose-response curve for ABA using ammonium sulphate as the separation technique. This curve was constructed using averaged data.


A
95
90
75
50
25
10
5
B/Bo (%)
0,01
0,05
0,1
0,5
1,0
5
10
ng ABA


B
95
90
75
50
25
10
5
B/Bo (%)
0,01
0,05
0,1
0,5
1,0
5
ng ABA

3.2.3.2 Effect of charcoal concentration

The charcoal dose-response curve is presented in Fig. 3.8. There was no concentration of charcoal that removed all the free (±)^{3}H-ABA and at the same time did not strip (±)^{3}H-ABA from the antibody complex. A compromise concentration was selected where stripping from the antibody complex was minimal and most of the free fraction removed. A charcoal concentration of 4mg per tube was used in all subsequent ABA RIA development and application.

3.2.3.3 Effect of pH

The effects of pH on the percentage binding are presented in Fig. 3.9. There was a marginal reduction in binding at pH 8,5. A buffer pH of 7,0 was used in the preparation of all primary reagents used in the ABA RIA.

3.2.3.4 Effect of incubation time

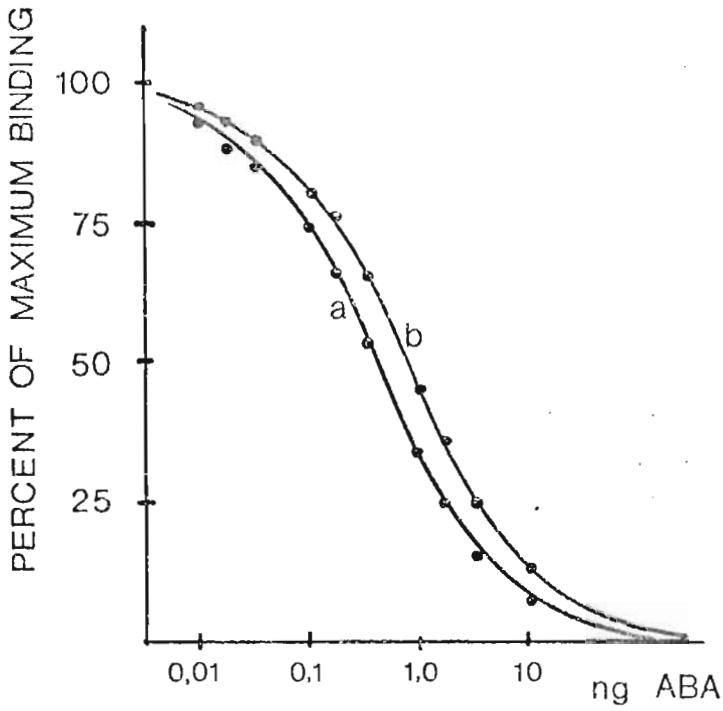
The incubation time-response curve is presented in Fig. 3.10 for serum from rabbit ABA-1. Maximum binding was achieved between 10 and 20 min, after which the antibody - antigen complex appeared to dissociate. An incubation time of 15 min was selected and this time was strictly adhered to. This relatively short incubation time led to consistently low NSB values, possibly because of reduced tracer damage.

3.2.3.5 Standard dose-response curve

Two typical standard dose-response curves for ABA are presented (Fig. 3.11), one with a normal slope and one with increased sensitivity (reduced binding and precision). Typically the assay had a measuring range from 25pg to 10ng.

Fig. 3.7 A comparison of dextran-coated charcoal (a) and ammonium sulphate as separation procedures for ABA and their effect upon the standard dose-response curve.

Fig. 3.8 Charcoal dose-response curve showing the percent of ^{3}H-ABA removed from the incubation volume (a) and the percent ^{3}H-ABA 'stripped' from the bound complex (b). A charcoal concentration of between 3 and 7mg per tube (c) removed all the free tracer without stripping tracer from the bound complex.


PERCENT OF MAXIMUM BINDING
100
75
50
25
a
b
0,01
0,1
1,0
10
ng ABA

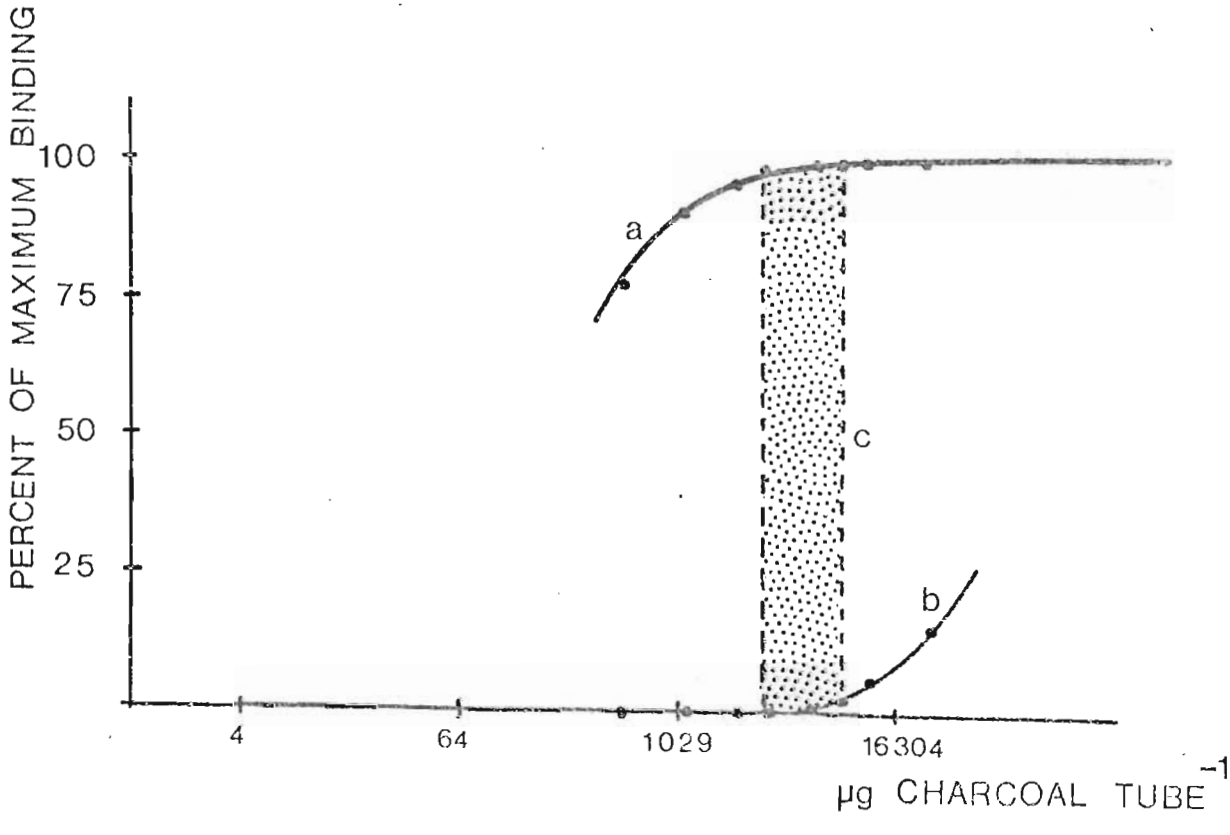
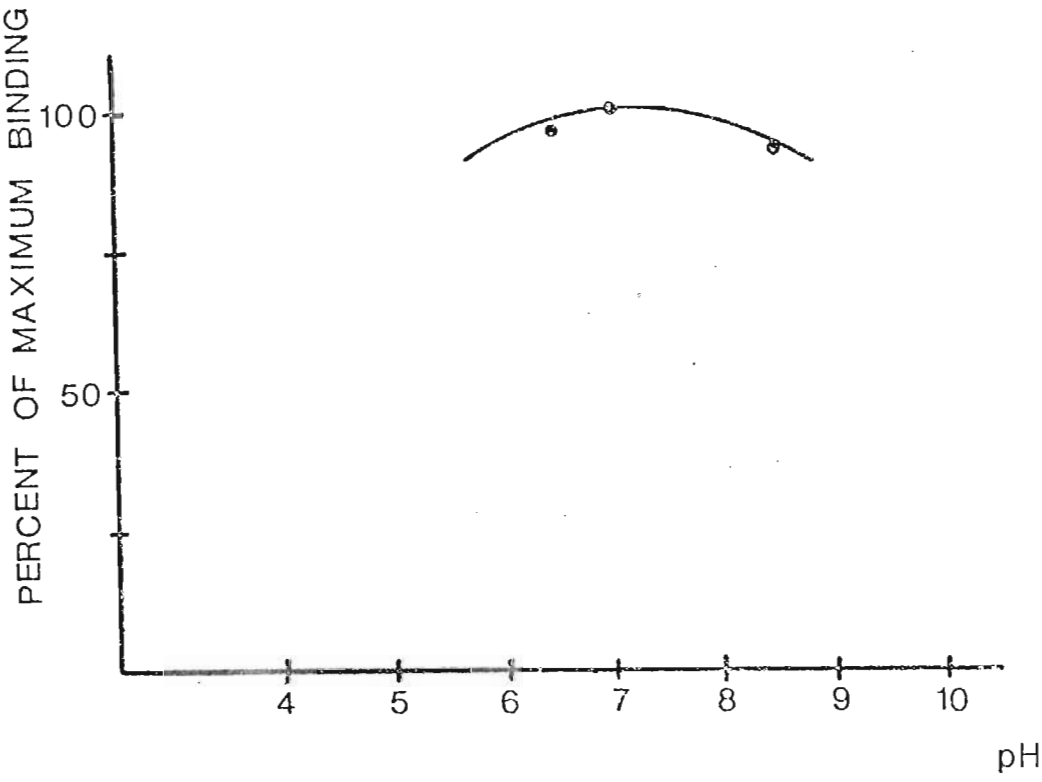

PERCENT OF MAXIMUM BINDING
100
75
50
25
a
b
c
4
64
1029
16304
µg CHARCOAL TUBE^{-1}

Fig. 3.9 The effect of the incubation pH on the percentage binding of anti-ABA-1.

Fig. 3.10 The incubation time-response curve for serum from rabbit ABA-1 at an incubation temperature of 37°C.


PERCENT OF MAXIMUM BINDING
100
50
4
5
6
7
8
9
10
pH

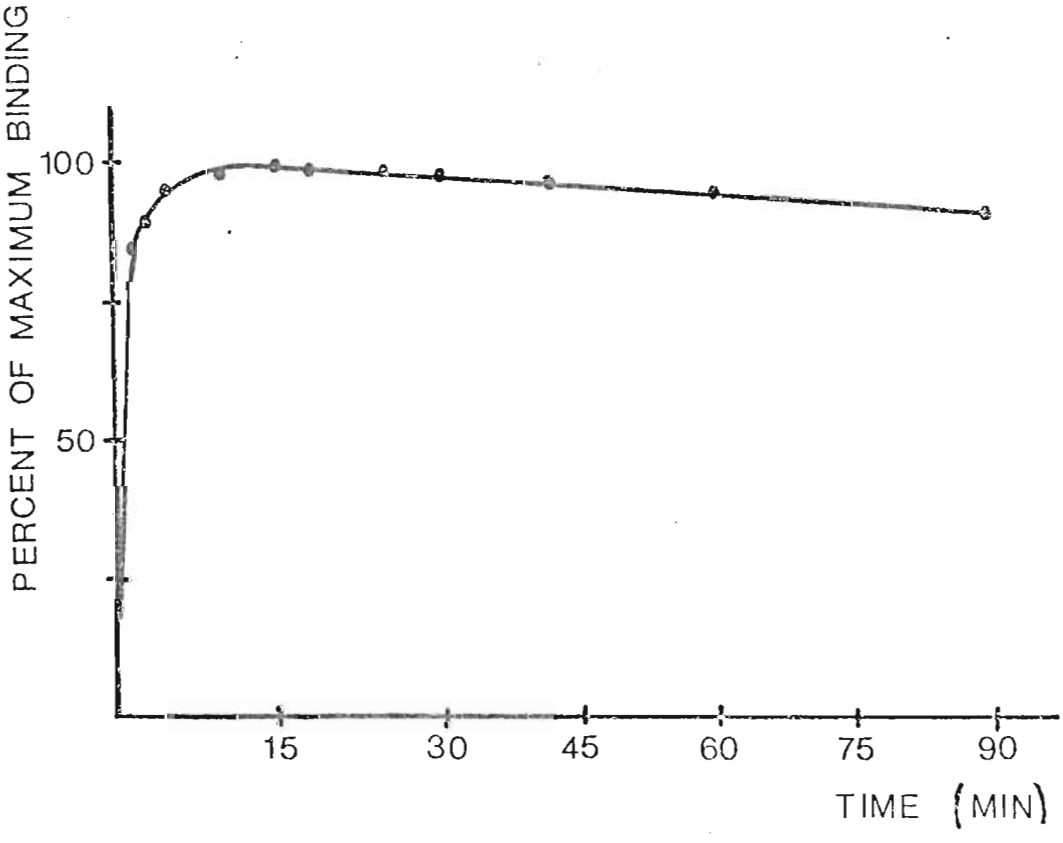
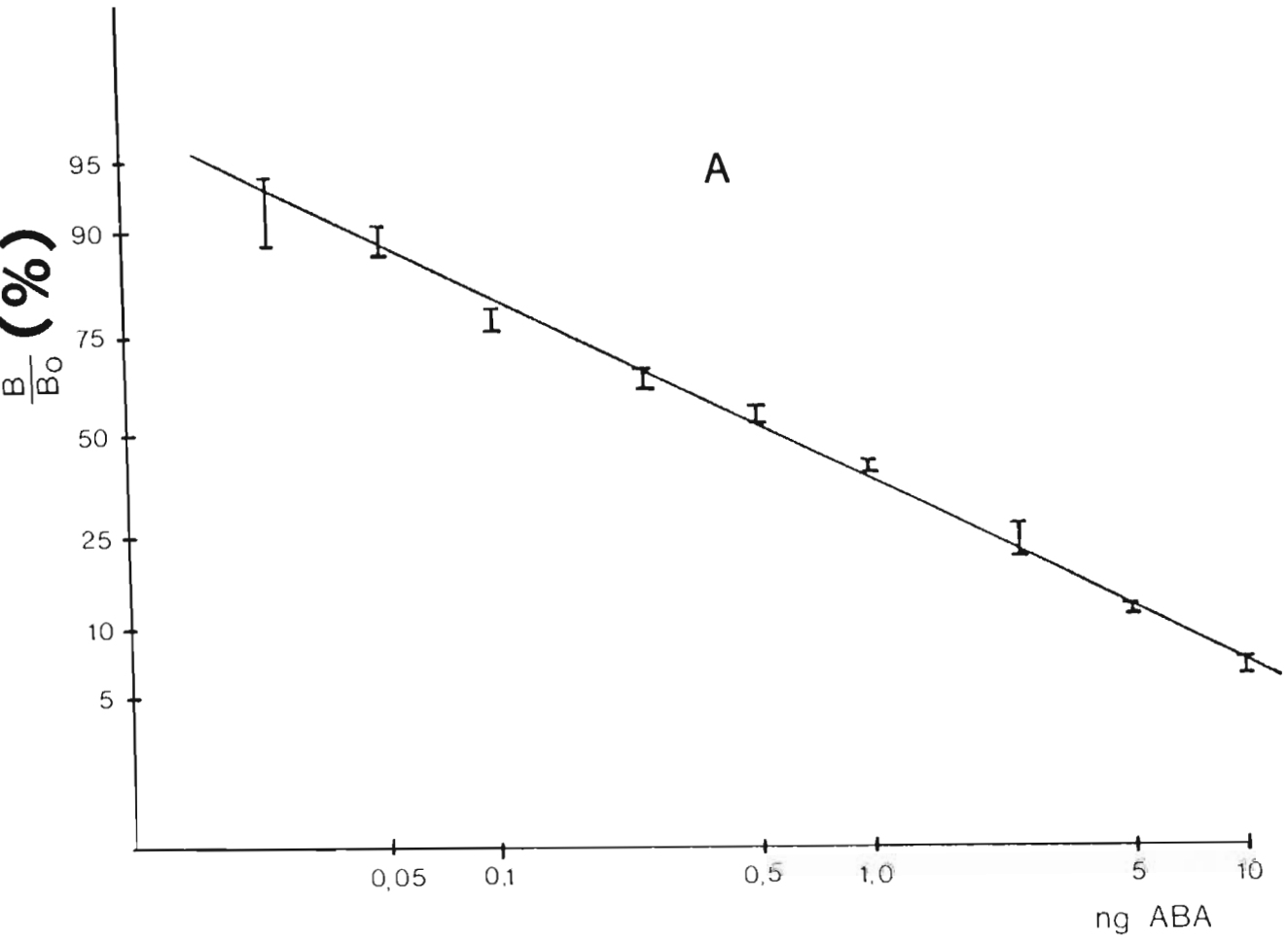
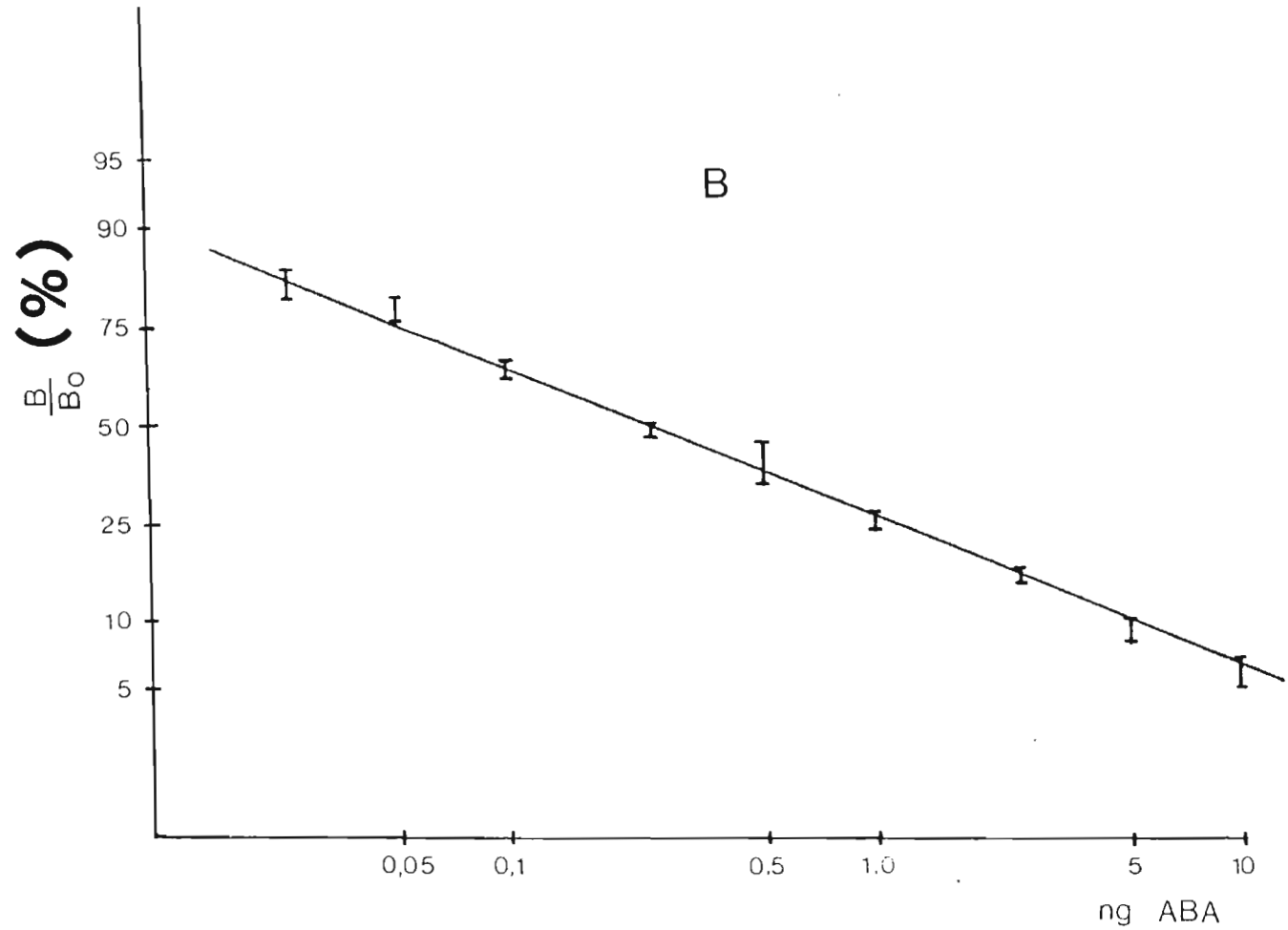

PERCENT OF MAXIMUM BINDING
100
50
15
30
45
60
75
90
TIME (MIN)

Fig. 3.11 Two typical standard dose-response curves for ABA, one with increased sensitivity and reduced precision (B) and the other with reduced sensitivity and increased precision (A).


A
95
90
75
50
25
10
5
B/Bo (%)
0,05
0,1
0,5
1,0
5
10
ng ABA


B
95
90
75
50
25
10
5
B/Bo (%)
0,05
0,1
0,5
1,0
5
10
ng ABA

3.2.4 Validation

3.2.4.1 Extract dilution curves

This RIA did not appear to be affected by contaminants present in the crude extracts. Data from the crude extract dilution showed that the curve was shallower than that of the standard (Fig. 3.12). However, the addition of standard ABA to the extract appeared to restore the slope to that of the standard dose-response curve. Results for ABA after TLC were very similar for the extract whether or not it had been subjected to a solvent partitioning cleanup. Therefore, only data from the solvent partitioned extract are presented (Fig. 3.13). The dilution curve for free ABA was again shallower than the standard dose-response curve.

3.2.4.2 Cross-reactivities to Anti-ABA-1

From the results in Table 3.2 it can be seen that the serum from rabbit ABA-1 was reactive with (±)ABA and both the methyl and glucosyl esters. However, none of the other compounds that were tested had more than 1% cross-reaction. This serum could therefore be used to quantitate any of the three ABA forms for which it exhibits high affinity.

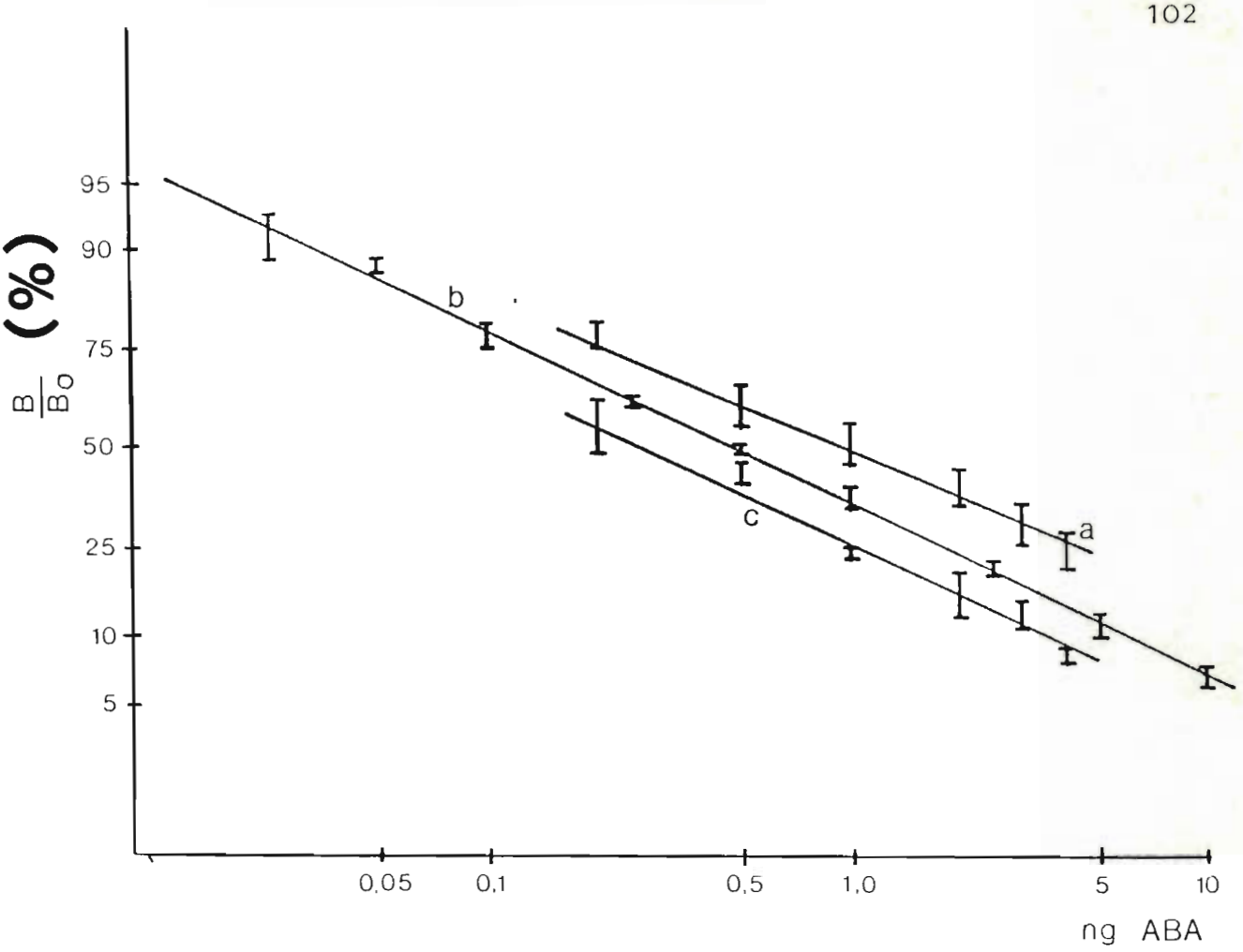
3.3 Discussion

ABA is known to occur in relatively high concentrations in the plant (Brenner, 1981). Due to the sensitivity of the ABA RIA, it was considered adequate for the detection of physiological levels of ABA. Small quantities of biological material could be used for the determinations, and the assay was relatively robust under normal laboratory conditions.

This RIA for ABA exhibited both positive and negative characteristics. The type of conjugate yielded by the

Fig. 3.12 Extract dilution curves for, a crude extract prior to TLC (a), authentic (±)ABA standard (b) and extract plus authentic standard (c).

Fig. 3.13 Extract dilution curves for, a solvent partitioned avocado extract after TLC (a), authentic (±)ABA standard (b) and purified extract plus authentic standard (c).


B/B0 (%)
95
90
75
50
25
10
5
a
b
c
0,05
0,1
0,5
1,0
5
10
ng ABA

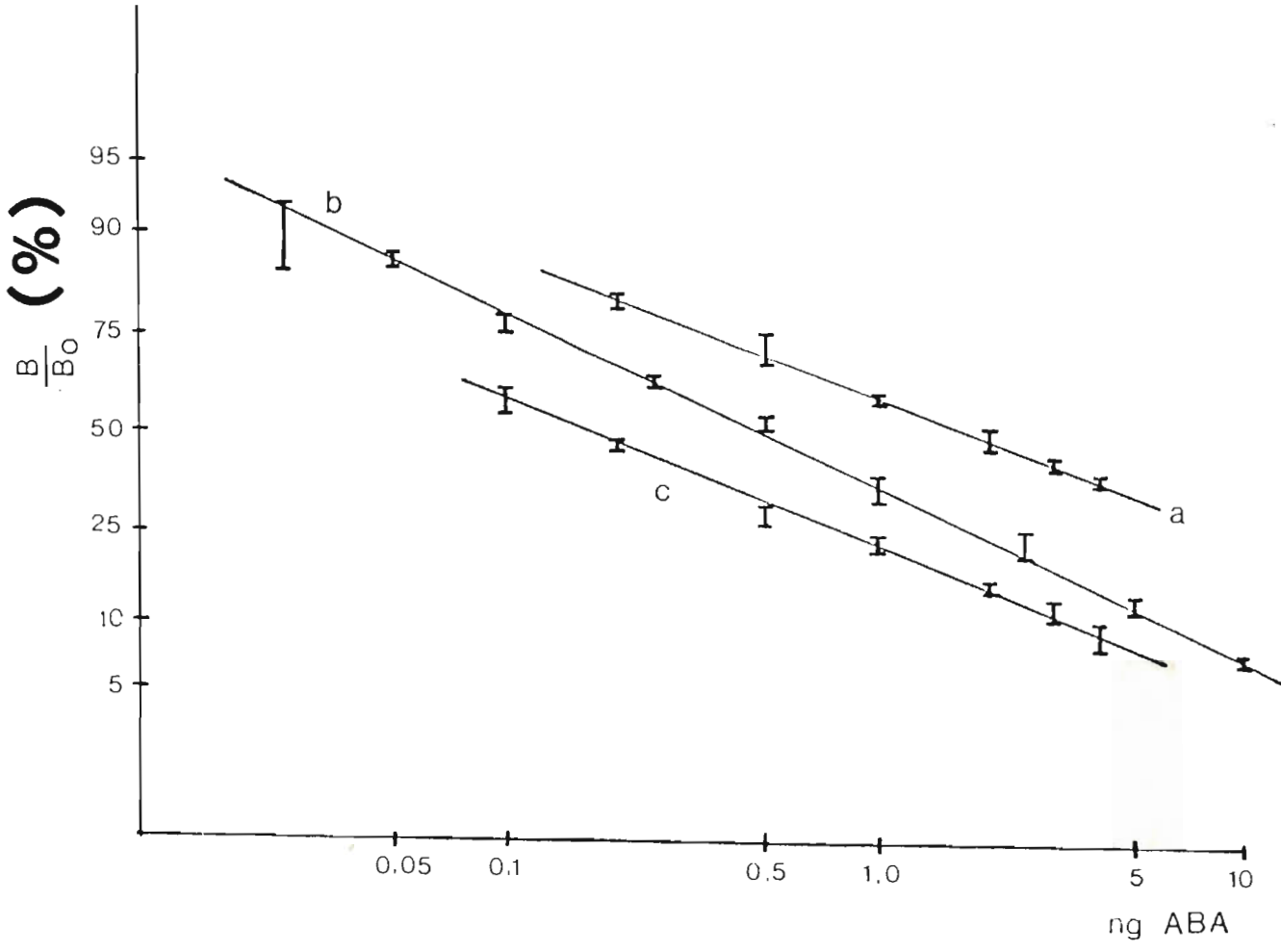

B/B0 (%)
95
90
75
50
25
10
5
a
b
c
0,05
0,1
0,5
1,0
5
10
ng ABA

Table 3.2 Cross-reaction of various ABA derivatives and similarly structured compounds to Anti-ABA-1 on a mass basis

Compound	% Cross-reaction
(±)ABA	100
(±)ABA glucosyl ester	100
(±)ABA methyl ester	142
Phaseic acid	0,2
Dihydrophaseic acid	0,1
(±)ABA mixed isomers	44
Farnesol	0

linking method gave rise to an antibody that was very specific for certain portions of the ABA molecule, but was totally non-selective for the carboxyl group. Because the carboxyl group on ABA was used as the site of attachment, this meant that the site was not recognized by the resultant antibody. Compounds with the basic ABA configuration but, esterified via the carboxyl group were therefore recognized as ABA. The antibody in fact actually displayed enhanced affinity for (±)ABA methyl ester. Structural changes to any other part of the molecule resulted in a near total loss of activity. It would thus appear that most of the ABA molecule with the exception of the carboxyl group was involved in the recognition response.

The conjugate had a high molar ratio of (±)ABA to BSA but a final titer dilution of over 1:500 was never achieved. Two possible reasons were that the ABA molecule is not very immunogenic, or that the ABA molecule was held within the folds of the BSA structure and the rabbit immune systems were never really challenged by ABA. An area that could

have been improved was serum production, but this would take excessive time as long rests between immunizations are important in the enhancement of titer (Hurn & Landon, 1971) and avidity (Parker, 1971).

Alternative conjugation sites on the ABA molecule have been investigated (Weiler, 1980b). These gave improved titer and specificity. More recently (Weiler, 1982b), the trend has been toward a carboxyl-linked conjugate and the separation of the various components prior to quantitation by immunoassay.

The ABA antibody appeared to be more robust than the other antibodies tested (Anti-IPA and Anti-IAA) and was not affected by contaminants except excessive avocado oil. However, the dissociation of the antibody complex with time was unusual and worrying in that a disequilibrium assay was developed. This phenomenon was not observed by other researchers working with immunoassays for ABA (Walton *et al.*, 1979; Weiler, 1979, 1980b, 1982b). Of the separation procedures tested, dextran-coated charcoal proved superior. As this technique is very rapid and simple, it is surprising that it is not implemented by other groups researching PGS RIAs.

In this study the rabbits produced antiserum against (±)ABA, which was also used in the construction of the standard dose-response curve. However, plants only contain (±)ABA (Weaver, 1972), and this could account for the non-parallelity of the serial dilution curves. It would appear that there is a difference in the affinity of the antibody for the two isomers and that the antibody exhibits less affinity for (±)ABA. However, this difference in affinity is marginal, as indicated by the linearity of the dilution curves. A solution would be to immunize with a (±)ABA-BSA conjugate, but costs militate against this at present.

As with the IPA RIA, the preparation of the biological material prior to RIA was the most time-consuming step. Some form of isolation was necessary due to the high cross-reactivity of the various ABA esters and the necessity to quantitate the free active ABA component. This was achieved by solvent partitioning and TLC. Recoveries were high and consistently around 80%. In nearly all cases it appeared that the antibody was unaffected by the high oil content present in avocado fruit extracts. Therefore, this assay could be used for most, if not all, plant extracts for the quantitation of free and conjugated forms of ABA.

CHAPTER 4
RADIOIMMUNOASSAY FOR INDOLE-3-ACETIC ACID

4.1 Materials and Methods

As with ABA, excessive repetition of methodology is avoided by reference to the relevant section in Chapter 2. Only differences that exist are described.

4.1.1 Conjugation of IAA to BSA

The literature search indicated that several options were available for the conjugation of IAA to BSA. Three different methods were selected which would yield conjugates having different sites of attachment to the carrier protein. The proposed immune response would differ and this would enable a comprehensive serum evaluation.

The method of Ranadive & Sehon (1967a) was modified slightly. This method would yield a conjugate with a peptide bond between the carboxyl group on IAA and the carrier protein. Three hundred mg IAA (Sigma Chemical Co.) was dissolved in 5ml of dioxane, followed by the addition of 350mg N,N´-dicyclohexylcarbodiimide. The solution was stirred for one hour at room temperature. The resultant precipitate was pelleted by centrifugation (2 000g at 4°C for 10 min). The clear supernatant was added dropwise to a solution of 500mg of BSA dissolved in borate buffer (pH 8,5; 0,15M). Stirring was maintained throughout the addition of the supernatant and continued for a further 24 hours at 4°C in the dark. The resultant solution was dialysed against a 1% solution of sodium bicarbonate for 24 hours, followed by a further 48 hour dialysis against a 0,15M borate buffer (pH 8,0). The solution was finally dialysed against distilled water for 24 hours, lyophilized and stored at -20°C.

The second method of conjugation was as above but using N-ethyl-N(3-dimethylaminopropyl)-carbodiimide-HCl (Bauminger & Wilchek, 1980), which was added in four portions of 50mg each with a 30 min interval between each addition. The IAA was initially dissolved in borate buffer (pH 8,5; 0,15M) as the ethyl carbodiimide is water soluble. Purification and lyophilization of the conjugate was as above.

The third method of conjugation achieved linkage utilizing a formaldehyde reaction which caused the formation of a covalent bond between the active nitrogen in IAA and the BSA carrier (Ranadive & Sehon, 1967b). This left the carboxyl group on IAA free. Five hundred mg of IAA was dissolved in 15ml borate buffer (pH 8,5; 0,05M). During dissolution the pH was maintained at 8,5 by additions of 1M KOH. After dissolution the pH was reduced to 7,0 with 1N HCl. Five hundred mg BSA was dissolved in 5ml distilled water followed by the addition of 4ml 3N sodium acetate and 5ml 7,5% formaldehyde solution with stirring. The IAA solution was then added dropwise to the stirred BSA reaction mixture. Further stirring took place for 24 hours in the dark under nitrogen at room temperature. Dialysis and lyophilization were as for the two carbodiimide methods.

The same procedure was used for determination of the linking ratios of the three IAA-BSA conjugates. Extinction coefficients were from Ranadive & Sehon (1967a). Solutions were prepared in borate buffer (pH 8,0; 0,15M). Standard IAA (2,66mg) was dissolved in 100ml buffer and 20mg quantities of the conjugates and BSA were each dissolved in 20ml buffer. The UV absorbance of all the conjugates was read at 295nm. The linking ratio was calculated using these data and the extinction coefficients.

4.1.2 Production of antiserum

4.1.2.1 Immunization

Ten 12 to 16 week old rabbits were selected for IAA immunization. Four rabbits were inoculated with the formaldehyde preparation while three rabbits were used for each of the carbodiimide prepared conjugates. The equivalent of 1mg of conjugate per rabbit was taken up in 1ml borate buffer (pH 8,0) and emulsified with Freunds complete adjuvant. The preparation of rabbits and immunization was as described earlier (refer to 2.1.2.1). Despite the good conjugate linkage ratios and the number of rabbits immunized, sera of a usable quality were never produced. It was therefore decided to inoculate sheep.

Four SA mutton merino wethers were selected and immunized with the formaldehyde-prepared conjugate. The sheep each received the equivalent of 2mg conjugate in a stable water-in-oil Freunds emulsion of 5ml. Subcutaneous injections on the inside of the hind legs were given to each animal in August, 1983. Animals were bled by jugular puncture 30 days later and then re-immunized using a Freunds incomplete based emulsion. Test bleeds for serum evaluation were drawn every week for 5 weeks, after which the sheep were rested. The animals were re-inoculated in December and bled 10 days later.

4.1.2.2 Purification and storage

The freshly drawn blood was allowed to stand for 2 hours in a water bath at 37°C for clot formation and contraction. The clot was separated from the wall of the collection tube using a metal spatula. Serum was decanted into 4,5ml tubes and centrifuged (2 000g at 4°C for 20 min) and the cleared supernatant removed using a Pasteur pipette. Serum was diluted 1:5 in 0,1% gelatine in PBS (pH 7,0) and stored at

4°C.

4.1.2.3 Titer determination

Titer determination was done by RIA. Anti-IAA serum was further diluted in 0,1% gelatine in PBS. Dilutions which ranged from 1:5 to 1:80 were prepared and the percentage binding relative to the total activity added to the system determined. The procedure was as for a standard RIA for IAA in the absence of standard or biological sample (refer to 4.1.3.2). Anti-IAA serum titer curves were established and all titers given are relative to 30% binding of the total ^{3}H-IAA added to the system.

4.1.3 Radioimmunoassay

4.1.3.1 Optimization

4.1.3.1.1 Effect of separation technique

Three methods of separation were assessed for IAA. The ^{3}H-IAA isotope was very unstable, therefore it was important that the separation technique was rapid and efficient.

Dextran-coated charcoal was the first method tested. A suspension of 500mg charcoal, 500mg dextran and 100ml distilled water was prepared using a magnetic stirrer, and the suspension temperature reduced to 4°C. After stabilization of the primary reaction mixture (refer to 4.1.3.2), an 800µl aliquot of the charcoal mixture was added to each assay tube. After vortexing the solution was allowed to stand for 10 min (4°C) and then centrifuged (2 000g at 4°C for 10 min). The supernatant was carefully decanted into counting vials to which 12,5ml of scintillant had been added. The radioactivity in the bound fraction was determined.

Ammonium sulphate precipitation was the second separation procedure evaluated. After incubation and stabilization of the primary reaction mixture (refer to 4.1.3.2), 300µl of a 90% saturated solution of ammonium sulphate at 4°C was added to each assay tube. After vortexing, the tubes were allowed to stand for 30 min at room temperature to allow the antibody to precipitate. The mixture was centrifuged (2 000g for 30 min at 4°C) and the supernatant (free fraction) assessed for activity. The precipitate (bound fraction) was dissolved in 400µl distilled water and assessed for activity. Data could be presented from either the free or the bound fraction.

Polyethylene glycol was the third separation procedure evaluated. A 20% (m/v) solution of polyethylene glycol 6 000 was prepared in PBS (pH 7,0) and stored at 4°C. The general procedure was very similar to that for ammonium sulphate except that 600µl of polyethylene glycol solution was added instead of the 300µl ammonium sulphate. Data from the free fraction was used to construct the standard dose-response curve.

4.1.3.1.2 Effect of charcoal concentration

Charcoal concentrations tested ranged from 1 to 20mg per tube. After incubation and stabilization of the primary reaction mixture (refer to 4.1.3.2), the various concentrations of charcoal were added to the relevant assay tubes. The addition of the charcoal solution and subsequent procedure were as described in 2.1.3.1.1.

4.1.3.1.3 Determination of incubation time

After addition of the primary assay reagents, various incubation times from 3 to 60 min were tested in a water bath at 37°C. After stabilization in ice for 60 min, 800µl of

charcoal slurry was added to each tube prior to centrifugation (2 000g for 10 min at 4°C). The bound fraction was assessed for activity. Results were calculated as a percentage of the highest value. The assumption was zero binding at zero time.

Due to the high NSB values encountered it was decided to dispense with a heated incubation and to test an incubation at 4°C for 12 to 15 hours. Except for this change the procedure was as above.

4.1.3.1.4 Effect of pH

Four different buffer pHs were tested, viz. three PBS buffers of 6,5, 7,0 and 7,4 and a borate buffer of 8,5. General procedure was the same as in 4.1.3.2. Results were calculated as the percentage binding of the highest binding value.

4.1.3.1.5 Effect of methanol

Various amounts of methanol were added to the primary reaction mixture prior to the addition of antibody and after the addition of buffer to test the effect upon antibody binding. Amounts of methanol tested ranged from 10µl to 200µl per tube (3,2 to 40% of the incubation mixture by volume). General procedure was the same as in 4.1.3.2. Results were calculated as the percentage reduction in binding of the zero level.

4.1.3.2 Standard procedure

After completion of the various parameter tests an optimized RIA procedure for IAA was formulated. Standard equipment was the same as for IPA (refer to 2.1.3.2).

All assay parameters were duplicated. Standard concentrations of IAA which ranged from 100pg to 500ng per 100µl or biological sample suspended in methanol were dispensed into the relevant assay tubes. The typical assay format used was similar to that presented in Table 2.1. Protein coating of the assay tubes and dispensing of the primary assay reagents was as in 2.1.3.2. After vortexing, assay tubes were incubated at 4°C for 15 hours and the tubes were then again vortexed.

Five hundred µl of dextran-coated charcoal (250mg charcoal and 500mg dextran slurried in 50ml distilled water) at 4°C was added to all assay tubes except those designated for Ta (Ta tubes had 800µl distilled water added). Centrifugation and preparation of the bound fraction for the determination of activity (refer to 2.1.3.2) and statistical analysis and calculation of results was as for IPA and 2iP (refer to 2.1.3.3).

4.1.4 Validation

4.1.4.1 Extract dilution curves

Two different extract dilution curves were run in order to detect the presence of any potentially interfering compounds in the biological extracts, and to determine if the antibody was able to distinguish between standard and biological ligand. A 40g sample of young avocado fruit material was homogenized and extracted in 90% methanol acidified with 1% acetic acid. Dilution curves were established for crude extracts as well as extracts that had undergone solvent partitioning (refer to 4.1.5.2).

After extraction for 48 hours in the dark at 4°C the sample was centrifuged (1 000g for 10 min at 4°C) and the supernatant split into two fractions and treated as follows :

(a) A crude fraction was suitably diluted and assayed directly to establish a crude extract dilution curve.

(b) The same as for (a) but with the addition of 250ng IAA standard.

(c) A crude fraction was solvent partitioned to remove possible contaminants and other interferants from the sample. Thereafter suitable dilutions were made and the sample subjected to RIA.

(d) The same as for (c) but with the addition of 250ng IAA prior to solvent partitioning.

The standard RIA procedure for IAA was used (refer to 4.1.3.2) for the establishment of dilution curves. Results for (a) and (b) were plotted on standard graph paper, while (c) and (d) were plotted on log-logit probability paper.

4.1.4.2 Antibody selectivity

Various IAA-related compounds and precursors were assessed for cross-reactivity. From this it was hoped to establish which components of the IAA molecule were involved in the recognition response. Ten mg standard preparations of the various compounds were individually suspended in 10ml of absolute methanol (refer to Table 4.2 for compounds tested). Dilutions used in this study ranged from 100pg to 10 000ng per 100µl.

The standard RIA procedure was followed (refer to 4.1.3.2) and cross-reactivity curves were plotted on 6 cycle log-logit probability graph paper. The percentage cross reactivity was calculated relative to 50% inhibition of

binding of ^{3}H-IAA on a mass basis.

4.1.5 Biological sample preparation

Unless otherwise stated the procedure described below was used as standard for the preparation of biological samples for measurement of IAA by RIA. Samples were rapidly frozen and stored at -20°C until required.

4.1.5.1 Extraction

Organic solvents such as chloroform, ethanol, methanol and diethyl ether can and have been used for auxin extraction (Weaver, 1972). Nitsch (1956) recommended the use of methanol as an excellent solvent for IAA extraction. Ten g samples were homogenized in 50ml 90% methanol acidified with 1% acetic acid and extracted for 24 hours at 4°C in the dark. After centrifugation (1 000g for 10 min at 4°C) the supernatant containing the extracted IAA was purified.

4.1.5.2 Purification

After the extract is obtained, preliminary purification is usually achieved using solvent partitioning (Weaver, 1972). A modification of the method of Dreher & Poovaiah (1982) was used.

Twenty five ml of extract (equivalent 5g fresh mass) was added to 20ml of potassium phosphate buffer (pH 8,0; 0,5M) and reduced to the aqueous phase under vacuum at 25°C. The aqueous phase was placed in a separating funnel and extracted with 40ml diethyl ether (2 x 20ml). The lower aqueous phase was retained and acidified to pH 3,0 with 2N HCl. After further extraction with diethyl ether (2 x 20ml) the ether phase was retained, reduced to 5ml

under vacuum at 30°C and stored at -20°C prior to RIA.

4.2 Results

4.2.1 IAA-BSA conjugates

The UV absorbance of the three conjugates and standards at 295nm is presented in Table 4.1.

Table 4.1 UV absorbance of IAA-BSA conjugates and standards at 295nm

Compound	Absorbance
IAA-BSA formaldehyde-catalysed conjugate	0,394
IAA-BSA ethyl carbodiimide-catalysed conjugate	0,0662
IAA-BSA cyclohexyl-carbodiimide catalysed conjugate	0,0776
IAA standard	0,667
BSA standard	0,030

The linking ratio was calculated as follows :

$$\frac{\text{Absorbance} \times 1\,000}{\text{Concentration (mg\%)}} = \text{extinction coefficient}$$

$$\text{Concentration (mg\%)} = \frac{\text{absorbance} \times 1\,000}{\text{extinction coefficient}}$$

(a) Formaldehyde-catalysed conjugate :

$$\text{Concentration IAA} = \frac{(0,394 - 0,030) \times 1\,000}{248}$$

$$= 1,47\text{mg } 100\text{ml}^{-1}$$

$$= 8,38 \times 10^{-5} \text{ mol } l^{-1}$$

Concentration BSA $= \frac{0,030 \times 1\,000}{1,19}$

$= 25,2\text{mg } 100\text{ml}^{-1}$

$= 3,6 \times 10^{-6} \text{ mol } l^{-1}$

Ratio IAA:BSA $= \frac{83,8 \times 10^{-6}}{3,6 \times 10^{-6}}$

$= 23,3:1$

(b) Ethyl carbodiimide-catalysed conjugate :

Concentration IAA $= \frac{(0,0662 - 0,030) \times 1\,000}{248}$

$= 0,146\text{mg } 100\text{ml}^{-1}$

$= 8,3 \times 10^{-6} \text{ mol } l^{-1}$

Concentration BSA $= 3,6 \times 10^{-6} \text{ mol } l^{-1}$

Ratio IAA:BSA $= \frac{8,3 \times 10^{-6}}{3,6 \times 10^{-6}}$

$= 2,31:1$

(c) Cyclohexyl carbodiimide-catalysed conjugate :

Concentration IAA $= \frac{(0,0776 - 0,030) \times 1\,000}{248}$

$= 0,182\text{mg } 100\text{ml}^{-1}$

$= 1,09 \times 10^{-5} \text{ mol } l^{-1}$

Concentration BSA $= 3,6 \times 10^{-6} \text{ mol } l^{-1}$

Ratio IAA:BSA $= \frac{10,9 \times 10^{-6}}{3,6 \times 10^{-6}}$

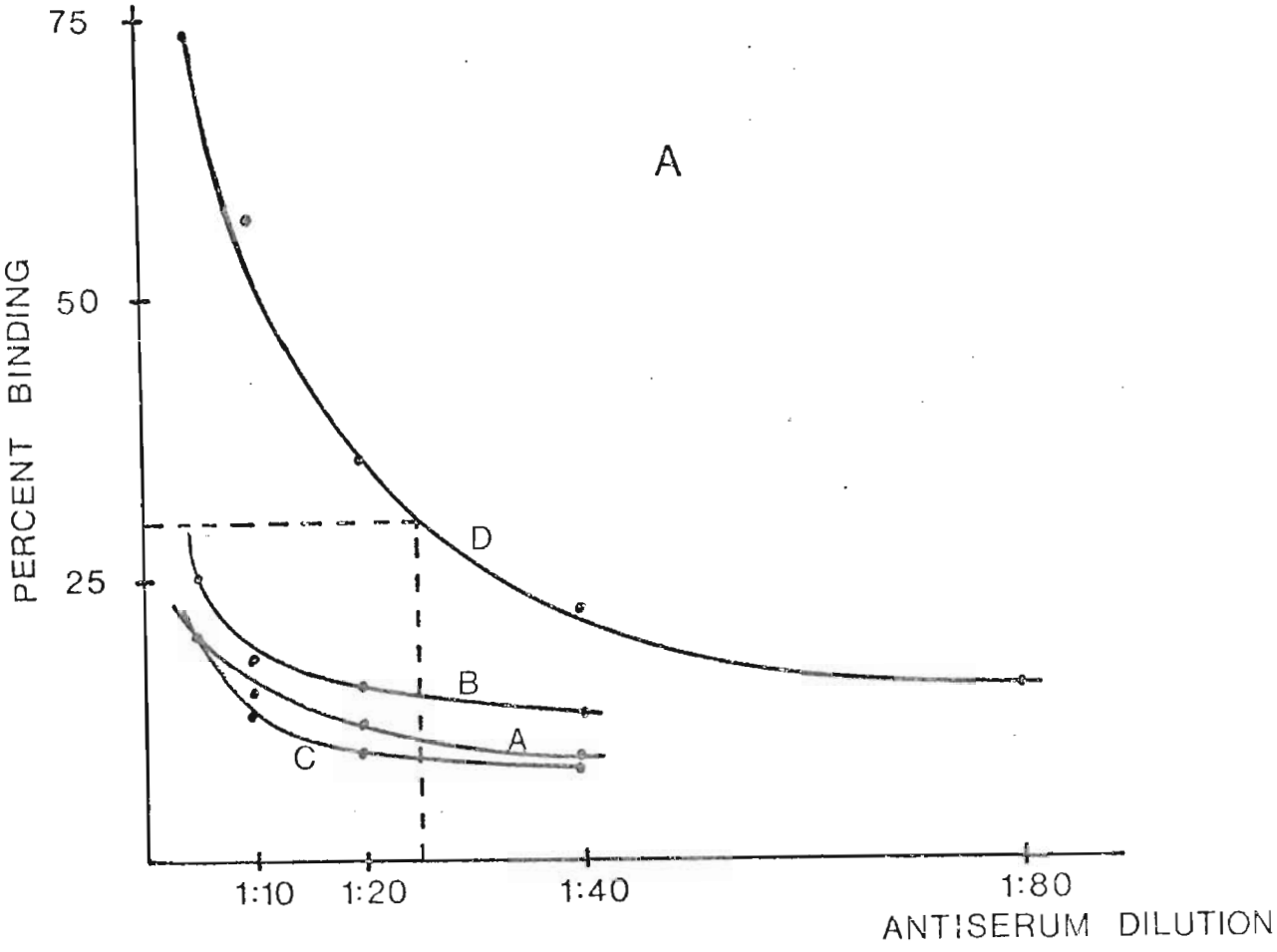
$= 3,0:1$

4.2.2 Response to immunization

None of the rabbits inoculated with the various IAA-BSA

Fig. 4.1A Antiserum dilution curves from sheep IAA-75 bled on the following days; 1983:11:07 (A), 1983:11:24 (B), 1983:12:19 (C) and 1983:12:14 (D).

Fig. 4.1B Antiserum dilution curves from sheep IAA-79 bled on the following days; 1983:11:07 (A), 1983:11:24 (B), 1983:12:19 (C) and 1983:12:14 (D).


A
75
50
25
PERCENT BINDING
D
B
A
C
1:10
1:20
1:40
1:80
ANTISERUM DILUTION

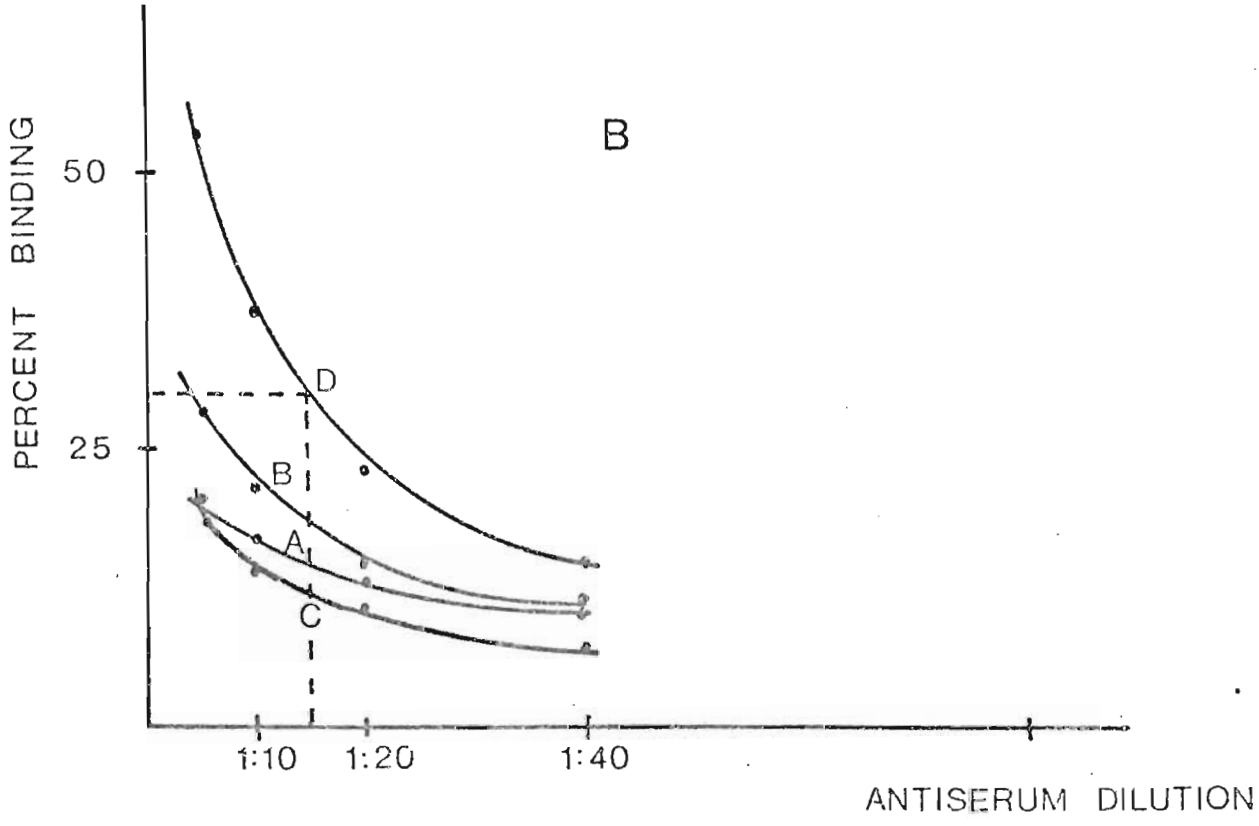
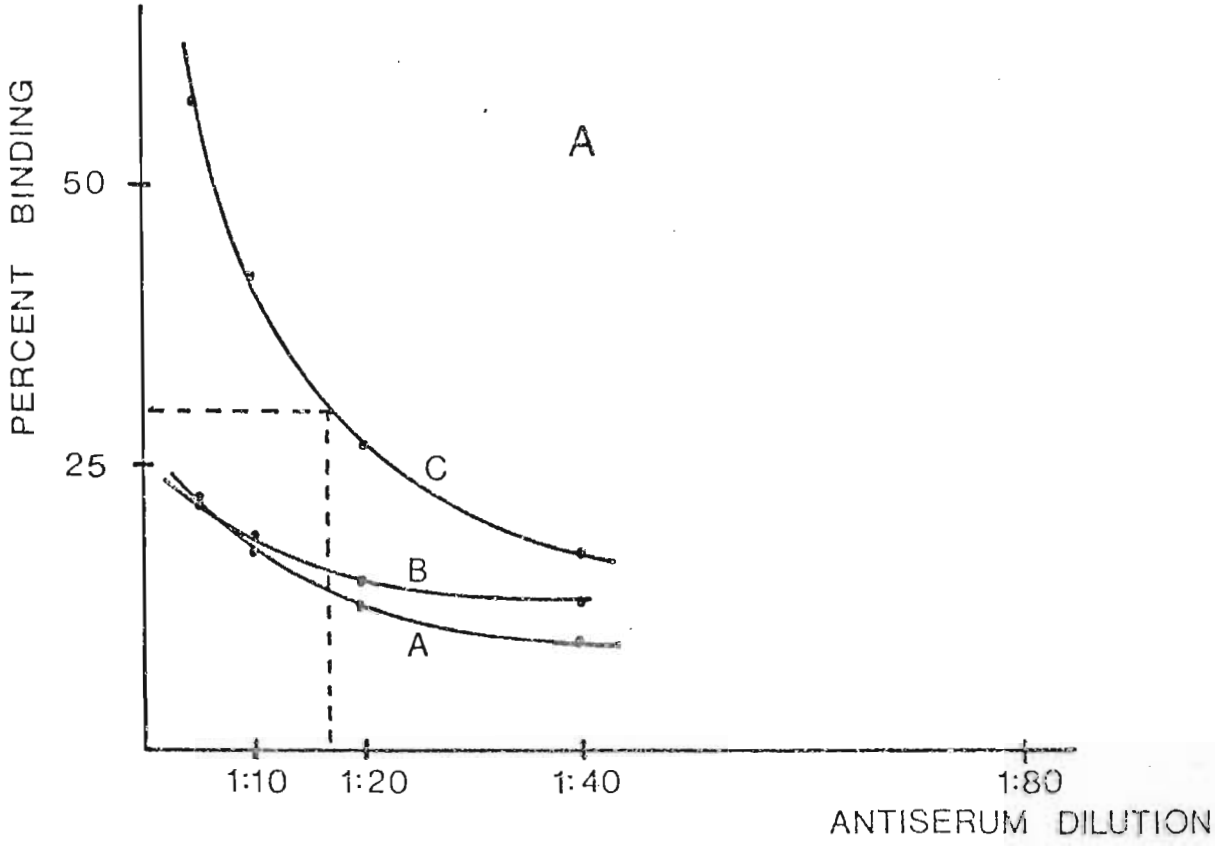
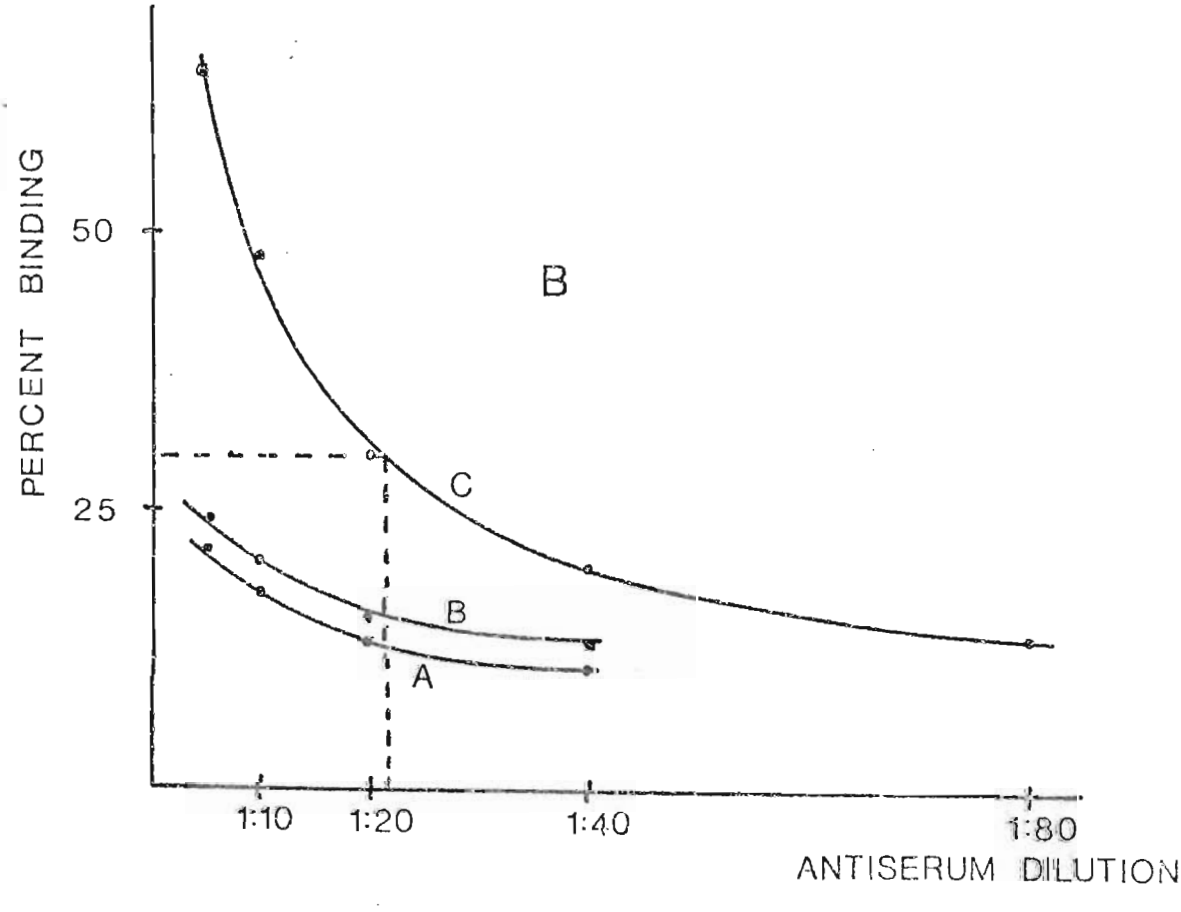

B
50
25
PERCENT BINDING
D
B
A
C
1:10
1:20
1:40
ANTISERUM DILUTION

Fig. 4.2A Antiserum dilution curves from sheep IAA-112 bled on the following days; 1983:11:07 (A), 1983:11:24 (B) and 1983:12:14 (C).

Fig. 4.2B Antiserum dilution curves from sheep IAA-115 bled on the following days; 1983:11:07 (A), 1983:11:24 (B) and 1983:12:14 (C).


A
PERCENT BINDING
50
25
C
B
A
1:10
1:20
1:40
1:80
ANTISERUM DILUTION


B
PERCENT BINDING
50
25
C
B
A
1:10
1:20
1:40
1:80
ANTISERUM DILUTION

conjugates produced sera of usable quality. Therefore only results from the sheep are presented (Figs. 4.1 & 4.2). The sheep responded similarly to the immunization, with sheep IAA-75 producing serum with a superior titer. Serum from this animal was used in the present study. The RIA for IAA was run at an initial dilution of 1:25 (final dilution of 1:75) which bound 30% of the total added tracer.

4.2.3 Radioimmunoassay

4.2.3.1 Effect of separation technique

Dextran-coated charcoal gave separation adjudged good. Regression analysis indicated good precision (r = 0,98) and the assay was suitable for linearization using the logit transformation (Fig. 4.3). NSB was low and ranged from 1,0 to 4,0 (n = 24) of the total tracer added to the system. The charcoal did not reduce the titer of the antiserum. The slope of the assay was relatively flat and the assay unusually sensitive.

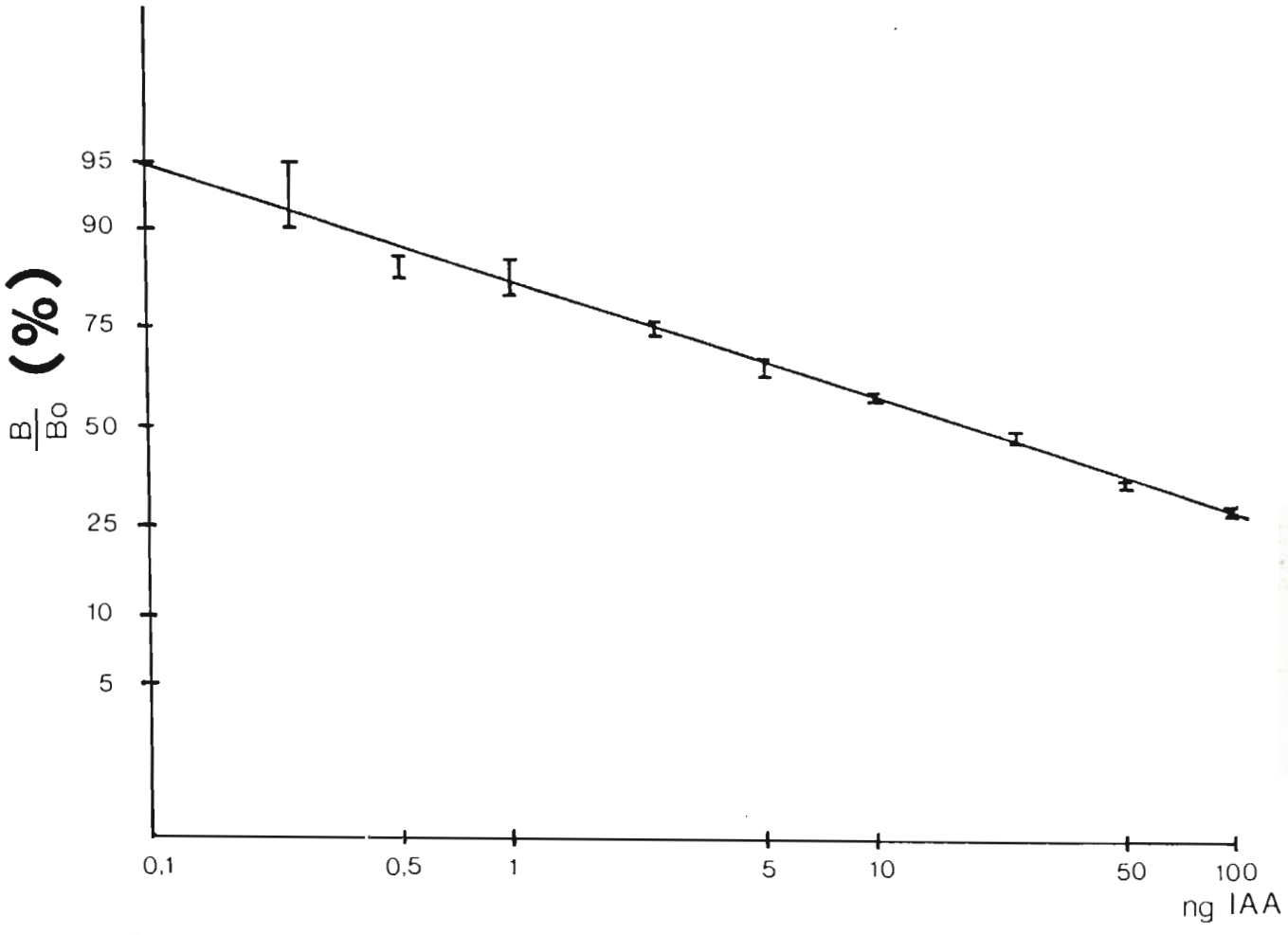
Separation of bound and free fractions using ammonium sulphate or polyethylene glycol precipitation was poor, with regression values of 0,94 and 0,93 respectively, indicative of poor precision. NSB values were high and ranged from 3 to 10% (n = 4) for both procedures. For these reasons, neither of the two techniques was investigated further.

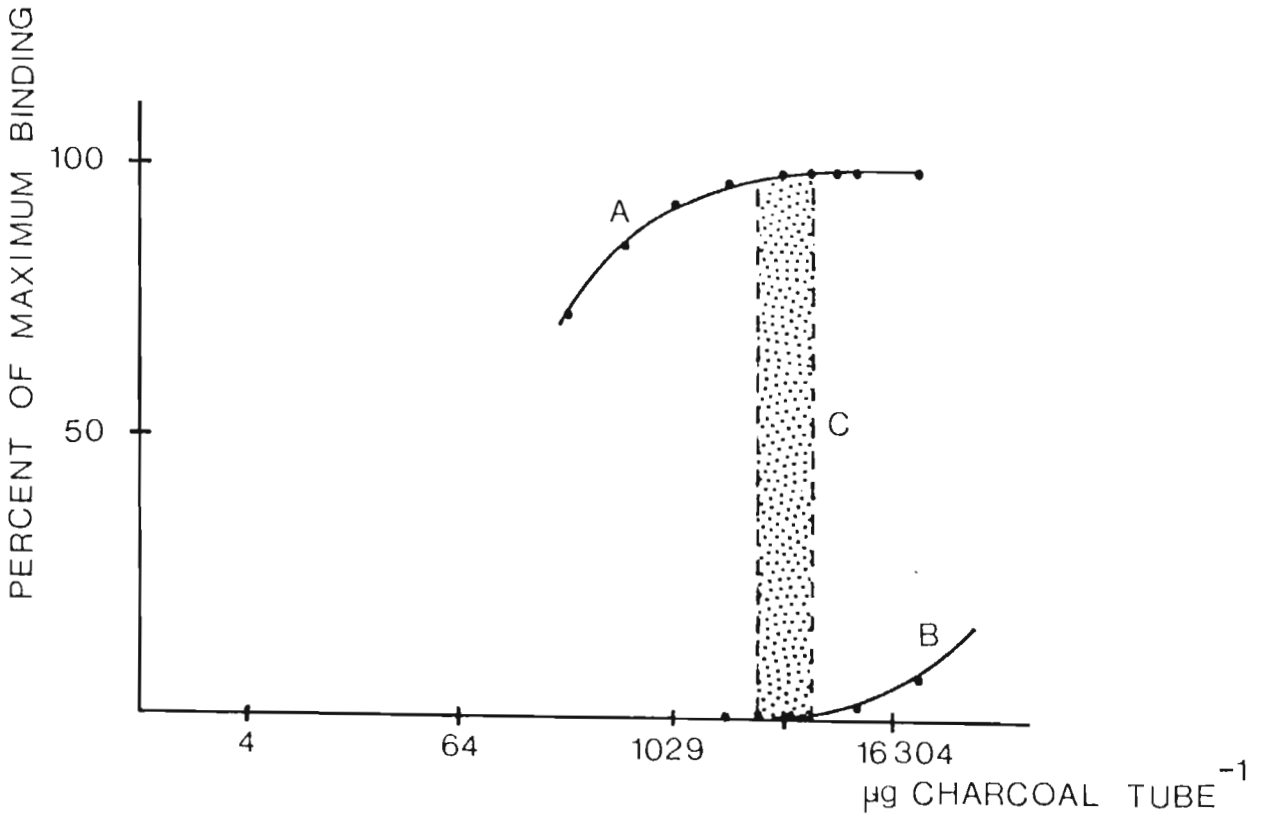
4.2.3.2 Effect of charcoal concentration

The charcoal dose-response curve is presented in Fig. 4.4. A charcoal concentration of between 3 and 6mg per tube removed all the free added tracer without stripping any tracer from the bound complex. A charcoal concentration of 4mg per tube was used in all subsequent IAA RIA development and application.

Fig. 4.3 Standard dose-response curve for IAA using dextran-coated charcoal as a separation procedure.

Fig. 4.4 Charcoal dose-response curve showing the percent of ^{3}H-IAA removed from the incubation volume (A) and the percent ^{3}H-IAA 'stripped' from the bound complex (B). A charcoal concentration of between 3 and 6mg per tube (C) removed all the free tracer without stripping tracer from the bound complex.


B/Bo (%)
95
90
75
50
25
10
5
0,1
0,5
1
5
10
50
100
ng IAA


PERCENT OF MAXIMUM BINDING
100
50
A
B
C
4
64
1029
16304
µg CHARCOAL TUBE^{-1}

4.2.3.3 Effect of pH

The effect of pH on the percentage binding is presented in Fig. 4.5. The antibody appeared to be pH insensitive as regards binding in the pH range tested. A buffer pH of 7,4 was used in the preparation of all primary reagents used in the IAA RIA.

4.2.3.4 Effect of incubation

The incubation time-response curve is presented in Fig. 4.6 for serum from sheep IAA-75. An increase of 10% binding was achieved when incubation took place at 4°C for 15 hours using the same titer concentration. Therefore, although equilibrium was achieved in the water bath (37°C) increased binding was possible with longer incubation at low temperature. NSB values were lower with the 15 hour incubation, thus it appeared that a temperature of 37°C was sufficient to cause tracer degradation.

4.2.3.5 Effect of methanol

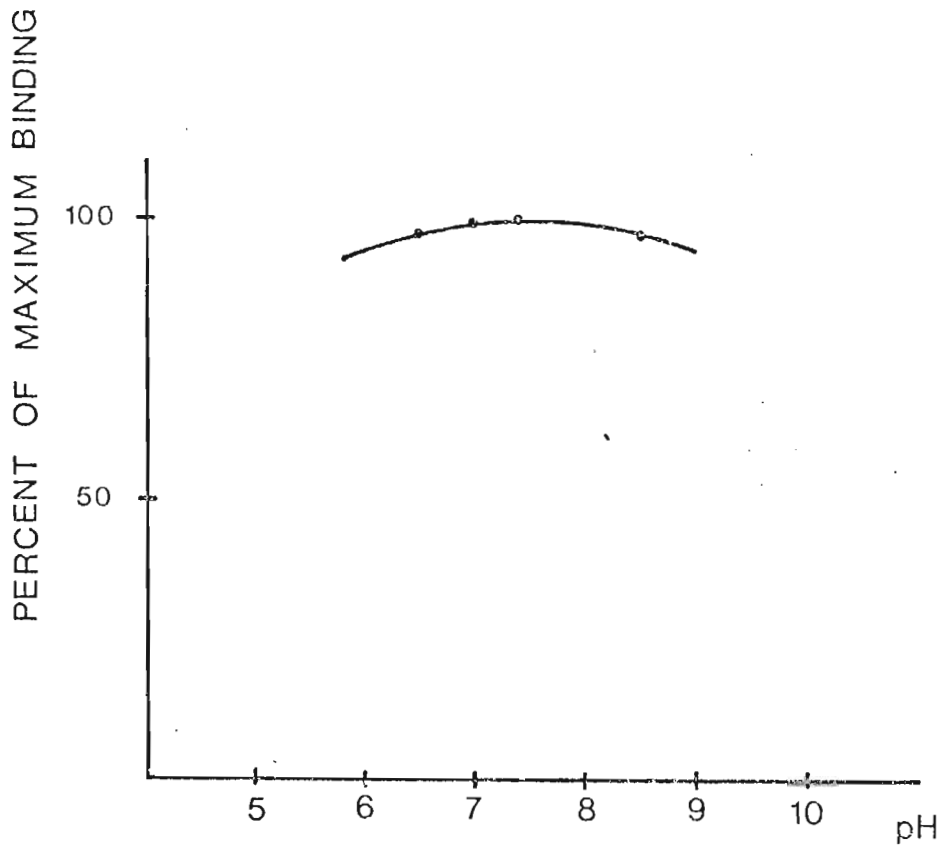
The effect of methanol on the percentage binding is presented in Fig. 4.7. Results are plotted as both the percentage methanol in the primary incubation volume or microliters methanol added to the 300µl primary reaction mixture. Even the smallest trace of methanol (3% of total volume) reduced the percentage binding. Increasing amounts of methanol further decreased the binding percentage. When methanol comprised 40% of the incubation volume, binding had been reduced by nearly half. The antibody was considered to be extremely methanol-sensitive.

4.2.3.6 Standard dose-response curve

A typical standard dose-response curve for IAA is presented

Fig. 4.5 The effect of incubation pH on the percentage binding of anti-IAA-75.

Fig. 4.6 The incubation time-response curve for serum for sheep IAA-75 at an incubation temperature of 37°C.


PERCENT OF MAXIMUM BINDING
100
50
5
6
7
8
9
10
pH

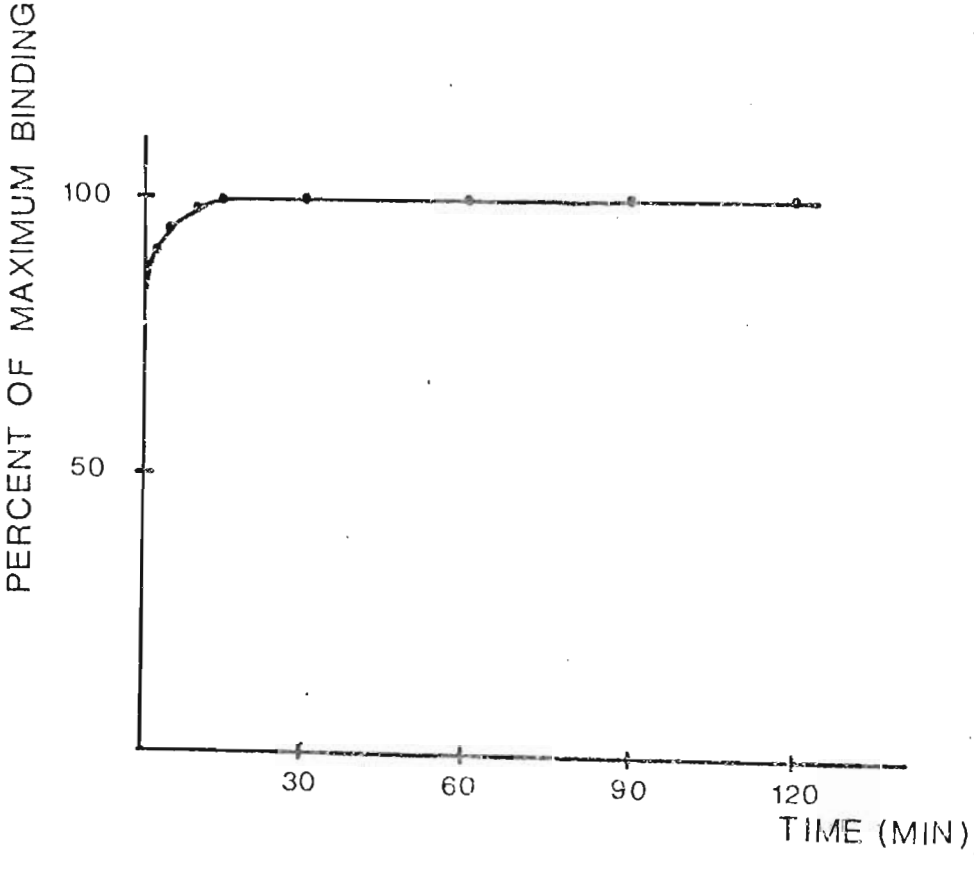
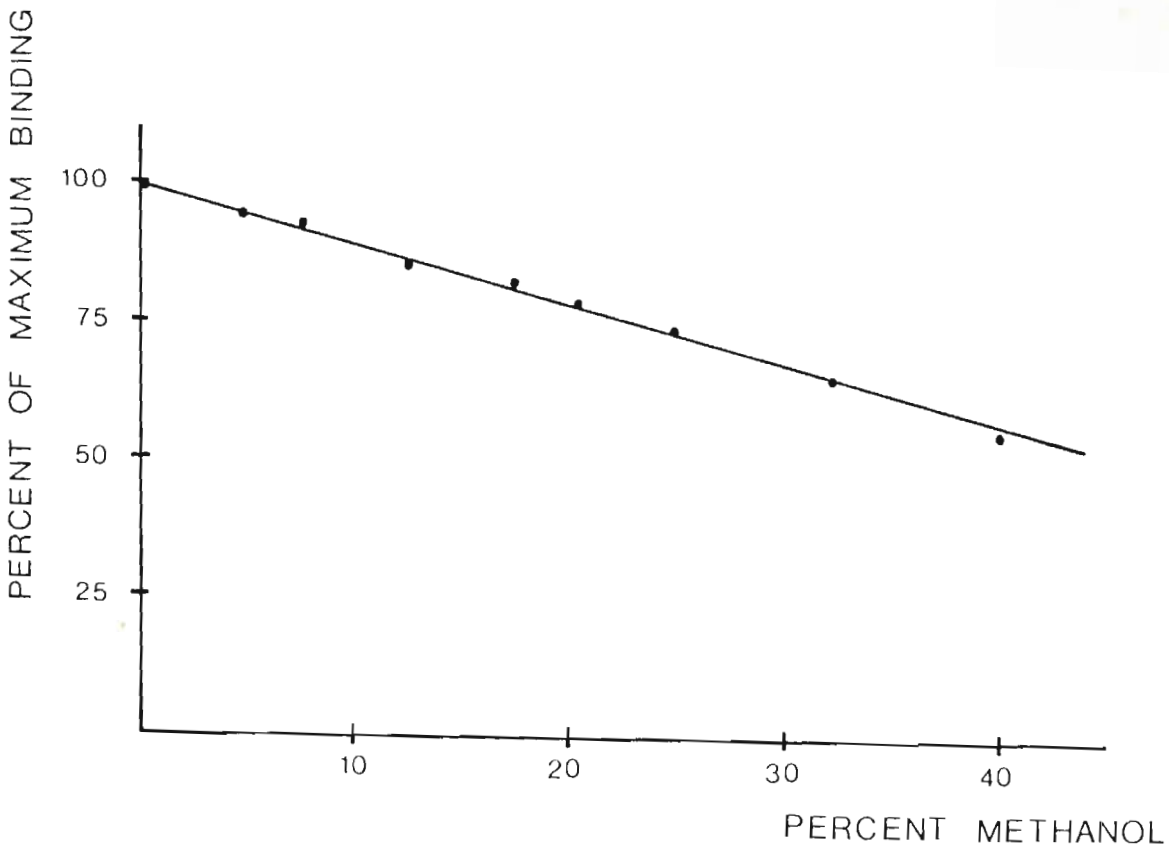
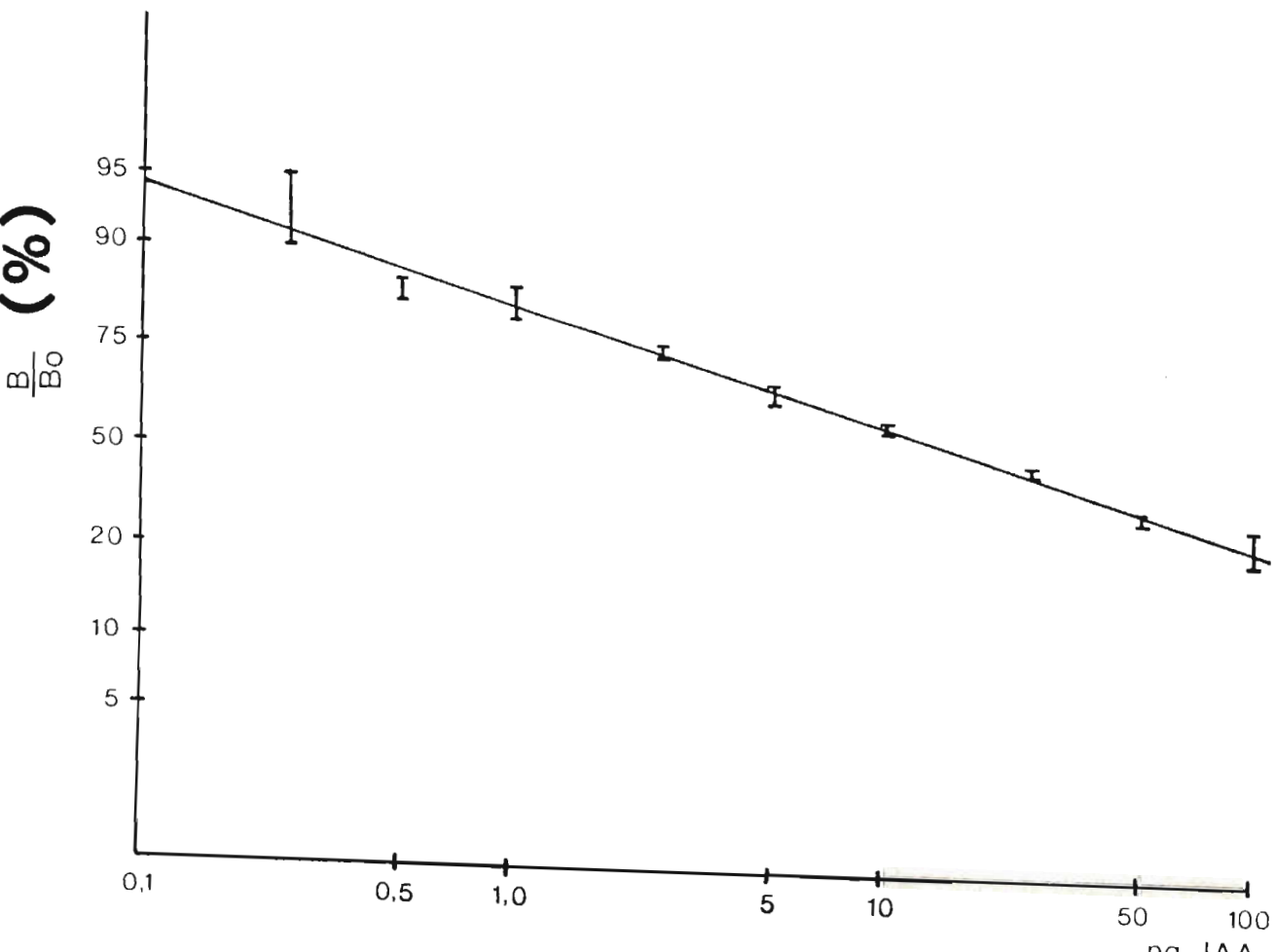

PERCENT OF MAXIMUM BINDING
100
50
30
60
90
120
TIME (MIN)

Fig. 4.7 The effect of methanol upon the percentage binding of serum from sheep IAA-75.

Fig. 4.8 A typical standard dose-response curve for IAA.


PERCENT OF MAXIMUM BINDING
100
75
50
25
10
20
30
40
PERCENT METHANOL


B/Bo (%)
95
90
75
50
20
10
5
0,1
0,5
1,0
5
10
50
100

in Fig. 4.8. The excessive sensitivity is a titer effect but precision remained good (r = 0,989). Typically the assay has a measuring range from 250pg to > 100ng.

4.2.4 Validation

4.2.4.1 Extract dilution curves

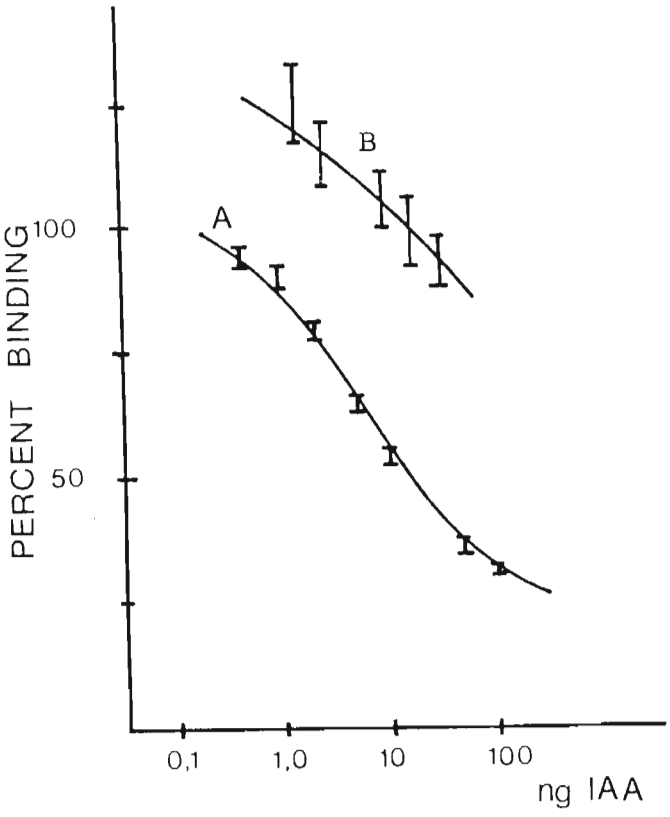
The crude extract contained some contaminant which bound the tracer, and was not separated from the antibody fraction by the charcoal separation procedure (Fig. 4.9). This contaminant displayed similar characteristics to that encountered with the IPA RIA. After solvent partitioning (Fig. 4.10) the contaminant appeared to have been removed as the extract and extract plus standard dose-response curves paralleled the standard dose-response curve. The antibody was unable to distinguish between standard IAA and biologically extracted ligand after purification. This was indicative of either good specificity or the removal of all cross-reactive components from the biological extract.

4.2.4.2 Cross-reactivities to Anti-IAA-75

The antiserum was very specific for the compounds tested (Table 4.2). The only compound which showed any cross-reaction was naphthalene acetic acid. No other compound tested had a cross-reaction greater than 1%. The estimation of cross-reactivities was by extrapolation, as 50% inhibition of binding was not encountered using 6 cycle log-logit probability graph paper. This serum could therefore be used to reliably quantitate IAA in the presence of structurally related compounds.

Fig. 4.9 Extract dilution curve for a crude extract prior to any purification showing the effect of a contaminant (B) by increasing binding above the preset level (A).

Fig. 5.10 Extract dilution curves for IAA after purification showing the curves for crude extract only (A), extract plus standard IAA (B) and the standard IAA dose-response curve (C).


PERCENT BINDING
100
50
A
B
0,1
1,0
10
100
ng IAA

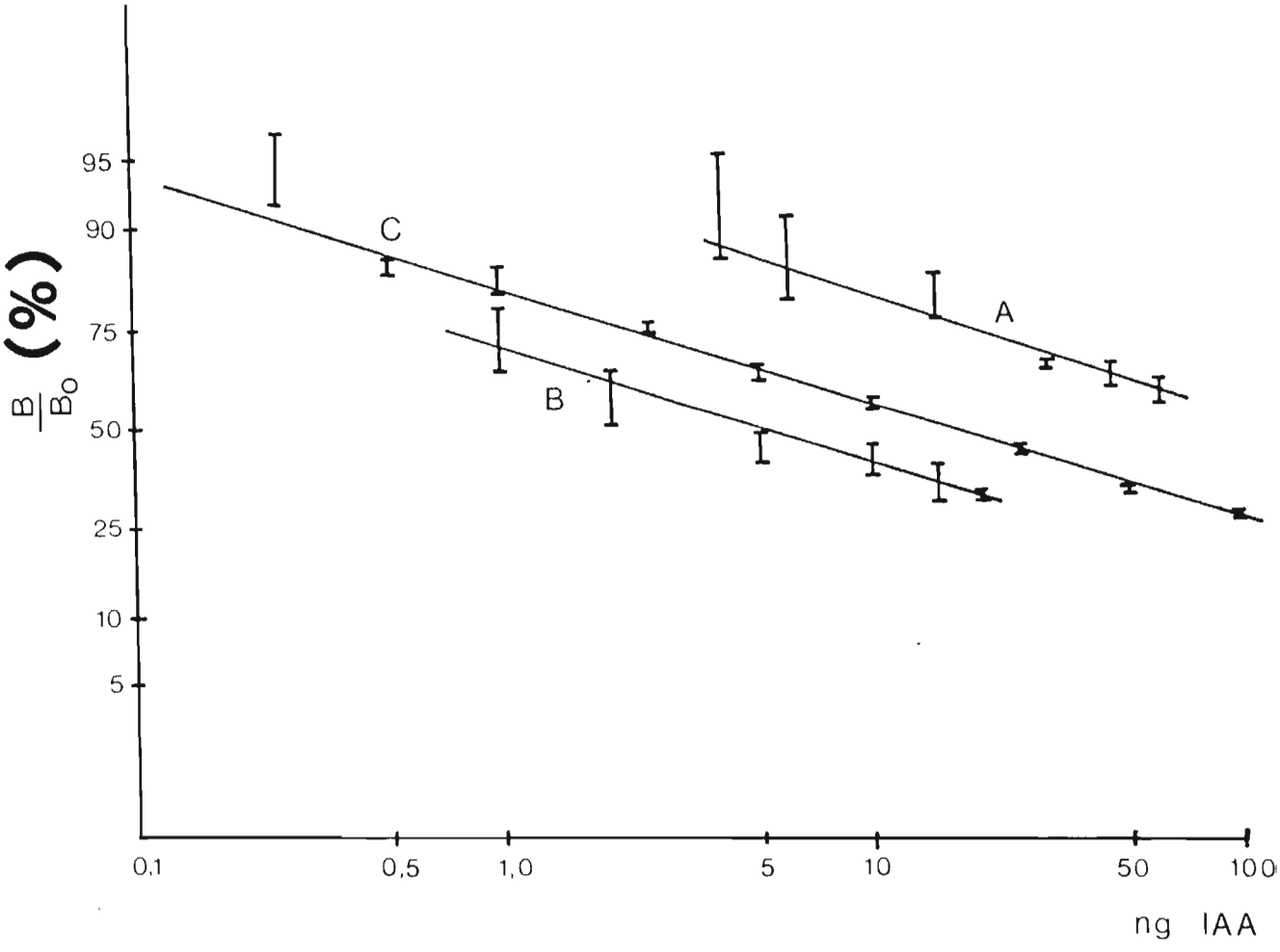

B/B0 (%)
95
90
75
50
25
10
5
C
A
B
0,1
0,5
1,0
5
10
50
100
ng IAA

Table 4.2 Cross-reaction of various IAA precursors and structurally related compounds to Anti-IAA-75 on a mass basis

Compound	% Cross-reaction
Indole-3-acetic acid (IAA)	100
Naphthalene acetic acid	0,5
Indole-3-acetaldehyde	0,1
Indole-3-acetone	0,1
Indole-3-acetamide	0,1
Indole-3-propionic acid	0
Indole-3-aldehyde	0

4.3 Discussion

Auxin levels show a considerable range in concentration in plants (Bandurski & Schultze, 1977). The wide measuring range of 0,5 to 500ng achieved in the RIA developed meant that it was possible to assay plant extracts from 1 to 10g, without resorting to differential dilutions to make the extract fall within the measuring range of the RIA.

The antibody used in the RIA was very specific. The specificity was considered to be a consequence of the linking method, whereby BSA was linked to the active nitrogen in the IAA molecule. The resultant conjugate probably left the carboxyl group intact, and cross-reactivity tests showed that this was most likely responsible for the antibody specificity. Similarly structural changes to any part of the molecule resulted in a near total loss of activity. It therefore appeared that most, if not all, of the IAA molecule was involved in the recognition response.

The conjugate had a very high molar ratio, however an antibody dilution titer of over 1:50 was never achieved. There are several possible reasons for this response. Firstly, the IAA molecule may not be very immunogenic. A second possible cause is that the IAA molecule has a very small molecular mass (175) which is lost or "clouded" in the folds of the BSA structure and is not exposed to the immune system of the host animal (Chard, 1981). Should this be the problem a possible solution is to produce a conjugate with a 'spacer' between the IAA and the BSA as done for ABA-BSA by Weiler (1980 b).

It was thought that the antibody obtained using the formaldehyde-derived conjugate and the immunization sequence outlined was possibly of the IgM class and not of the desired IgG class, resulting in an antibody of low titer and poor stability. Strict adherence to temperature and incubation times was necessary to maintain high assay precision. However, the use of low temperature incubation (4°C) appeared to improve stability.

Once again the dextran-coated charcoal proved to be superior to ammonium sulphate and polyethylene glycol as a separation method, particularly when working with authentic standard. However, the crude avocado extracts contained some contaminant that interfered with either the binding ability of the antibody or the absorptive properties of the charcoal. Alternatively, the contaminant, thought to be avocado oil, could have itself served as a non-specific binder. Some form of purification was therefore necessary to remove the contaminant from the biological extract before extract dilution curves could be obtained. This was achieved by solvent partitioning.

After removal of the contaminant, the RIA was both reliable and reproducible, and the endogenous compound performed in

the same manner as authentic IAA. Following the recommendations of Yalow & Berson (1971) the assay was considered validated. This RIA was thought suitable for most plant extracts, provided any interfering contaminants (should they be present) were removed.

The RIA for IAA should be seriously considered as an alternative to the more established and older techniques of IAA quantitation, as the technique is superior in terms of simplicity and speed of execution (Weaver, 1972) and similar to physicochemical methods as regards sensitivity (Stoessl & Venis, 1970) and specificity (Feung, Hamilton & Mumma, 1975).

CHAPTER 5
PLANT GROWTH SUBSTANCE TRENDS IN AVOCADO FRUIT

This study was undertaken to follow pre- and post-harvest PGS levels of the 'Fuerte' avocado fruit. It was of particular interest to relate PGS levels to growth phases of the fruit flesh and seed, in what is believed to be the first multi-PGS study of avocado fruit. Whenever an endogenous compound displayed the same immunochemical and chromatographic properties as authentic standard it was considered to be the compound of interest. The construction of PGS trend models would hopefully give an indication as to which PGSs were involved in critical periods in avocado fruit growth and ripening. The results are compared with those of other researchers studying individual PGSs using older techniques.

5.1 Fruit Growth and Development

5.1.1 Materials and methods

5.1.1.1 Fruit material

Avocado (*Persea americana* L. cv 'Fuerte') fruits were obtained from an inland escarpment farm at Claridge, northwest of Pietermaritzburg. The orchard was east-facing with a steep aspect, at an altitude of about 930m. The trees were grown under dryland conditions on a deep dystrophic soil of the Hutton form and an intergrade of Balmoral and Farningham series (Macvicar, Loxton, Lambrechts, le Roux, de Villiers, Verster, Merryweather, van Rooyen & Harmse (1977). The farm is representative of the inland Natal high rainfall mistbelt (Bioclimatic group 3) with a moderately cool climate and later-than-average fruit maturity. Mean annual rainfall is about 1 100mm.

The trees did not all flower at the same time, and there was a staggering of physiological stages on different trees at any one time. Therefore, sampling was done from a single representative early-flowering tree (to avoid masking the PGS peaks). Fruit development in the selected tree was considerably in advance of the mean for 'Fuerte' in the orchard.

Fruit sampling began in September, 1983 and continued through to the end of March, 1984. A minimum composite sample of 30g was collected approximately every two weeks depending upon the growth stage of the crop. The samples were rapidly frozen and stored at -20°C prior to extraction. Sampling was terminated at the end of March when the testa had shrivelled in typical fruits from the test tree.

5.1.1.2 Preparation of material and RIA

As soon as development was sufficiently advanced to enable separation into the various morphological components, this was done. The first four samples were either too small or had not clearly differentiated into the tissues of interest. They were therefore treated as pooled or whole fruit samples. At each sample date the individual fruits were massed and fruit length measurements also taken for the fruit growth curve.

Material used for the determination of free ABA and IAA was treated as follows: a 10g sample was homogenized in 50ml 90% methanol acidified with 1% acetic acid (Weiler, 1979), and extracted for 48 hours in the dark at 4°C. The supernatant was reduced under vacuum so that 1ml contained the equivalent of 1g of fresh mass material. Five ml of extract was added to 10ml of 0,5M potassium phosphate buffer (pH 8,0) and the solution mixed. After reduction to the aqueous phase under vacuum at 30°C the buffer was placed in a 100ml separating funnel and extracted twice with 40ml

diethyl ether (2 x 20ml). The bottom fraction was retained and acidified to pH 3. This was extracted twice with a further 40ml diethyl ether (2 x 20ml) and the top fraction retained (Tillberg & Pinfield, 1982). After being reduced to dryness under vacuum at 30°C, the residue was suspended in 2,5ml absolute methanol. For the determination of free ABA a portion of the extract was subjected to TLC (refer to 3.1.5.2) and then assayed by RIA (refer to 3.1.3.2). For the determination of IAA the extract was suitably diluted in methanol and subjected to RIA (refer to 4.1.3.2). Data were corrected for losses which occurred during purification and isolation.

Material used for the determination of IPA and 2iP was treated as in 2.1.5. A sample of 10g fresh mass was used in all cases. Refer to 2.1.3.2 for the RIA procedure. Results were corrected for purification and isolation losses.

5.1.2 Results

5.1.2.1 Fruit growth morphology

The fruit growth curve, on a mass basis, for 'Fuerte' is presented in Fig. 5.1 and shows that for the first five weeks after flowering there was only very slow growth. A typical single sigmoid curve was obtained. This is in accordance with other workers (Valmayor, 1964; Robertson, 1971; Barmore, 1977). Twenty six weeks after the closed flower stage the testa had changed in appearance from thick, white and fleshy to thin, brown and membranous, tightly enfolding the seed. In accordance with Erickson (1966) the fruit was considered "legally" mature, i.e. with approximately 8% oil content or 80% moisture content. Fruit from the last sampling date softened normally within about 7 days at room temperature, and had an acceptable flavour. The organoleptic (taste) test can also be considered an indication of maturity (Lee, 1981). However,

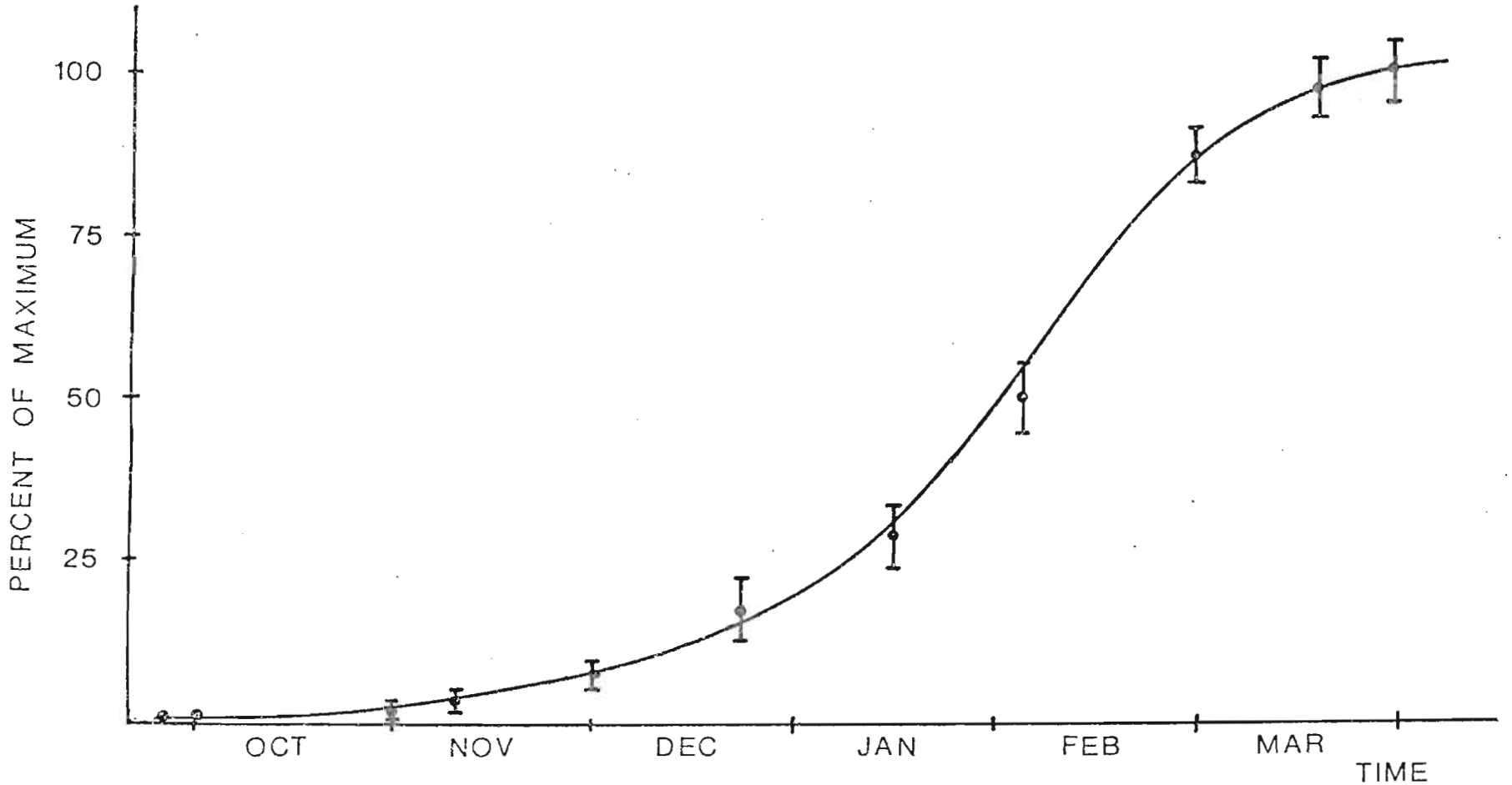


Fig. 5.1 Fruit growth curve for 'Fuerte' avocado on a mass basis. The SE for each sampling date is presented.

more typical 'Fuerte' fruits from the same orchard in earlier studies by McOnie & Wolstenholme (1982) and van den Dool & Wolstenholme (1983) continued slow growth through autumn and the following spring, with a plateau in the two coldest months of June and July.

5.1.2.2 Auxin (Indole-3-acetic acid)

Whenever results of seed PGS concentrations are given, reference is made to the seed minus the surrounding testa which was assayed separately. All results presented are in ng g^{-1} fresh mass.

The levels of IAA equivalents in developing fruits are presented in Fig. 5.2 with the growth curve superimposed. IAA concentration was highest (about 150ng g^{-1}) when the fruits had a mass of approximately 10g in late October/early November. The concentration fell rapidly during the next month, and then declined more slowly from mid-December until the end of March, when the IAA concentration in the whole fruit had dropped to less than 10ng g^{-1}.

The levels of IAA in the fruit components are presented in Fig. 5.3, with the growth curve superimposed. The three fruit components displayed markedly differing trends. The testa had an IAA concentration of about 20ng g^{-1} during mid-December, which then rose and plateaued during February at a concentration of 70ng g^{-1}. Levels then fell very rapidly during March until IAA was no longer detected by the end of March.

Levels of IAA in the seed (minus testa) peaked during mid-January and then declined slowly to a level of 22ng g^{-1} by the end of March. The seed together with its testa maintained high levels of IAA from December until mid-March. Concentrations of IAA in the flesh (meso- plus endocarp) declined with increasing development from 30ng g^{-1} in mid-

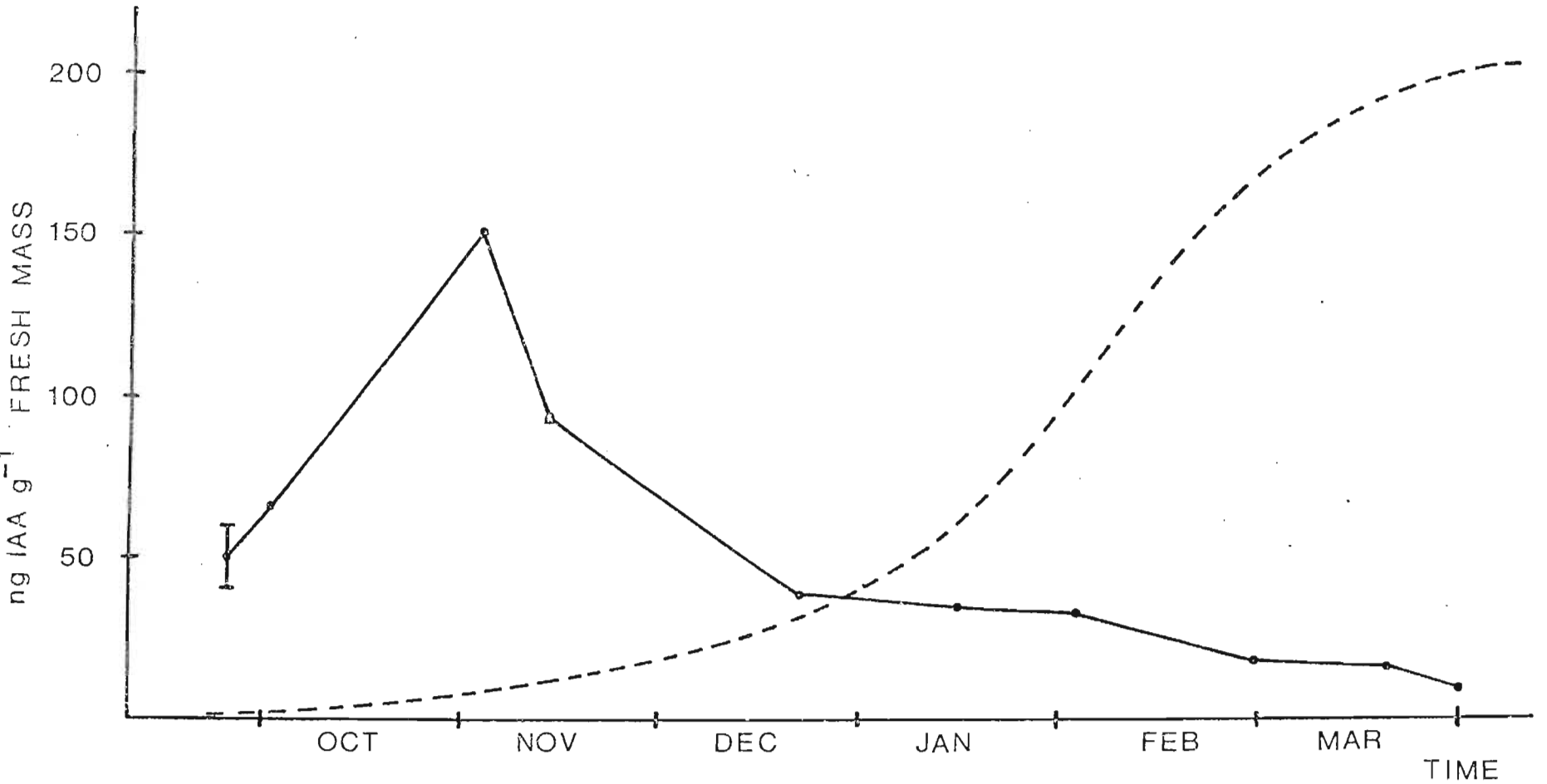
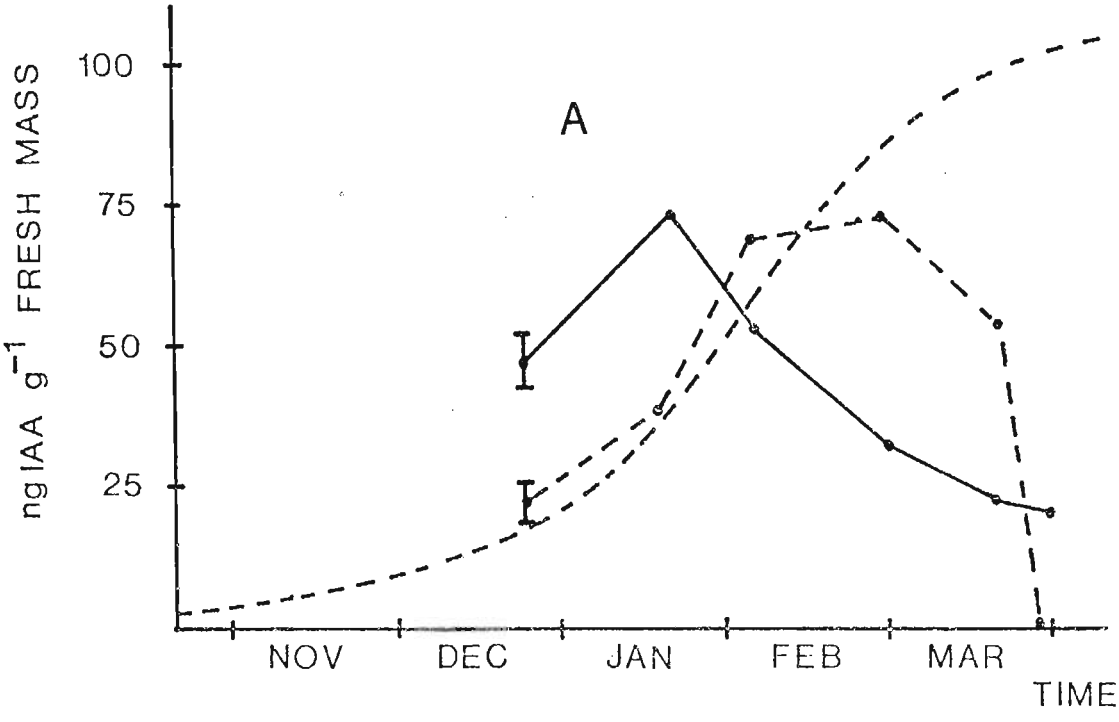
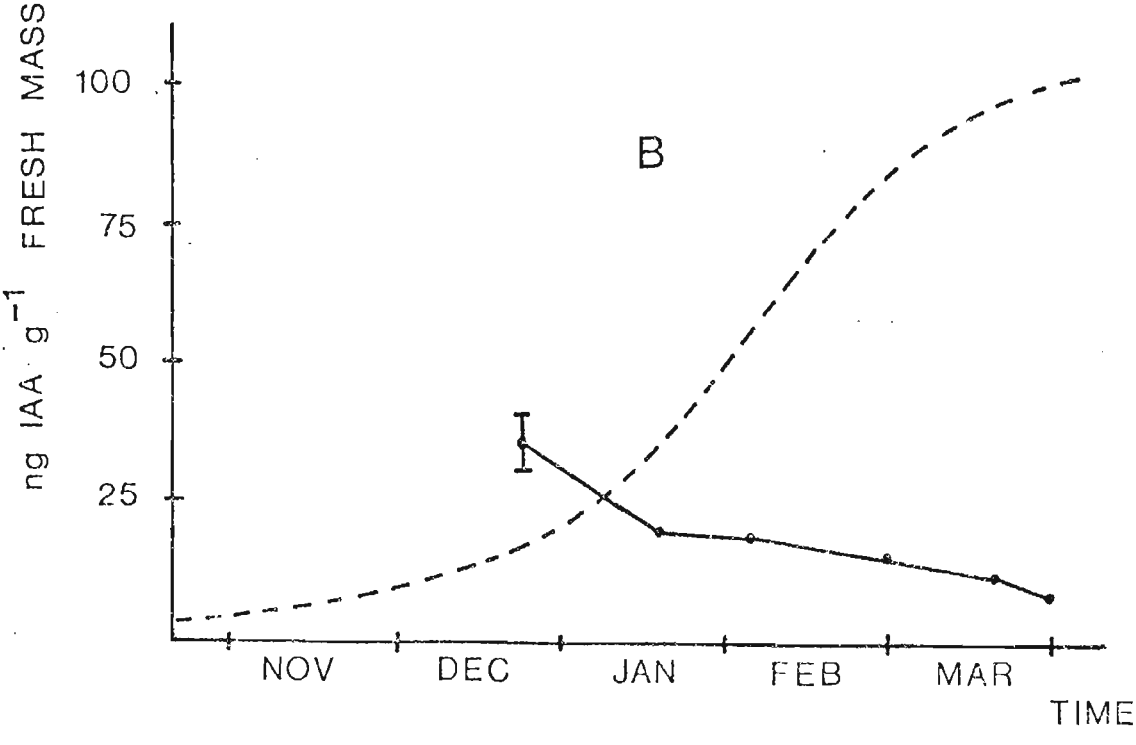


Fig. 5.2 Changes in IAA levels in developing 'Fuerte' fruit (solid line) in relation to growth (broken line). The results are for pooled whole fruit. The assay SE is presented.

Fig. 5.3A The levels of IAA in the 'Fuerte' seed minus testa (solid line) and testa (broken line) with the growth curve superimposed. The assay SE for seed and testa are given.

B The levels of IAA in the 'Fuerte' flesh (solid line) with the growth curve superimposed. Assay SE for the flesh is presented.


A
100
75
50
25
ng IAA g^{-1} FRESH MASS
NOV
DEC
JAN
FEB
MAR
TIME


B
100
75
50
25
ng IAA g^{-1} FRESH MASS
NOV
DEC
JAN
FEB
MAR
TIME

December until less than 10ng g^{-1} by the end of March.

5.1.2.3 Isopentenyl adenine and isopentenyl adenosine

After fruit set in early October the level of 2iP equivalents rose very rapidly to a peak of 32ng g^{-1} by the end of October. Thereafter, the concentration remained high for approximately 70 days (until mid-January). The fruit sample assayed in early February showed a marked decline to 5ng g^{-1}, the approximate level which was maintained until the end of March (Fig. 5.4).

The 2iP equivalents in the various fruit components are presented in Fig. 5.5. The concentration of 2iP in the testa was 25ng g^{-1} during December and peaked in early February after having risen to a level of over 30ng g^{-1}. Thereafter the concentration fell rapidly until no 2iP was detected by the end of March. The seed (minus testa) had a concentration of 36ng g^{-1} during December, the level declining to 18ng g^{-1} by the end of January. The level of 2iP further declined to about 14ng g^{-1} by the end of March.

During December the concentration of 2iP in the flesh was 22ng g^{-1}, this being only slightly less than the level in the testa. However, the concentration fell very rapidly to 5ng g^{-1} by the end of January. This level remained constant until the end of March.

The levels of IPA detected in the fruit were low when compared to 2iP. Concentration of IPA in the fruit remained fairly constant during development (Fig. 5.6), never rising above 5ng g^{-1} and never less than 1ng g^{-1}. Similar trends were observed for both the flesh and seed (minus testa) components. However, levels in the seed were marginally higher than in the flesh as can be seen in Fig. 5.7. The concentration of IPA in the testa showed a changing trend with development. Levels increased from about 5ng g^{-1} in

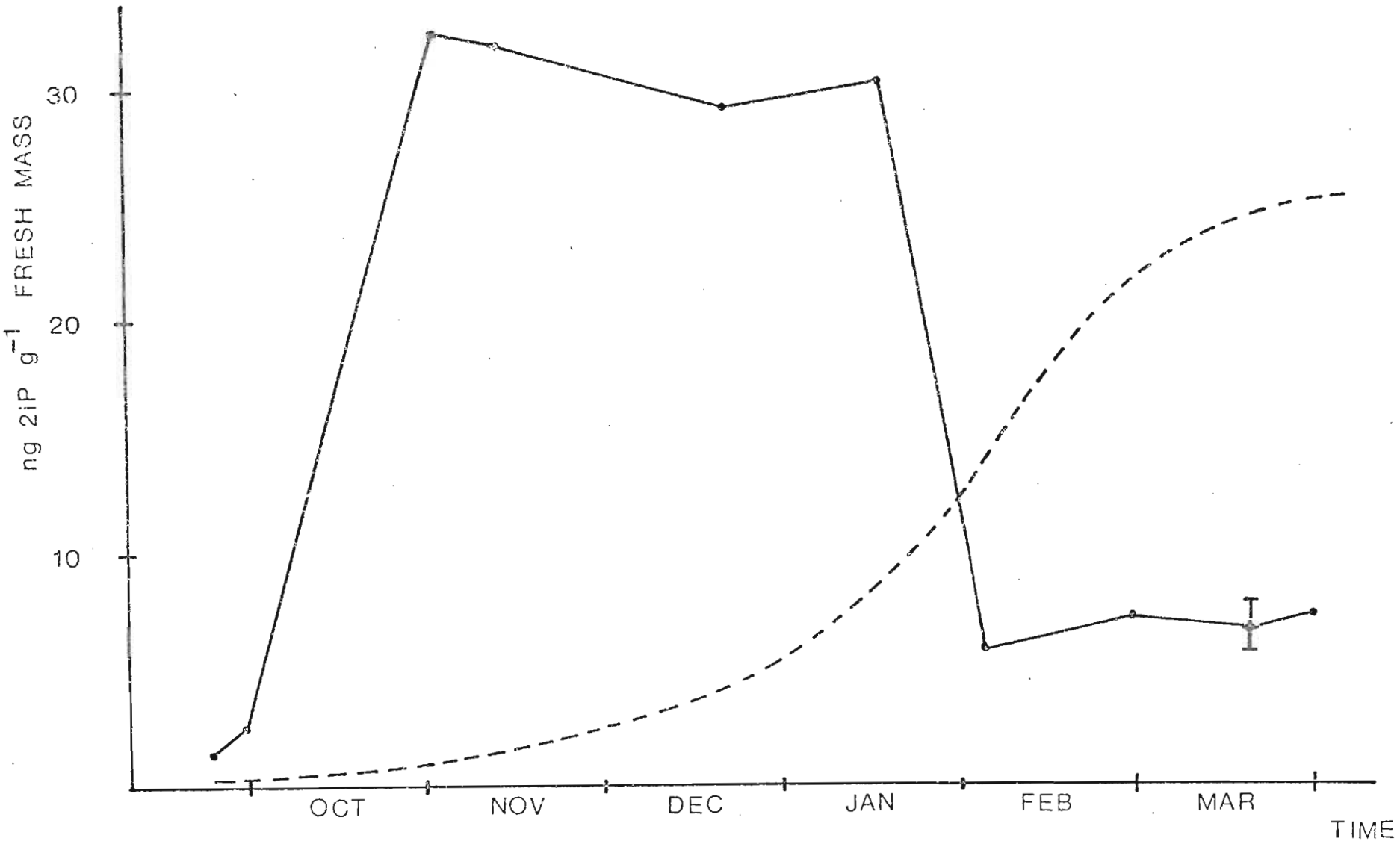
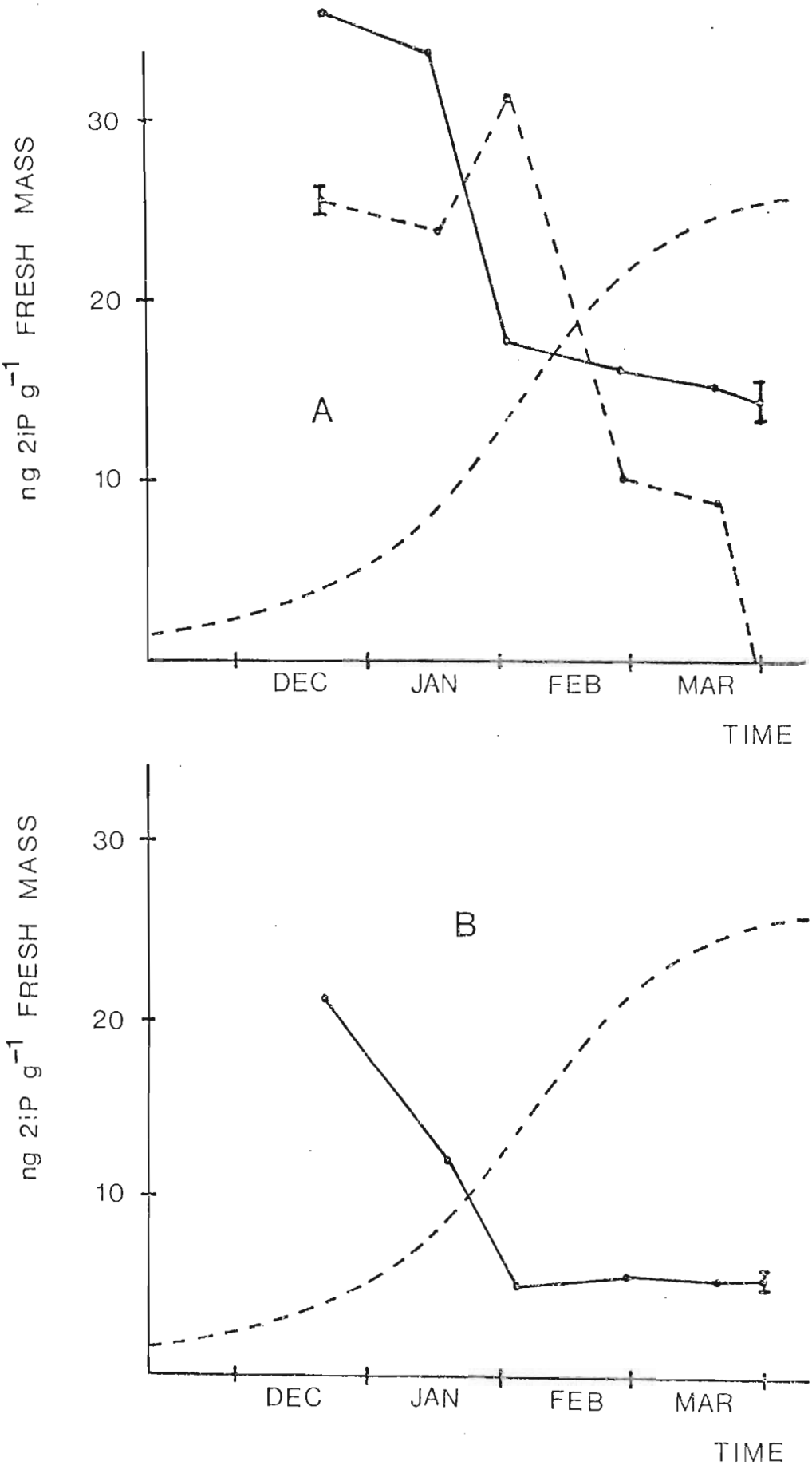


Fig. 5.4 The levels of the cytokinin 2iP in 'Fuerte' fruit (solid line) in relation to growth (dotted line). Results are for pooled whole fruit. The assay SE is

Fig. 5.5A 2iP levels in seed (minus testa) (solid line) and testa (dotted line) in relation to stage of growth (dotted line). The assay SE is presented.

Fig. 5.5B 2iP levels in the flesh (solid line) in relation to growth (dotted line) with the assay SE presented.


ng 2iP g^{-1} FRESH MASS
30
20
10
A
DEC
JAN
FEB
MAR
TIME
ng 2iP g^{-1} FRESH MASS
30
20
10
B
DEC
JAN
FEB
MAR
TIME

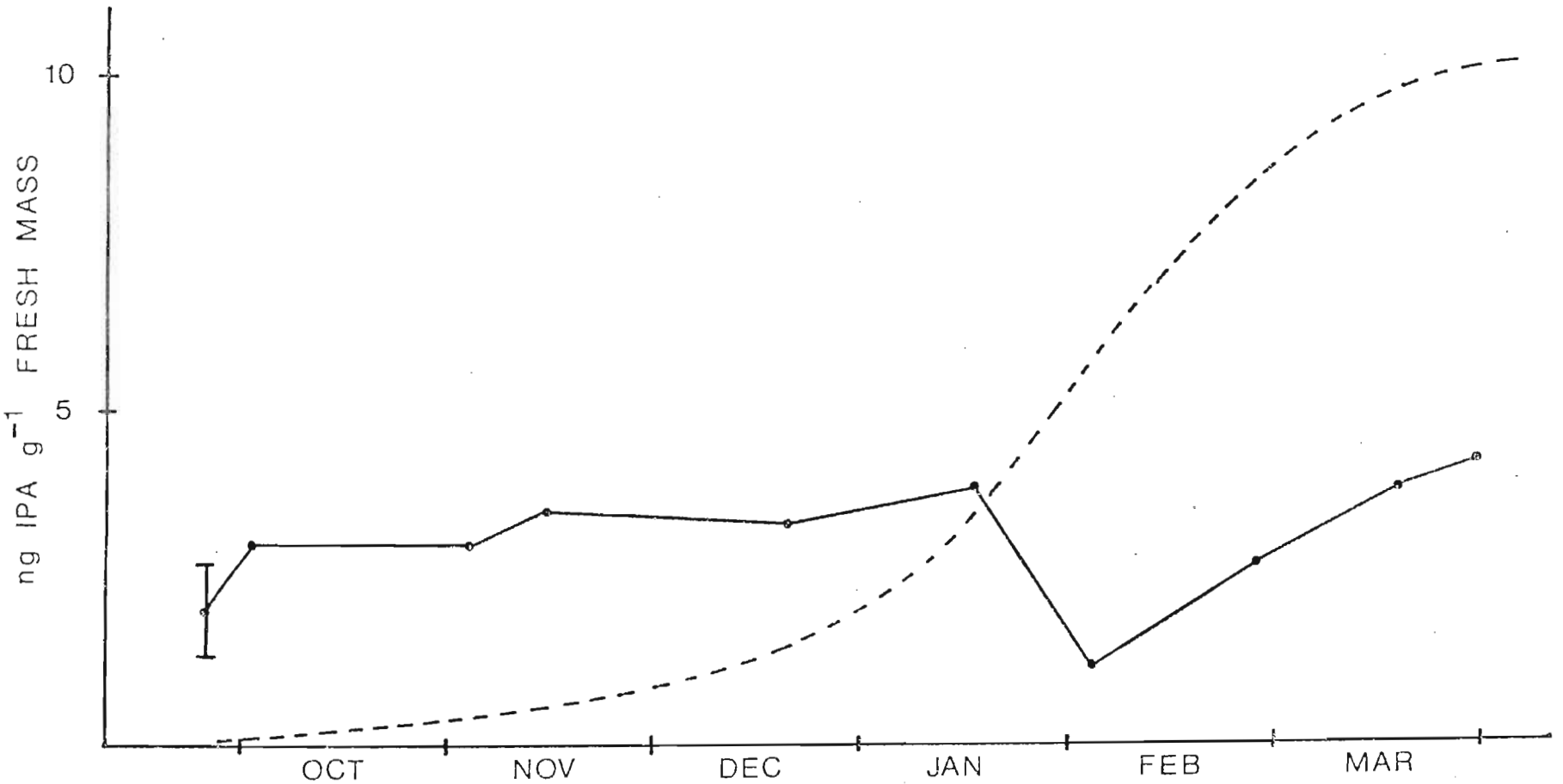
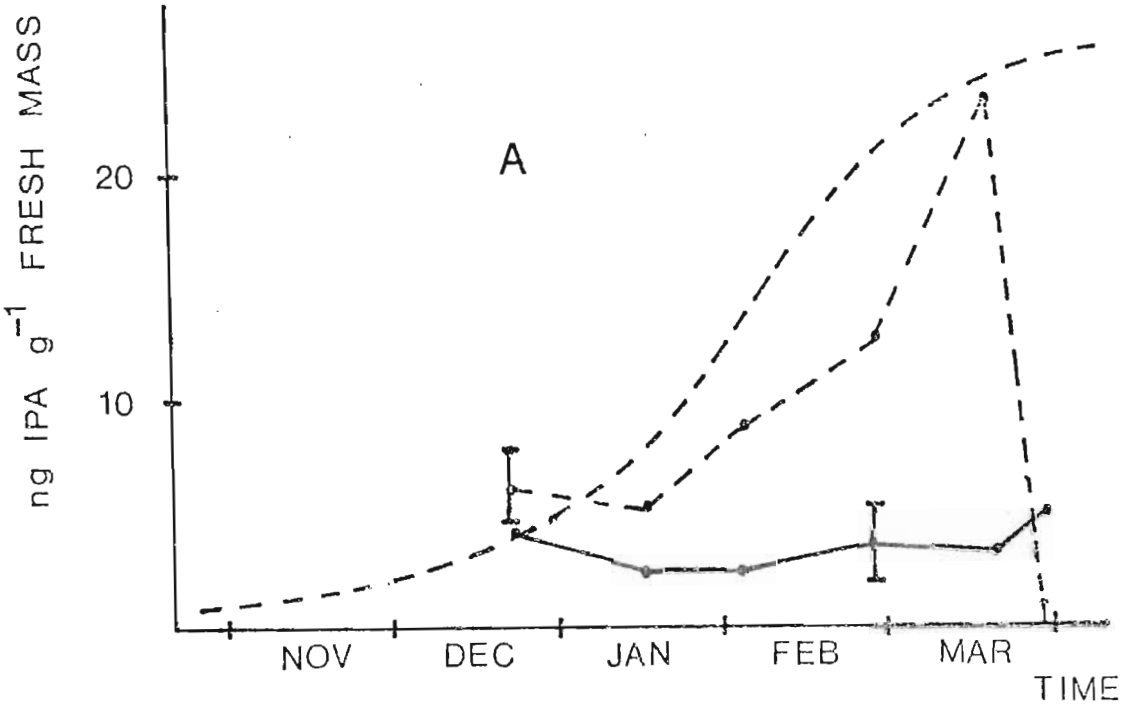
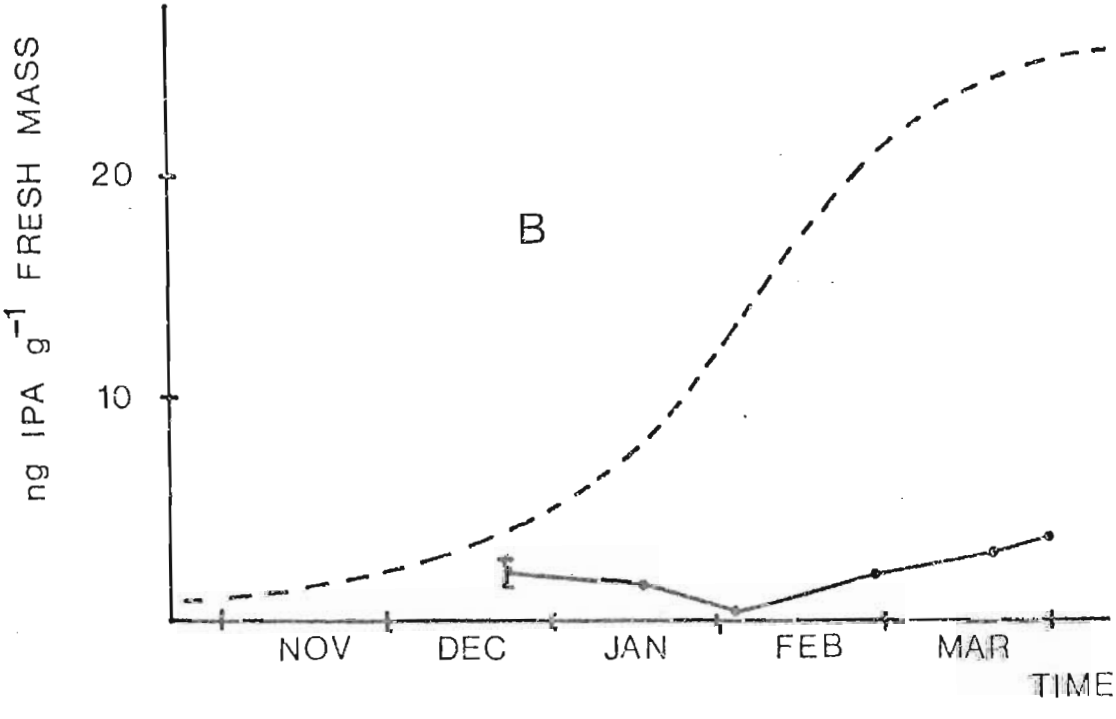


Fig. 5.6 IPA levels in 'Fuerte' fruit (solid line) in relation to growth (dotted line). Results are for pooled whole fruit. The assay SE is presented.

Fig. 5.7A IPA levels in seed minus testa (solid line) and testa (broken line) in relation to stage of growth (broken line). The assay SE is presented.

Fig. 5.7B IPA levels in the flesh (solid line) in relation to stage of growth (broken line) with the assay SE presented.


ng IPA g^{-1} FRESH MASS
20
10
A
NOV
DEC
JAN
FEB
MAR
TIME


ng IPA g^{-1} FRESH MASS
20
10
B
NOV
DEC
JAN
FEB
MAR
TIME

mid-January to about 23ng g^{-1} in mid-March just prior to testa browning and shrivelling. IPA then fell very rapidly over the next week and was not detected in the testa by the end of March (Fig. 5.7).

5.1.2.4 Free abscisic acid

The ABA equivalents given in Fig. 5.8 showed a gradual increase with fruit development, the levels approaching 90ng g^{-1} in late March. The concentration of ABA increased most rapidly during the latter phase of fruit growth, from January till late March.

ABA equivalents in the various components of the developing fruit are presented in Fig. 5.9. The ABA concentration in the testa fell during December from about 50ng g^{-1} to 30ng g^{-1} and then rose to about 60ng g^{-1} by mid March. Thereafter the concentration fell very rapidly and ABA was not detected in the testa by the end of March.

Levels of ABA in the seed (minus testa) varied between 35 and 65ng g^{-1} but showed a general inconsistent increase with development. The level of ABA in the flesh (meso- plus endocarp) remained constant from mid-December to early February (Fig. 5.9) at about 40ng g^{-1}. Thereafter levels rose consistently until late March when a concentration of just under 100ng g^{-1} was detected. The high ABA levels in the flesh coincided with a slowing down of fruit growth.

5.1.3 Discussion

In reviewing the literature on the relationships between endogenous PGSs and fruit development remarkably few complete studies are found. This is surprising since the interactions of different PGSs in the regulation of growth and development are known, and the possibility of such interactions in the regulation of plant development has often

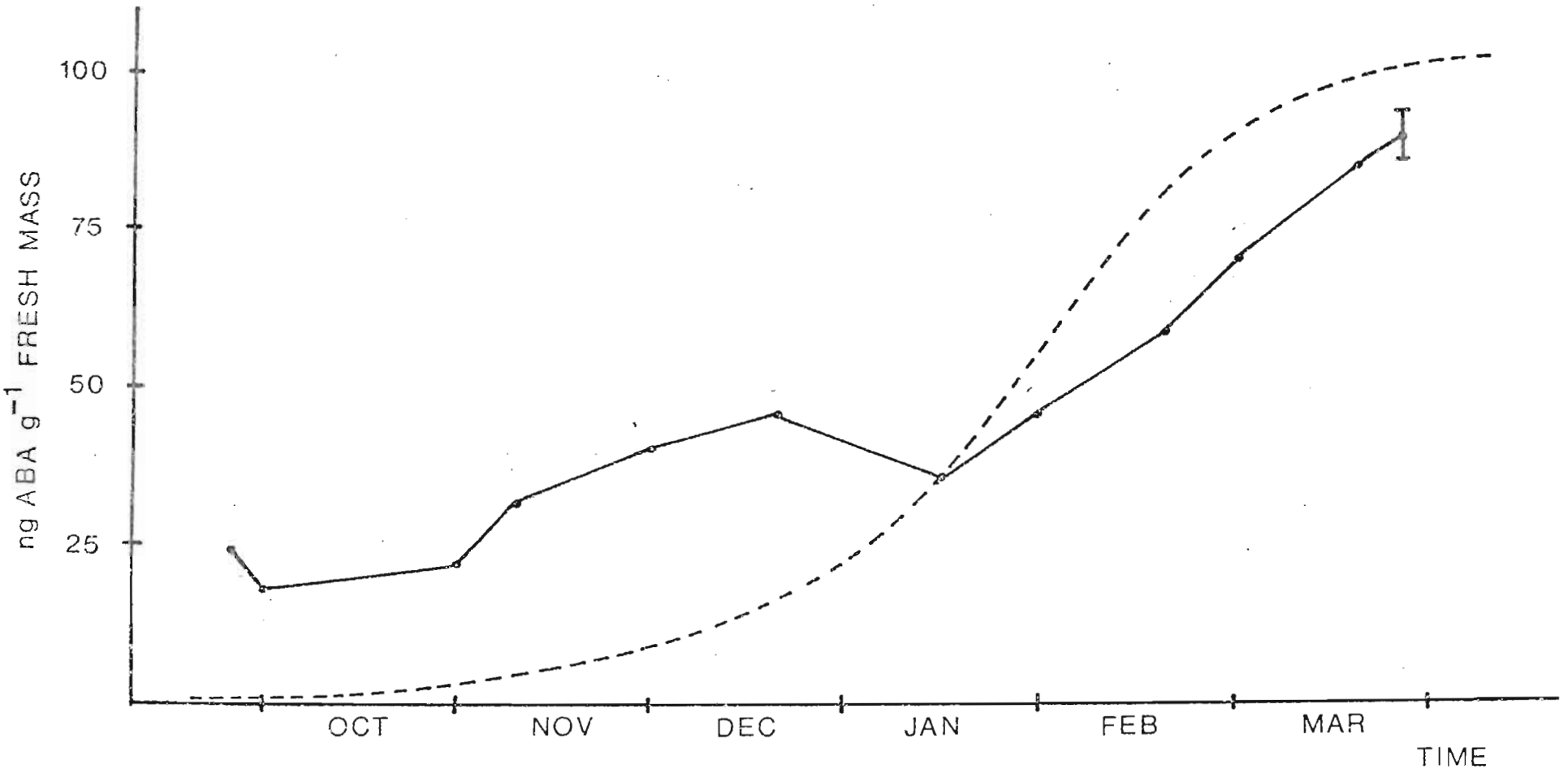
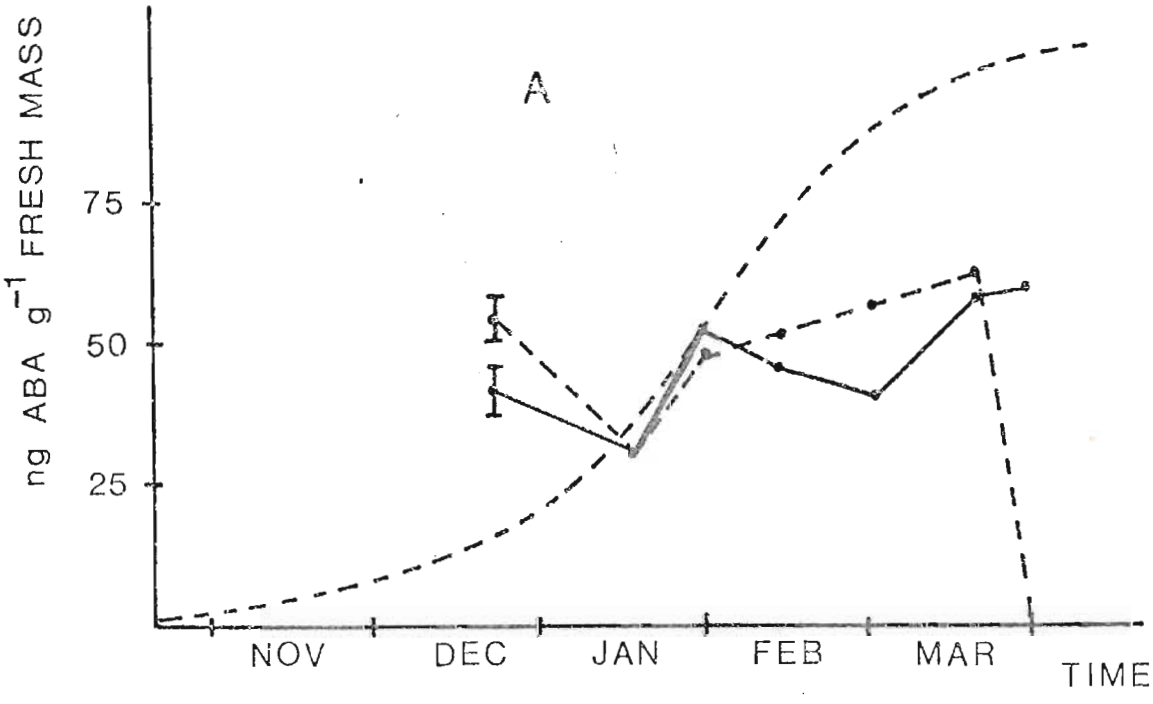
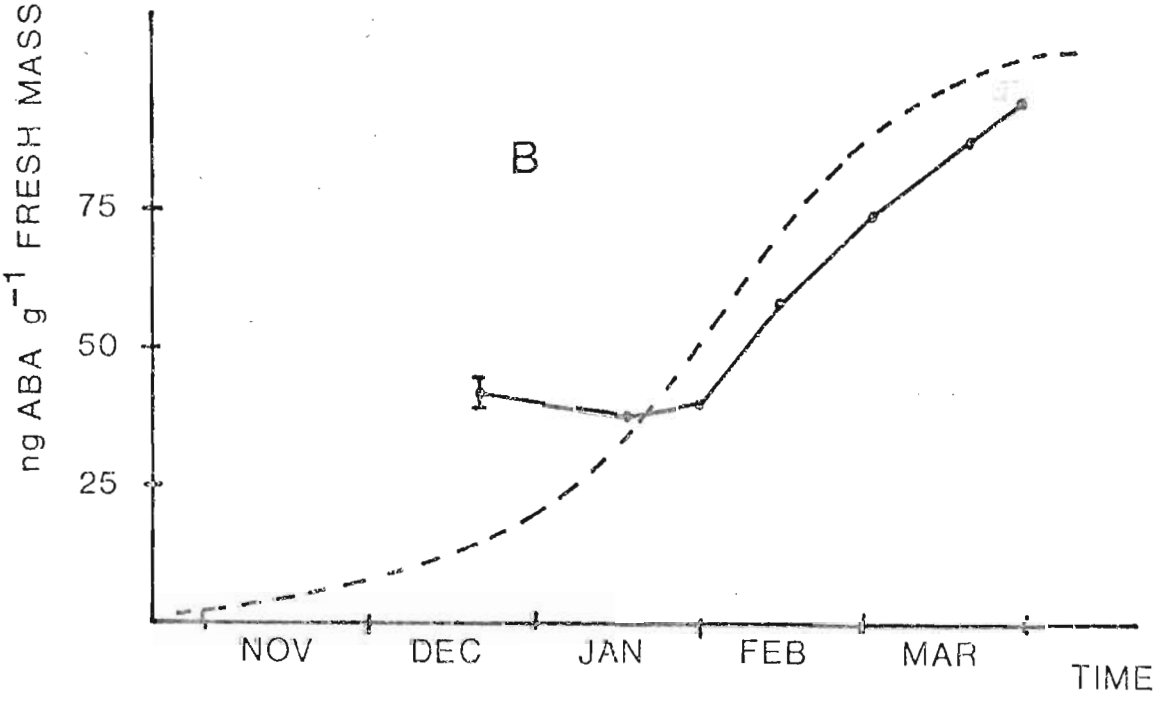


Fig. 5.8 ABA levels in 'Fuerte' fruit (solid line) in relation to growth (dotted line). Results are for pooled whole fruit. The assay SE is presented.


A
ng ABA g^{-1} FRESH MASS
75
50
25
NOV
DEC
JAN
FEB
MAR
TIME


B
ng ABA g^{-1} FRESH MASS
75
50
25
NOV
DEC
JAN
FEB
MAR
TIME

been mentioned (Leopold & Kriedemann, 1975; Goodwin, 1978).

The levels of IAA equivalents found in the various fruit tissues was low when compared to IAA levels in similar plant components from different plant species (Bandurski & Schultze, 1977). The highest levels recorded in this study were 140ng g^{-1} in young whole fruit. Bandurski & Schultze (1977) detected free IAA levels of up to 1 000ng g^{-1} in air dried *Zea mays* seed.

The overall trend for IAA equivalents was in some respects similar to that reported by Gazit & Blumenfeld (1972), who gave no indication of the levels of IAA in the fruit. A major difference between this study and that by these workers was the concentration of IAA found in the testa. In late January, when IAA in the seed (minus testa) started to decline, levels of IAA in the testa were still rising, peaking in the late February sample when the testa was most prominent and fleshy. It appeared that the testa served to maintain IAA levels as the fruit entered the late stages of development and the onset of "legal maturity". In contrast to the earlier Israeli study, IAA was detected in the flesh throughout development but declined as the fruit approached maturity. The IAA trend supported the proposal that auxin enhanced fruit "sink" strength (Crane, 1964; Gazit & Blumenfeld, 1972). The fruit attached to the tree had very high IAA levels in early November, a period notorious for fruit drop in South Africa (Bower, 1984. Citrus and Subtropical Fruit Research Institute; personal communication).

In general, endogenous cytokinin levels can occur in the range from 1 to 1 000ng g^{-1} fresh mass in plant tissues (Brenner, 1981). The IPA and 2iP equivalents detected by RIA in avocado fruit fell well within this range, although IPA equivalents were always very low.

Cytokinins are known to play a role in cell division (Hill, 1980). The high 2iP levels could therefore have been necessary for this process. However, in contrast to most other fruits, in the avocado, cell division continues during the latter growth phase (Barmore, 1977). It is probable that the seed (excluding testa), and later the fleshy testa itself served as a cytokinin source for this cell division, as 2iP levels remained high in these two components until the fruit was nearly mature.

Levels of IPA equivalents were always very low in the whole fruit, particularly in the flesh and seed (excluding testa). Levels in the testa rose during cell division and expansion stages, but fell sharply when growth slowed down during March. The avocado may utilize these low levels of IPA by modulation of tissue sensitivity, a concept reviewed by Kende (1982). If both IPA and 2iP function in a similar manner then tissue sensitivity to these two cytokinins must vary. Alternatively they could be involved in different responses in the fruit. Zeatin and zeatin riboside levels were not studied, and their trends could be equally, if not more, important than the two cytokinins studied. However, this study showed that different cytokinins displayed marked differences in both trends and levels. It was therefore not possible to give a general cytokinin trend in avocado fruit development.

While the fruit can be supplied with cytokinins from non-fruit tissues (Kende, 1965; Letham, 1969), the role of seed and testa must not be overlooked. From this study it appeared that these tissues with their high levels of promotive PGSs could be of great importance in the regulation of fruit growth via the production and/or release of promotive PGSs. This study has not, however, thrown any further light on the site of actual cytokinin synthesis, considered by van Staden & Davey (1979) to be the roots of the plant.

The trends of ABA equivalents in the developing avocado fruit fell within the general plant tissue range of 10 to 4 000 ng g^{-1} fresh mass (Milborrow, 1978). The rise in ABA equivalents in December coincided with a dry spell (trees were grown under dryland conditions) which was alleviated by heavy rains in early January. Fruit growth slowed down as ABA levels rose during the latter half of February and the whole of March. Whether this was a causal response due to higher ABA and declining levels of promotive PGSs needs further investigation.

Of interest was the general rise in ABA equivalents in the flesh with the development of the fruit, with the pattern somewhat interrupted in mid-January. This relationship was not observed by Gazit & Blumenfeld (1970), who found a constant concentration of ABA during avocado fruit development. It is perhaps noteworthy that ABA concentrations were fairly high in November and December, when fruit drop is marked. It would be useful to analyse separately abscissing fruitlets for ABA and promotive PGS concentrations, in comparison with fruits retained by the tree. The possibility of ABA involvement in opening and closing of flowers, and in the very heavy abscission of flowers, warrants further investigation.

The rise in free ABA during December (Fig. 5.10) coincided with a reduction in 2iP equivalents. Exogenous ABA has been observed to inhibit cytokinin metabolism (Letham & Palni, 1983) and it is thought that ABA has a cytokinin-sparing action (Letham, 1978). The implication was that water stress caused an increase in free ABA and a reduction in 2iP and perhaps IAA as well. This would tend to reduce the rate of cell division (Hill, 1980). Therefore, any stress during this November and December period caused an irretrievable loss in fruit growth, as there was only one 2iP peak. The effect would tend to be masked to some extent, as the avocado fruit continues with cell division,

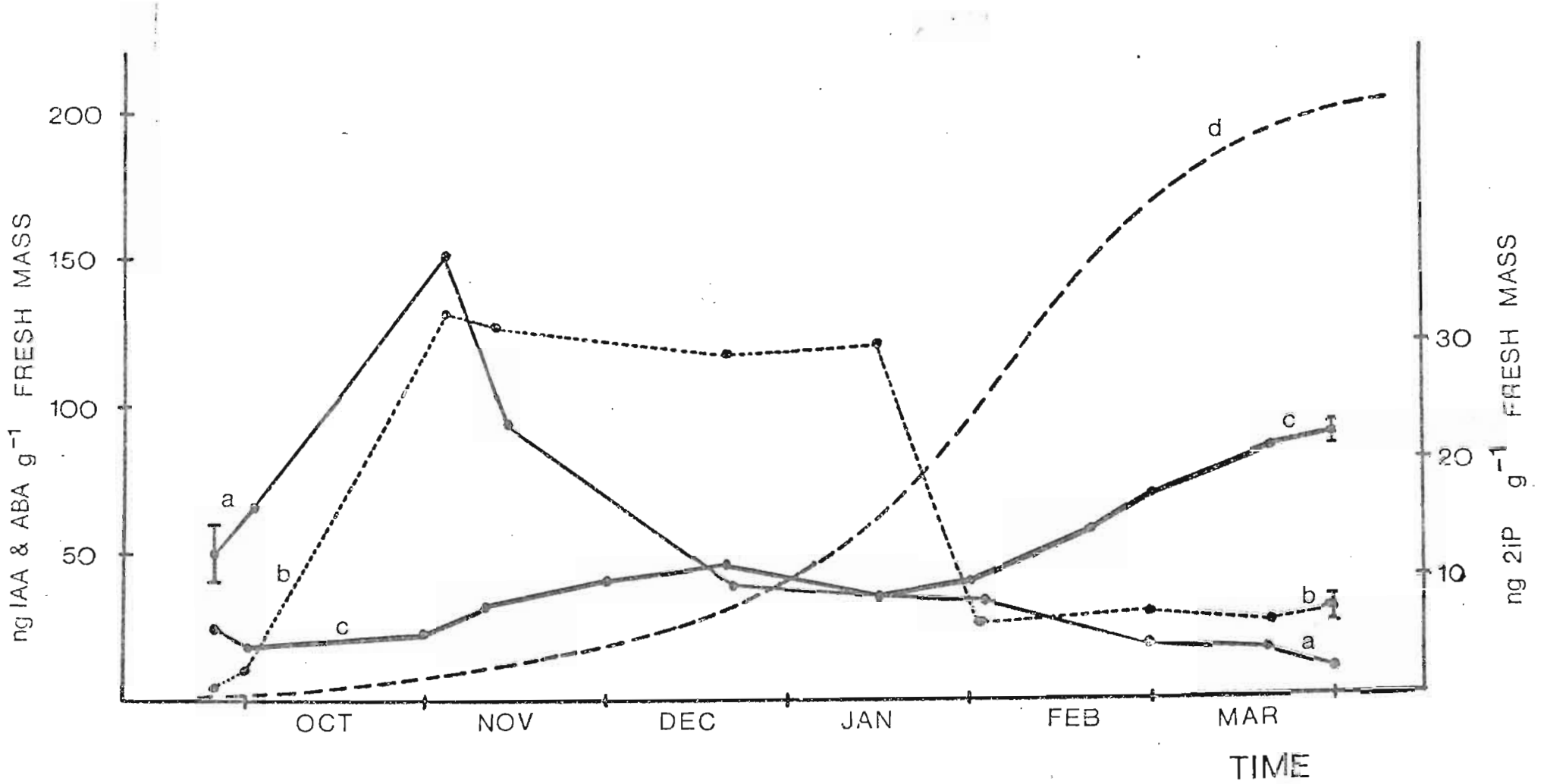


Fig. 5.10 Possible IAA (a), 2iP (b) and ABA (c) interaction in developing avocado fruit superimposed on the fruit growth curve (d).

although at a reduced rate, until maturity. The role of ABA in avocado fruit growth is an avenue that deserves much future attention.

Unfortunately the development of a RIA for gibberellic acid was unsuccessful, therefore a comparison with gibberellic trends in the literature was not possible. The trend for gibberellin activity in avocado fruit (Gazit & Blumenfeld, 1969) was very similar to that for auxin in the present study. A gibberellin study would be desirable to complete the present investigation.

5.2 Post-harvest PGS Trends in Avocado Fruit

5.2.1 Materials and methods

5.2.1.1 Avocado material

Avocado fruit cv 'Fuerte' material was obtained from the University of Natal research farm, Ukulinga, near Pietermaritzburg. This farm is situated in a relatively warm, low rainfall (710mm mean) area in Bioclimatic Group 6. The avocado orchard is planted on a soil of the Westleigh form, Devon series, characterized by soft plinthite in the B horizon (Macvicar *et al.*, 1977). Such a soil does not meet the requirements of exceptionally good drainage for the cultivation of avocado, but its depth has been improved by the construction of half terraces. The 'Fuerte' trees sampled were water-stressed for much of the season, especially during the autumn and winter. This was due to drought, severe water restrictions limiting irrigation and the droughty nature of the soil itself.

5.2.1.2 Preparation of material and RIA

Thirty 'Fuerte' fruit were harvested on 1983:04:15 and stored at room temperature. Each day 2 or 3 fruit were

destructively sampled. Firmometer readings were taken daily, and when remaining fruit reached a firmometer reading of 100, indicating eating ripeness (Swarts, 1981), the determinations were terminated. After each firmometer reading, avocado rind (exocarp) was removed and extracted separately. Meso- and endocarp (flesh) was pooled after removal of any attached testa. Material was homogenized in 50ml 80% methanol (acidified with 1% acetic acid) per 5g fresh mass. After extraction at 5°C for 48 hours in the dark the material was centrifuged at 1 000g for 10 min. The supernatant was reduced to dryness under vacuum at 30°C, taken up in 5ml methanol and centrifuged at 1 000g for 10 min.

Duplicate 100µl subsamples of the supernatant were assayed for total ABA. For the determination of free ABA, 50µl of supernatant was applied to a silica TLC plate, dried and developed (solvent toluene: ethyl acetate: acetic acid:: 50:30:4). The R_f zone corresponding to authentic ABA was removed and the ABA eluted with methanol. After concentrating the volume to 1ml the sample was split and assayed in duplicate. The above procedure was repeated for a second group of 'Fuerte' harvested on 1983:08:01, which was well after the optimum harvest date for such a relatively warm area. The RIA was as described in 3.1.3.2.

Material used for the determination of IPA and 2iP equivalents was treated as in 2.1.5. A 10g fresh mass sample of distal flesh was used in all cases. Refer to 3.1.3.2 for the RIA procedure.

Ethylene evolution of 'Fuerte' avocado fruit obtained from Burgershall Research Farm, Eastern Transvaal lowveld, was determined by gas chromatography in May, 1984. During the ripening phase, percentage ripeness was estimated daily by firmometer for fruit from a water-stressed and an unstressed irrigation experiment (irrigation at 80kP and 55kP

tensiometer readings respectively). Each fruit was placed in a 1,5l glass container, the bottom or open end being sealed by standing in water. Fruit was left for 30 or 60 min, depending on the physiological state of the fruit, before withdrawal of the gas sample. A® Carlo Erba 4200 gas chromatograph with an injection temperature of 160°C, oven temperature of 125°C and detector temperature of 160°C was used. Gas flow was: air 300µl min, and the helium carrier gas 40µl min and hydrogen 50µl min. A 2m long activated alumina column with flame ionization detector was used. A 1ml gas sample was injected. After calibration, results were computed on a® Spectra Physics 4100 computing integrator.

5.2.2 Results

5.2.2.1 General

The times required by 'Fuerte' fruit to reach various firmometer readings are given in Tables 5.1 and 5.2.

Table 5.1 Mean time (days) required by water-stressed 'Fuerte' fruit from Ukulinga to reach various firmometer readings

Firmometer reading	1983:04:15	1983:08:01
30	3	1
50	7 ± 1	3
100	9 ± 1	4

It is apparent from Table 5.1 that the late harvested (tree-stored) fruit softened very rapidly as compared with the fruit harvested at optimum maturity in mid-April. This effect was probably enhanced by extreme autumn and winter water stress.

Table 5.2 Mean time (days) required by water-stressed and unstressed 'Fuerte' fruit from Burgershall to reach various firmometer readings

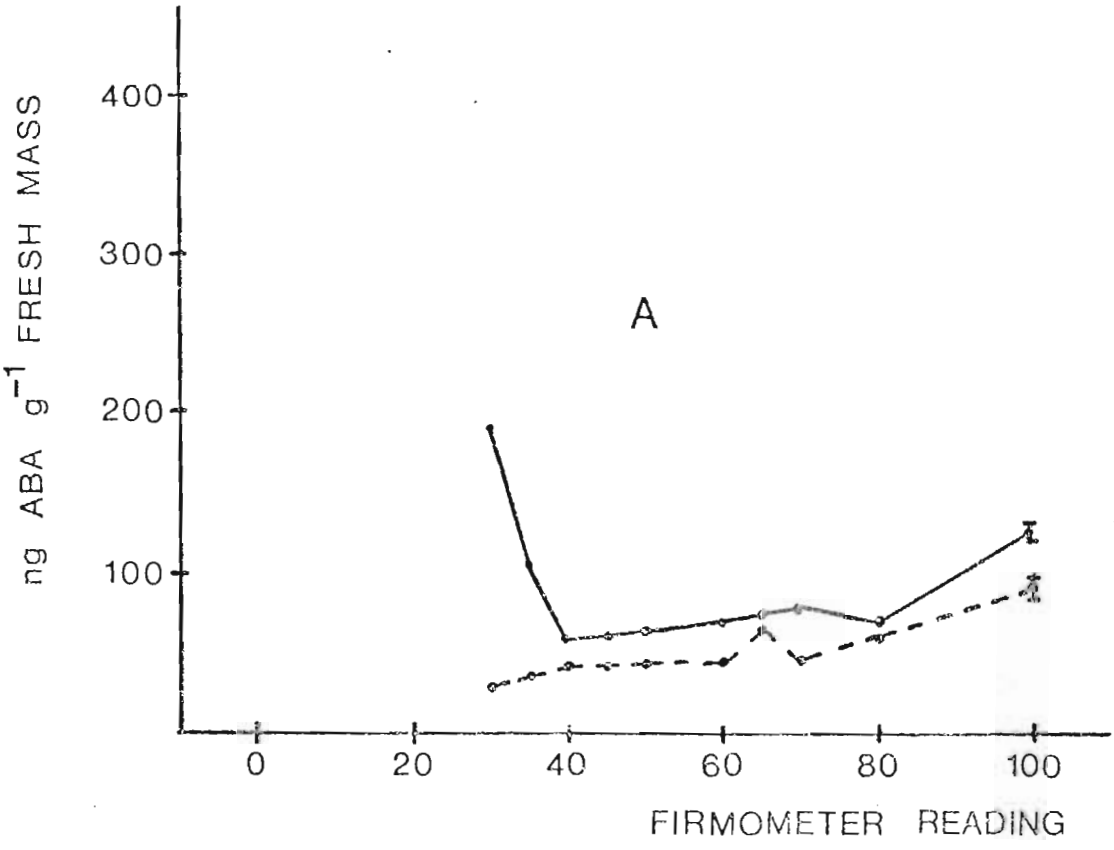
Firmometer reading	Stressed (80kP)	Unstressed (55kP)
30	7 ± 2	6 ± 1
50	8 ± 3	7 ± 1
100	10 ± 3	8 ± 1

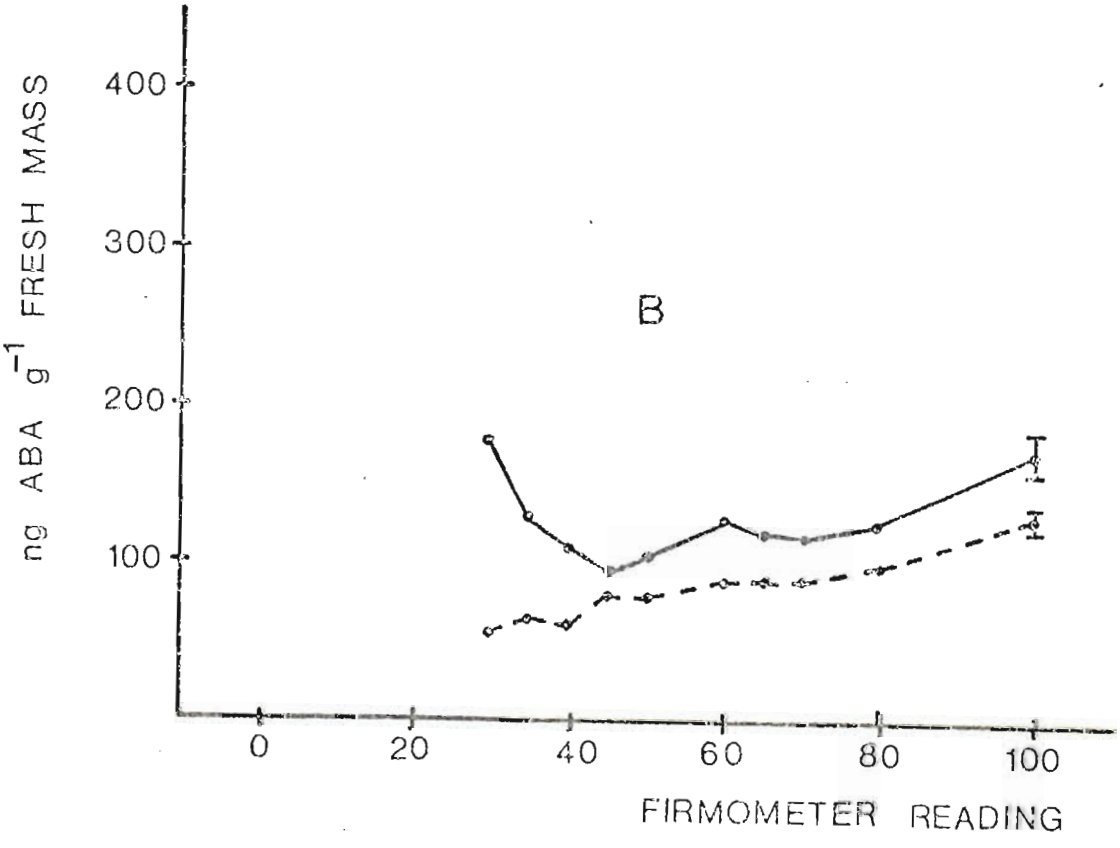
From Table 5.2 it can be seen that the unstressed fruit took a shorter time to ripen while the ripening pattern was fairly uniform. In contrast, the stressed fruit were inconsistent in their ripening pattern and took longer on average to ripen.

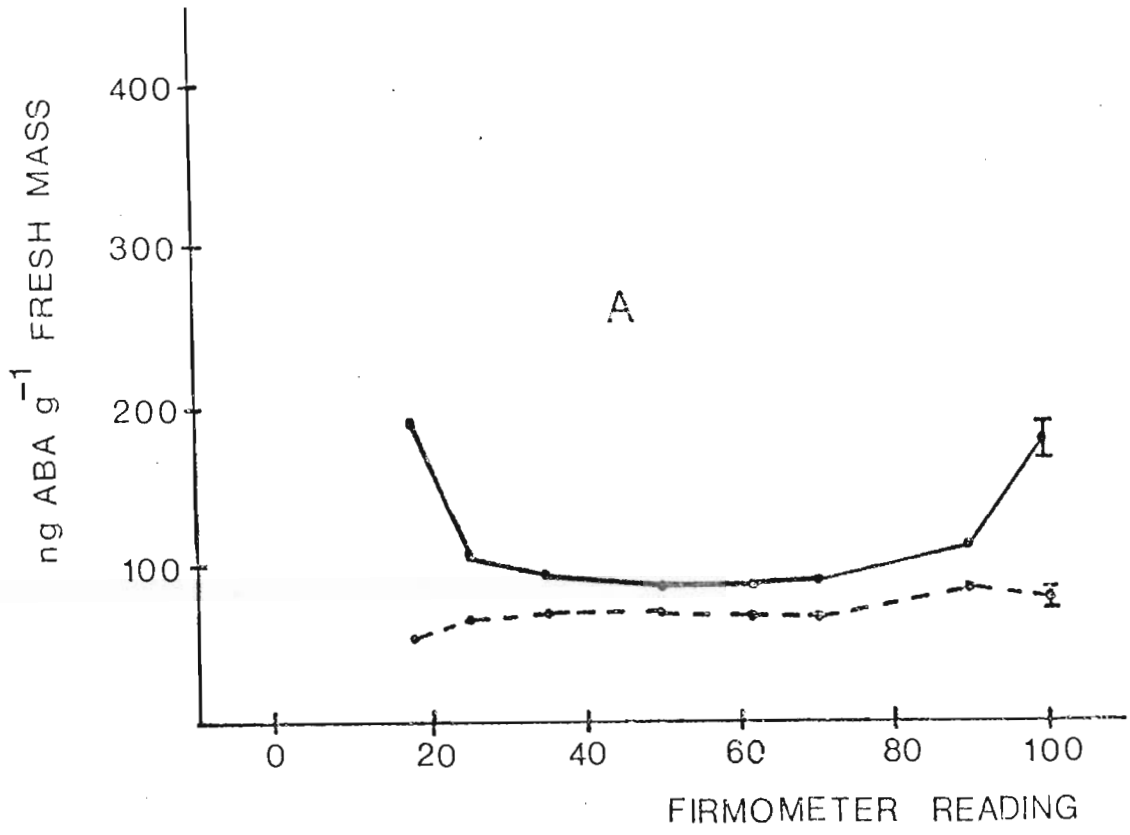
5.2.2.2 Abscisic acid

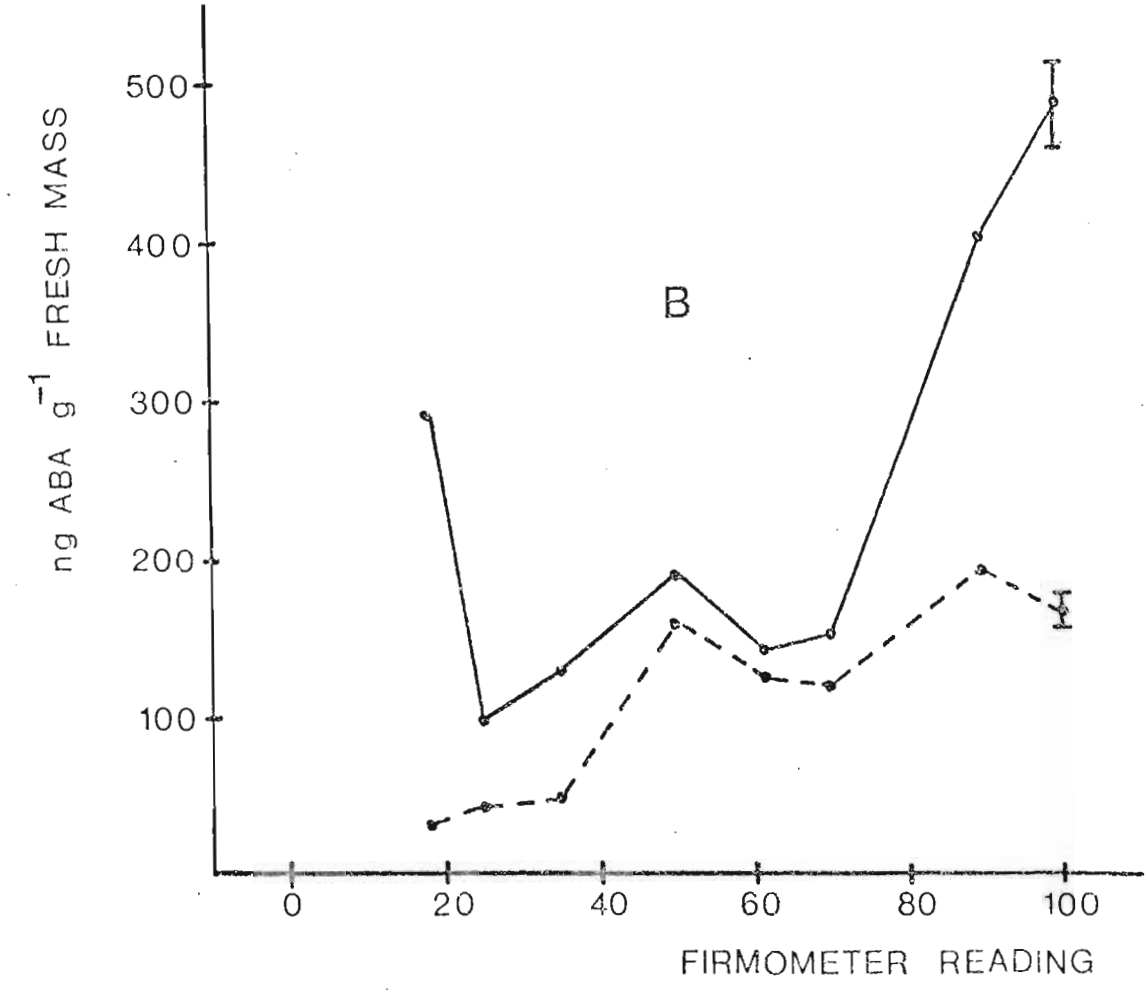
Changes in ABA equivalents that occurred with softening as monitored by firmometer are presented in Figs. 5.11 and 5.12. In April harvested (slow-ripening) fruits, the distal flesh contained about 170ng g^{-1} total ABA at a firmometer reading of 25, two days after harvesting. At this stage the free ABA concentration was however only about 55ng g^{-1}. With fruit softening the total ABA level declined to about 80ng g^{-1} and then slowly began to increase up to 170ng g^{-1} at eating ripeness. In contrast, free ABA levels increased from around 55ng g^{-1} ABA as softening proceeded to about 120ng g^{-1} at a firmometer reading of 100, rising up to about two thirds of the total ABA concentration in fully ripe fruits (Fig. 5.11).

The pattern for late-harvested (fast-ripening) fruit was essentially similar, but with total ABA concentrations in the distal flesh always higher than in early harvested


ng ABA g^{-1} FRESH MASS
400
300
200
100
A
0
20
40
60
80
100
FIRMOMETER READING


ng ABA g^{-1} FRESH MASS
400
300
200
100
B
0
20
40
60
80
100
FIRMOMETER READING


400
300
200
100
ng ABA g^{-1} FRESH MASS
A
0
20
40
60
80
100
FIRMOMETER READING


500
400
300
200
100
ng ABA g^{-1} FRESH MASS
B
0
20
40
60
80
100
FIRMOMETER READING

fruits. In soft fruits (firmometer readings of 80 to 100), levels of total ABA rose to between 450 and 500ng g^{-1} more than twice the level in April harvested fruit. Free ABA concentrations were again comparatively low in firm unripe fruits (40ng g^{-1}), followed by a rising trend (proportional to total ABA) until a firmometer reading of 70, when the concentration of free ABA declined relative to that of total ABA. Generally free ABA concentrations were higher in late-harvested than in early-harvested fruit.

Concentrations of both total and free ABA in the distal rind showed similar overall trends to those for distal flesh, but with less pronounced peaks of total ABA in firm unripe and soft ripe fruits. Free ABA in the rind was initially low (about 25ng g^{-1}) in April fruits, and appeared to gradually increase as softening proceeded to a concentration somewhat above 75ng g^{-1}. Free ABA trends in the rind were remarkably similar in both April and August harvested fruits with the concentrations being slightly higher in the August fruits.

5.2.2.3 Isopentenyl-type cytokinins

IPA and 2iP equivalent trends in softening avocado fruits from the August sampling are presented in Fig. 5.13. Data are corrected for losses during extraction and isolation. The general trend was a decline in 2iP equivalents until a firmometer reading of 50 to 60 followed by an increase in 2iP until a level of 18ng g^{-1} was reached at eating ripeness. The IPA equivalent trend was similar to that for 2iP, but displayed greater variation and range in absolute levels. Fig. 5.14 shows a possible cytokinin:ABA interaction for fruit harvested in August.

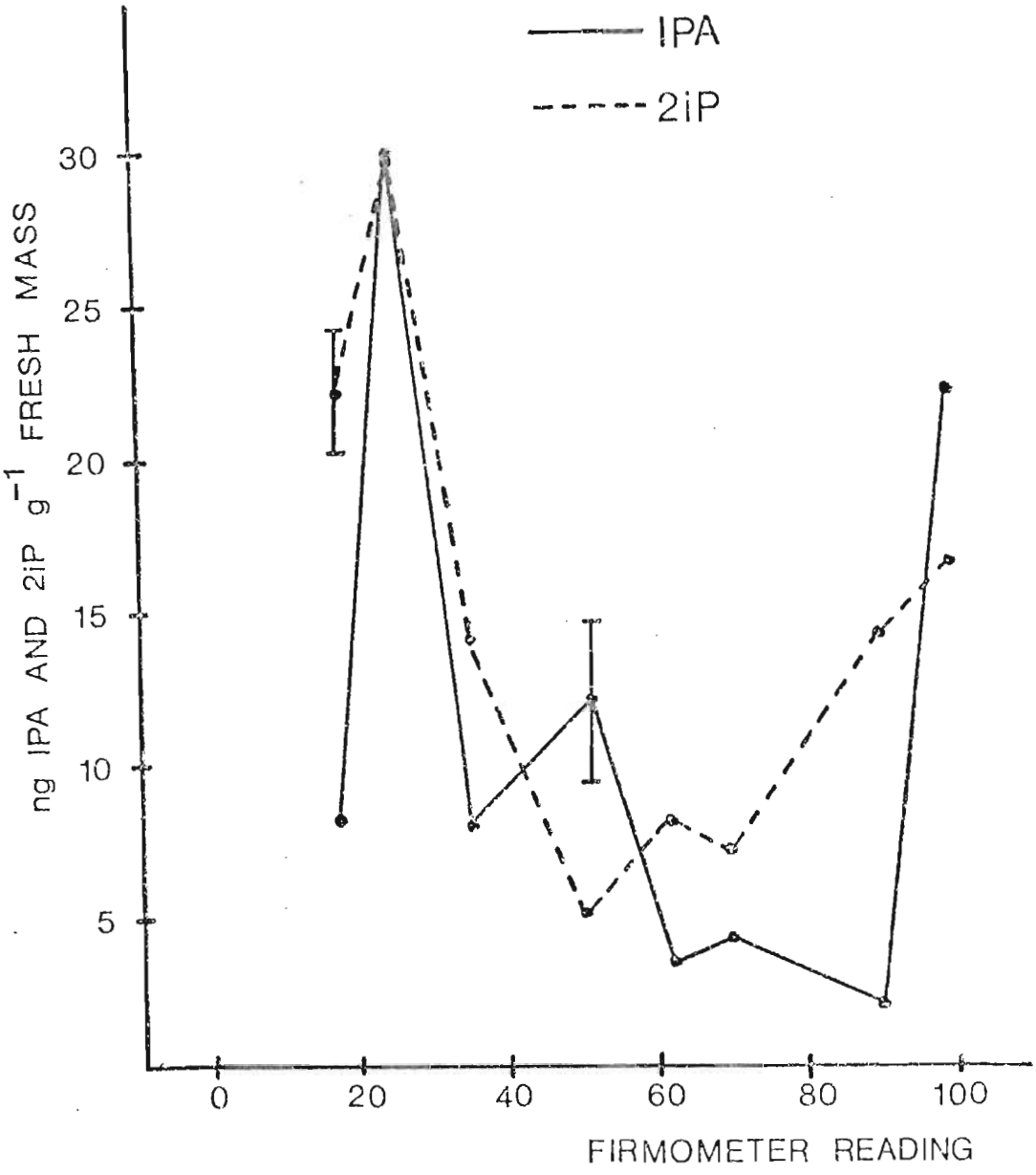


Fig. 5.13 Changes in the levels of 2iP and IPA in ripening 'Fuerte' flesh from fruit harvested in August. Assay SE are presented.

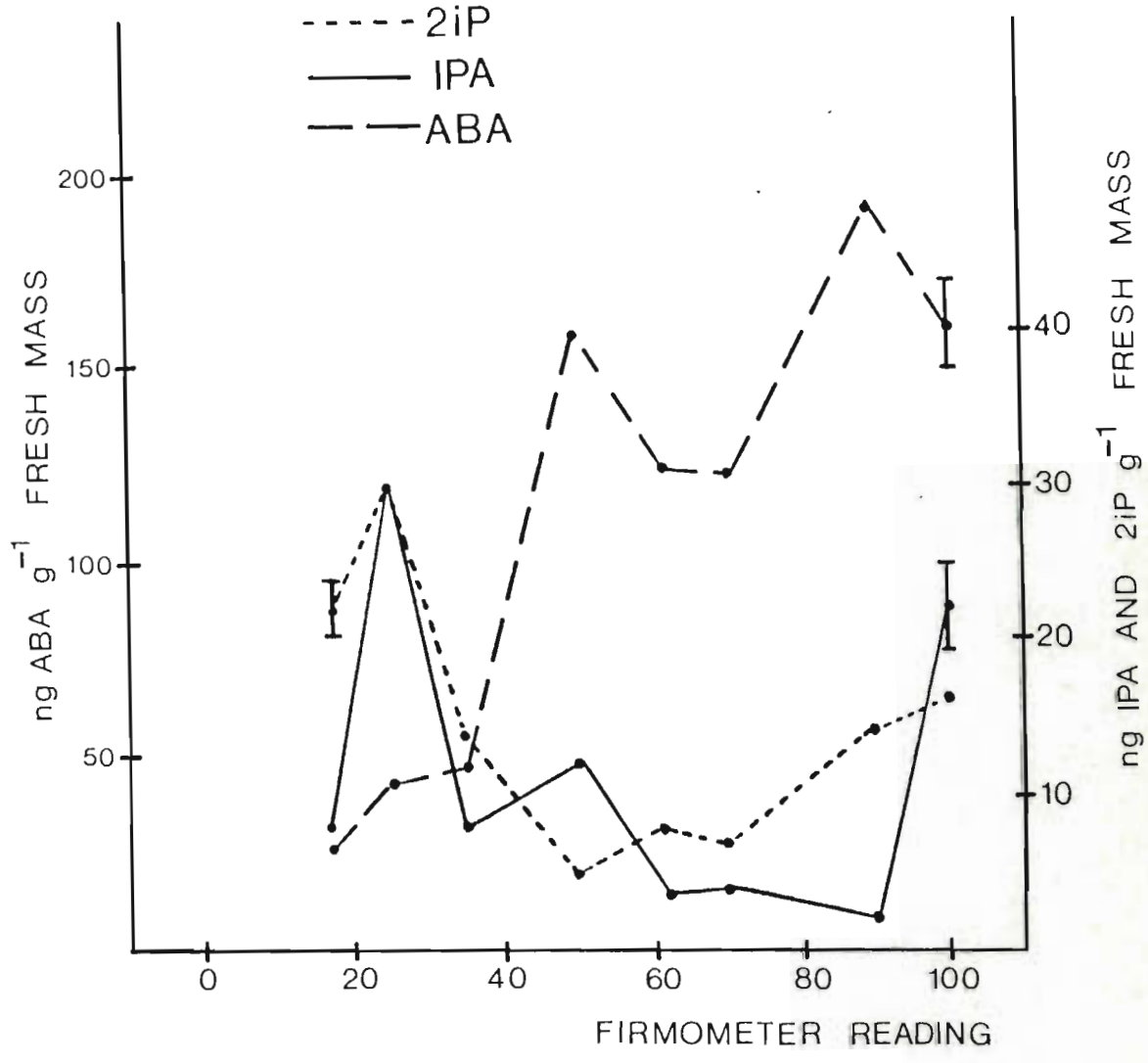


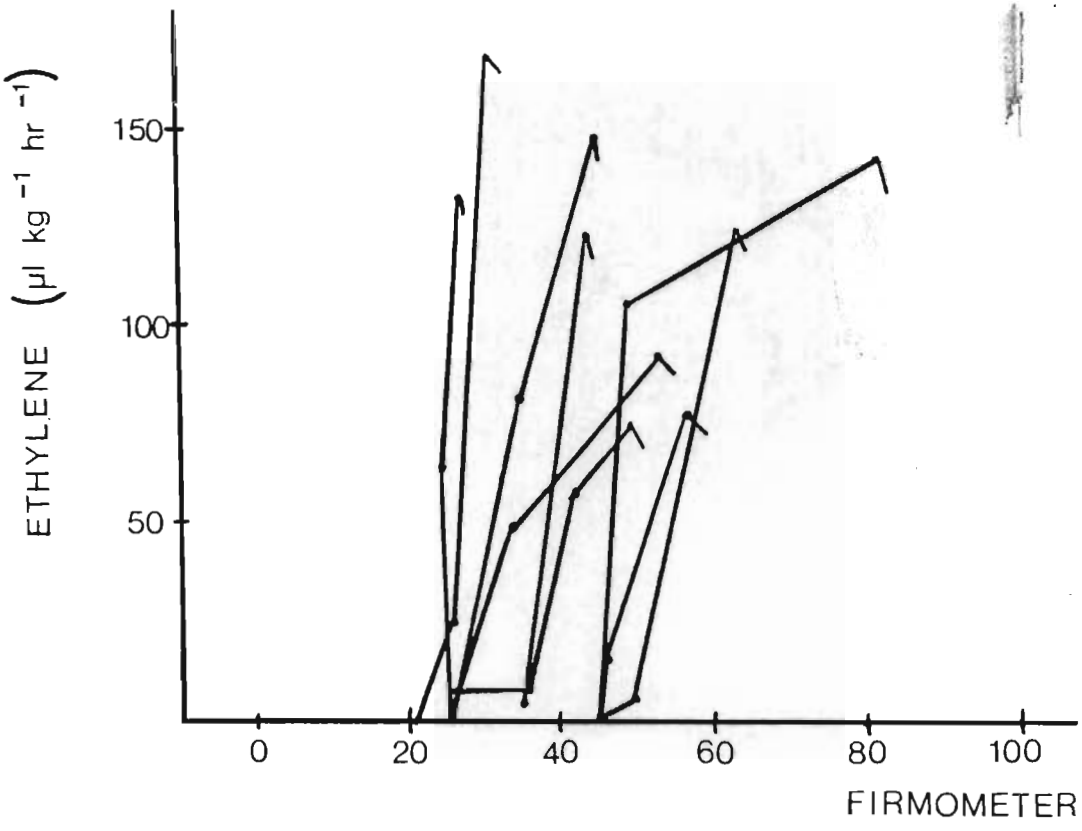
Fig. 5.14 Changes in the levels of IPA, 2iP and free ABA with ripening in 'Fuerte' flesh from fruit harvested in August. Assay SE are presented for data from the distal portion of the fruit.

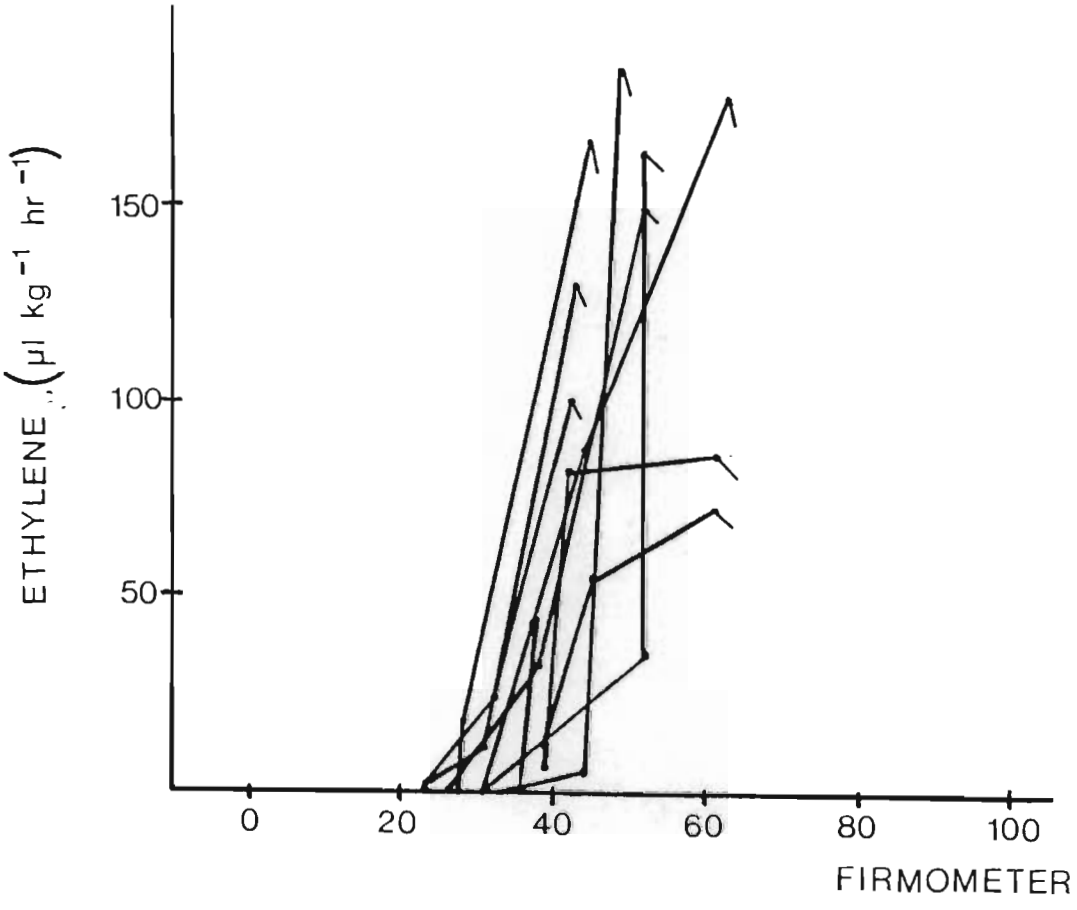
5.2.2.4 Ethylene

The pattern of ethylene evolution in 'Fuerte' is presented in Figs. 5.15 and 5.16 for fruit from the unstressed and stressed irrigation treatments respectively. Although there was considerable variation in ethylene evolution as softening proceeded, especially in stressed fruit, there was a pronounced tendency for the latter to give off more ethylene at lower firmometer readings, i.e. harder fruit. Fruits from the unstressed irrigation treatment evolved most ethylene between firmometer readings of 45 and 65. Since the peak of ethylene evolution and the respiratory climacteric peak nearly coincide in avocado fruits (Kosiyachinda & Young, 1975), it can be inferred that unstressed fruit underwent a climacteric at a later stage of softening than stressed fruit. Of interest was that two stressed fruits failed to produce an ethylene climacteric.

5.2.3 Discussion

In this study the ABA equivalent levels were in the same range as reported earlier for avocado fruits (Weiler, 1979) using similar RIA techniques. The decline in bound (total minus free) ABA observed after harvest must have been due to either utilization or degradation. The subsequent rise in free ABA is a response associated with the avocado climacteric (Adato, Gazit & Blumenfeld, 1976) and follows the onset of ethylene production (McGlasson, Wade & Adato, 1978) which is thought to stimulate ABA synthesis. In the present study only stressed fruit showed an increase in ethylene evolution prior to a firmometer reading of 45, but results were very variable with some fruits requiring 12 days before reaching an ethylene evolution peak. However, once ripening had been set in motion, the autocatalytic response (Adato & Gazit,1974 b)was observed.


ETHYLENE (µl kg⁻¹ hr⁻¹)
150
100
50
0
20
40
60
80
100
FIRMOMETER


ETHYLENE (µl kg⁻¹ hr⁻¹)
150
100
50
0
20
40
60
80
100
FIRMOMETER

The accumulation of bound (total minus free) ABA is consistent with other findings, and lends support to the opinion that these conjugates may be the end products of ABA metabolism. These are stored in the cell and accumulate with age (Weiler, 1980 b). However, this does not account for the rapid decline in bound ABA between firmometer readings of 20 and 40, and the slow increase in free ABA which occurred over the same period. Adato & Gazit(1974 a)found that water stress occurred in ripening avocado fruit (due to significant transpiration losses from the rind, flesh and fruit stalk) and concluded that this was a determining factor in the rate of ripening. The ABA and ethylene trends found in the present study would tend to support this claim if it could be shown that the increase in free ABA was generated in response to water stress.

Concomitant with the increase in oil content of maturing avocado fruits is a significant decrease in water content (Pearson, 1975). This could explain the reduced ripening time of the August harvested fruit as they experienced greater stress, due to reduced water content. However, it must be remembered that the water-stressed fruit from Burgershall Experimental Station took longer than the unstressed fruit to ripen. The unstressed fruit showed ethylene much later than their stressed counterparts, but were more uniform in their response. This made it difficult to associate firmometer readings and ethylene evolution. The presence of surrounding fruit even when evolving large amounts of ethylene did not appear to influence the time required before the onset of the ripening climacteric. The fruit from the water-stressed treatment are, to some extent, conditioned to withstand stress, while those from the unstressed water treatment do not have this pre-conditioning. Thus after harvest it appeared that unstressed fruit experienced greater stress due to transpiration water losses leading to the generation of ABA.

Associated with this response is an earlier softening of the fruit (Climacteric at a higher firmometer reading) and faster ripening times. Unfortunately, the fruits sampled for ethylene were from a different locality to those sampled for ABA, and any comparison between PGSs for these fruits, based on firmometer alone, might lead to erroneous conclusions. A preliminary hypothesis from the present study is that an increase in internal water stress and related ABA levels could be the natural ripening trigger in avocado fruit. ABA could also continue to play a role in the regulation of the rate of ripening in the fruit.

Speculation that ABA is a fruit-ripening PGS is supported mainly by exogenous application experiments (McGlasson *et al.*, 1978). However, Bangerth (1980) believes a direct role for ABA in fruit ripening is doubtful as ethylene appears to be the dominant PGS in fruit ripening (Bangerth, 1978; Bruinsma, 1983). The higher levels of free ABA and the greatly reduced ripening time for fruit harvested in August, 1983 could be due to ABA modulating the sensitivity of flesh tissue to ethylene, thereby accelerating the ripening process. This is in accordance with the role suggested for ABA in fruit ripening by Bruinsma (1983), and lends support to the theory of modulation of sensitivity of the target tissue by other PGSs (Kende, 1982).

ABA synthesis must take place in the fruit as the levels of free ABA (total tends to decrease initially and then increases) increase after detachment from the tree, although the actual site of synthesis is not obvious from the present study. As ABA and ethylene interactions are perhaps only one factor in the ripening process, the role of other PGSs must be evaluated. From Fig. 5.14 it was seen that in general, the trends for the cytokinins IPA and 2iP tended to oscillate when superimposed on the ABA trend. ABA is known to modify cytokinin metabolism (Letham & Palni, 1983) and could well play a role together with various cytokinins in

fruit ripening, particularly in the latter stages when ripening proceeds very rapidly. However, further investigation is needed before any definite conclusions can be drawn. It must also be remembered that zeatin, zeatin riboside, the gibberellins and inhibitors other than ABA could also play a role in avocado fruit ripening. However, these PGSs were not investigated.

OVERALL DISCUSSION AND CONCLUSIONS

The experiments reported in this study had two main objectives in mind :

(a) To establish RIAs for as many PGSs as possible, to critically validate them, and to determine if they were suitable for use with avocado fruit material.

(b) To establish general PGS trends in developing and ripening 'Fuerte' fruit using RIA. The formulation of new avocado research projects could then consider these PGS trends as one of the controlling mechanisms in growth and development.

The use of any new technique in the field of PGS analysis is bound to meet with some initial resistance. This is both justified and desirable until the technique has proved itself to be superior in some way to the more established methods of PGS analysis. Failing this, it should be discarded. RIA, which relies heavily on a largely theoretical specificity, needs stringent validation before general acceptance. The obvious advantages of sensitivity, sample volume and speed have been well lauded by various immunoassay developers (Landon & Moffat, 1976; van Vunakis, 1980; Chard, 1981; Weiler, 1982 a).

Research reported in this thesis has shown that PGSs are not very immunogenic, even when linked to BSA. Titers were very low when compared to titers achieved using animal hormones of a similar molecular mass. The selection of a suitable separation procedure to separate bound and free fractions was found to be one of the most critical decisions in RIA development. In contrast to most other researchers (Pengelly & Meins, 1977; Weiler,

1979; Walton, *et al.*, 1979; Weiler, 1980 a; Ernst *et al.*, 1983), data obtained during this study indicated the superiority of dextran-coated charcoal as a separation procedure, particularly when working with pure standards. Charcoal was particularly effective as regards reproductibility and efficiency. However, in reviewing the results, a potential problem could be experienced with charcoal when applied to biological extracts with a high oil content. Oil could interfere with the results by preventing adsorption of a portion of the free fraction. This could account for the problems experienced in the establishment of extract dilution curves. Clearly, this aspect requires much further investigation, and perhaps the development of a completely new separation technique.

The theoretical specificity of the antibody would appear to be largely justified. Results obtained from this study indicated that even the smallest structural change to the compound of interest invariably destroyed binding activity. The exception was ABA antiserum, which displayed cross-reactivity for several ABA metabolites.

This study reports on the development of four RIAs. However, several others, notably zeatin, zeatin riboside and gibberellic acid were attempted. The development of RIAs for these compounds was unsuccessful, due primarily to the inability to obtain suitable radioactive tracer, even from commercial institutions which specialize in radiotracer production.

As a technique, RIA is both rapid and sensitive but already a cheaper and faster related technique, enzymeimmunoassay (EIA), is available. However, this technique has several developmental disadvantages. In EIA there is virtually no control over antibody and only limited control over tracer. In addition, the antibody has to be purified before suitable for use in EIA. Therefore, RIA would still

appear to be superior for developmental work. However, once such an assay has been established it would be advantageous to convert to EIA as this technique does not have the health hazards (enzyme tracer versus radioactive tracer), and is faster and less expensive.

The first objective of this study, the RIA developmental aspects, was successful for the auxin IAA, two cytokinins 2iP and IPA, and the inhibitor ABA. In view of the results obtained, particularly as regards assay time, sample volume potential, automation potential, ease of execution, sensitivity and specificity, the immunoassay procedures warrant recognition as a PGS assay technique. There appears to be no reason why immunoassay should not be used in more multi-PGS studies where the analysis of large numbers of samples are required.

The second part of this study dealt with PGS analysis in avocado fruit material. Results pertaining to the problems experienced in avocados and the aims of this study as stated in the introduction are now discussed.

The collection of data from fruitlets which would have either abscissed or have been retained by the tree is nearly impossible, as the abscission process in avocado is apparently random (Adato & Gazit, 1977), and the assay technique destructive. The collection and determination of PGS levels in abscissed fruitlets could be misleading for the following reasons: the fruitlet is already senescent; there has been the formation of an abscission layer; and in all likelihood, there has been transport of promotive PGSs out from the fruitlet into the tree. The only feasible option at present is, therefore, to assay attached fruitlets for PGS levels and trends and to see which PGSs, if any, coincided with the fruitlet abscission stage. However, the results obtained from the determination of endogenous PGS levels often pose new questions as the

elucidation of other problems is attempted, e.g. the role of IAA in fruitlet abscission, which is considered below.

There is strong evidence that ethylene is involved in the abscission of young avocado fruitlets (Davenport & Manners, 1982) even after ovule fertilization (Sedgley, 1980). Abscission appears to be a random process and is not related to fruitlet size within the fruit cluster (Adato & Gazit, 1977). Results from the present study have shown that there is a very sharp IAA peak in attached fruit during the fruitlet drop stage. Fruit sampled two weeks prior to this drop period had much reduced IAA levels, as was the case with fruit sampled two weeks after the main fruitlet drop period. In addition, ABA levels were low in attached fruit before, during and after this drop period.

Davenport & Manners (1982) noted that an ethylene 'shock' did not occur in fruit that failed to absciss. There is obviously some regulatory mechanism which determines which determines which fruitlets absciss and which are retained by the tree. Evidence from the current study indicates that IAA could be a controlling factor. Associated evidence would tend to support this hypothesis. Other studies have shown that the seed often contains the highest concentrations of IAA, e.g. in apple (Luckwill, 1953) and avocado (Gazit & Blumenfeld, 1972). The high IAA levels could have been involved with ethylene promotion as auxin has been shown to stimulate ethylene synthesis (Dilley, 1969; Leopold & Kriedemann, 1975; Goodwin, 1978). The prevention of the ethylene 'shock' and associated fruitlet abscission could, therefore, be under the control of other promotive PGSs such as 2iP which showed a strong peak during this period. In this case IAA would exert a promotory effect upon ethylene. Alternatively, the IAA could have been necessary to reduce or prevent the ethylene 'shock', i.e. the IAA had an antagonistic effect upon ethylene evolution. Similarly, while it was noted that the

cytokinin, 2iP, showed a strong peak during this period, it might have been erroneous to relate this to fruitlet retention, as this peak was maintained for considerably longer than the critical abscission period.

The ripening model proposed by Dilley (1969) has been taken and enlarged upon to include development. However, this new model is specific for avocado as this tree shows some unusual features, such as the inability of the fruit to ripen while still attached to the tree. Similarities to Dilley's model are not described but major alterations or additions are discussed. The addition of gibberellin and the other cytokinins would be necessary before this model can be considered complete. The avocado fruit development and senescence PGS model as proposed by the writer is presented in Fig. 6.1.

A 'lag' phase was introduced which occurred after harvest. This was because of the markedly changed levels of 2iP, IPA and ABA which occurred in 'legally' mature fruits as opposed to fruits which had been harvested for two or three days. During this period there was some form of metabolism conversion, as there could no longer have been PGS transport into the now detached fruit. Any increase in PGS levels was from either release from bound forms or synthesis of new PGSs within the fruit.

The two cytokinins assayed, namely 2iP and IPA, gave markedly differing trends during fruit development. Therefore, the presentation of a single general cytokinin trend is undesirable. In addition, other cytokinins not assayed in the present study could well have shown still further differing trends, although they may have also been very similar. This is obviously an avenue that deserves further attention. The actual role of the differing cytokinins in fruit development remains an unanswered, but interesting topic.

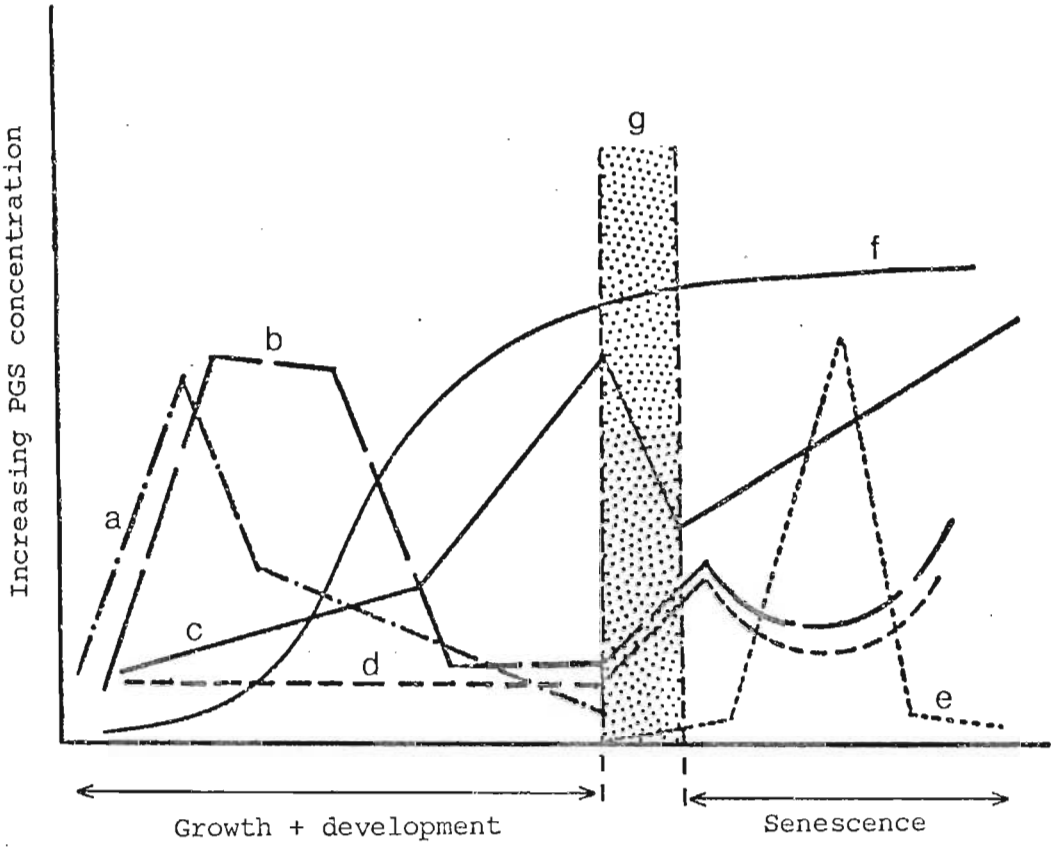


Fig. 6.1 Hypothetical model for the PGS control of fruit development and senescence in the avocado. Trends for IAA (a), the cytokinin 2iP (b), ABA (c), the cytokinin IPA (d), ethylene (e) as well as the growth curve (f) and the 'lag' phase between harvest and the onset of ripening (g) are presented.

A vague but perhaps inaccurate indication of cytokinin synthesis in young fruit may be obtained from the detection of 2iP in these extracts. This compound is thought to be a component of the cytokinin synthetic pathway (Letham, 1978). There is evidence for the involvement of cytokinins in the regulation of assimilate partitioning (Richards, 1980; Goussard, 1981). The high 2iP levels detected in young fruitlets (Fig. 5.4) may have actively

assisted in increasing the 'sink' strength of the fruit. This prolonged high 2iP peak is therefore presented in Fig. 6.1.

Levels of ABA showed two general increases, one during fruit development and the other during ripening. There also appeared to be a marked utilization, conversion or degradation of ABA after harvest and prior to the onset of ripening.

Fruit development, growth and senescence processes and associated assimilate partitioning and stress are just a few of many processes thought to be under the integrated control of PGSs (Trewavas, 1981, 1982; Wareing, 1982). Trewavas (1982) proposed that these processes are mediated by the tissue sensitivity to the various PGSs, and that this sensitivity varies with time. This allows more for a threshold level concept and not as much for a dose-response concept. Should this hypothesis be true however, it would still be desirable to know the threshold level for a particular PGS to be activated for a specific response at a fixed growth stage. This would allow for possible manipulation of growth by either maintaining certain PGS levels above a threshold requirement, or suppressing a certain response by the addition of an antagonistic PGS, a response for which there are many examples (Leopold & Kriedemann, 1975; Goodwin, 1978).

Although the present study has improved, to a greater or lesser extent, upon the current knowledge of PGS levels and trends in avocado fruit development and senescence, and possible PGS regulation thereof, there is undoubtedly scope for extensive further investigation, using, among other techniques, RIA. There is, in particular, the possibility of using this new technique to investigate, in greater depth, some of the pre- and post-harvest problems facing the local export-oriented avocado industry.

SUMMARY

The objective of the present study was to develop, optimize and validate several RIAs for various PGSs. The completed RIAs were then used to determine PGS trends in 'Fuerte' avocado fruit from flowering through development to ripeness. This would give an indication as to which PGSs were involved in the various developmental and senescent processes in the avocado fruit.

Antibodies to IPA were obtained from rabbits inoculated with a periodate-derived IPA-BSA conjugate. The serum dilution titer which bound 30% of the added tracer was 1:250. The antiserum was relatively specific cross-reacting with only one other tested compound, 2iP, for which it displayed 25% cross-reactivity. The developed IPA RIA had a measuring range from 0,1 to 100ng. Because of the high cross-reactivity to 2iP the anti-IPA serum was used for the development of a RIA for 2iP. To compensate for reduced sensitivity a serum titer dilution of 1:50, which bound 45% of the total added tracer, was used. The assay measuring range for 2iP was from 0,5 to 100ng. Purification of the avocado extract was necessary for validation, primarily to remove a contaminant which interfered with either the antibody or the dextran-coated charcoal separation procedure. This was achieved by Dowex 50W-X8 and cellulose TLC. The preparation of plant extract prior to RIA was the rate-determining step and under the conditions stated a maximum of 20 samples a day could be processed.

A RIA for ABA was established from antibodies produced by rabbits inoculated with a carbodiimide-derived (±)ABA-BSA conjugate. The serum dilution titer which bound 20% of the total added tracer was 1:125. High cross-reactivity for several ABA metabolites was displayed by this particular antiserum. Therefore, isolation of the free active ABA

component from the composite biological sample was necessary prior to quantitation by RIA. This was achieved by solvent partitioning and silica gel TLC. A contaminant present in the crude avocado fruit extracts interfered with either the antibody or the separation procedure. This was removed by solvent partitioning. Due to the TLC step only 30 samples could be processed a day.

The RIA for IAA was established using antibodies produced in a sheep inoculated with a formaldehyde-derived IAA-BSA conjugate. This particular conjugate was not very immunogenic and antibodies were only obtained after repeated re-inoculations. The resultant antibodies were unstable and did not store well. The serum dilution titer which bound 30% of the total added IAA-tracer was 1:25. The serum was very specific and did not display cross-reactivity for any of the compounds tested. A contaminant, present in some of the crude avocado extracts, interfered with the validation of the RIA and was removed using solvent partitioning. Preparation of plant extract was the most time-consuming step, therefore only 30 samples could be processed a day.

Based on mass the 'Fuerte' avocado displayed a typical sigmoid growth curve. In general the PGSs tested coincided well with important growth stages in the avocado fruit. IAA showed a peak of about 150ng g^{-1} just after fruit set, whereafter the level of endogenous IAA steadily declined. The level of IPA was relatively constant throughout development. In contrast a second cytokinin, 2iP, showed a peak in late October, the level of which was maintained for almost 7 weeks before rapidly declining. This 2iP peak coincided with the exponential growth phase. The level of ABA rose consistently throughout development and reached a concentration of 100ng g^{-1} as the fruit approached maturity. Indications are that all the PGSs tested (with the possible exception of IPA) interacted to

play a role during development of the avocado fruit.

Firmometer readings were used as a constant in all ripening studies. Avocado fruit harvested later in the season had reduced ripening times at room temperature. Stressed fruit took longer to ripen than their unstressed counterparts. Water-stressed fruit showed an ethylene climacteric at an earlier stage of softening than did unstressed fruit. However, the water-stressed avocado fruits were more inconsistent in the time taken to the ethylene climacteric.

In ripening avocado fruit, the level of free ABA rose as softening progressed. In contrast, total ABA levels fell initially, but then also proceeded to rise after a firmometer reading of between 50 and 60. In the later harvested fruit the concentration of ABA at eating ripeness was approximately double that of the early harvested fruit.

This study has shown that although the establishment of RIAs was relatively routine, certain problems were experienced with optimization and validation of the assays. In contrast to most other PGS isolation and quantitation techniques, RIA has shown that it is well suited to multi-PGS studies and batch-type analysis. In avocado fruit development there appeared to be a critical period in November and December when three of the PGSs tested, namely IAA, 2iP and ABA, interacted to either retain or abscise the fruit. It would appear that in avocado fruit the stimulus to ripening is involved with water stress and associated ABA levels. Water stress and ABA also appear to control the rate of ripening.

LITERATURE CITED

ADATO, I. & GAZIT, S. 1974a. Water-deficit stress, ethylene production and ripening in avocado fruits. Plant Physiol. 53: 45-46.

_____ 1974b. Post harvest response of avocado fruits of different maturity to delayed ethylene treatments. Plant Physiol. 53: 899-902.

_____ 1977. Role of ethylene in avocado fruit development and ripening. 1. Fruit drop. J. Exp. Bot. 28: 636-643.

_____ & BLUMENFELD, A. 1976. Relationship between changes in abscisic acid and ethylene production during ripening of avocado fruit. Aust. J. Plant Physiol. 3: 555-558.

ALEXANDER, J.W. & GOOD, R. 1977. Fundamentals of clinical immunoassay. Philadelphia: Saunders Press.

ANON., 1979. The Farr technique. New England Nuclear Catalogue.

_____ 1980. Research products. Amersham: Amersham International.

ARMSTRONG, D.J., BURROWS, W.J., EVANS, P.K. & SKOOG, F. 1969. Isolation of cytokinins from tRNA. Biochem. Biophys. Res. Comm. 37: 451-456.

ATZORN, R. & WEILER, E.W. 1983a. The immunoassay of gibberellins. 1. Radioimmunoassay for the gibberellins A_1, A_3, A_4, A_7, A_9 and A_{20}. Planta 159: 1-6.

_____ 1983b. The immunoassay of gibberellins. 2. Quantitation of GA_3, GA_4 and GA_7 by ultra sensitive solid phase enzyme immunoassay. Planta 159: 7-11.

_____ 1983c. The role of endogenous gibberellins in the formation of α-amylase by aleurone layers of germinating barley caryopses. Planta 159: 289-299.

BAILE, J.B. 1941. The climacteric rise in respiration of the 'Fuerte' avocado fruit. Proc. Amer. Soc. Hort. Sci. 39: 137-142.

BAILE, J.B. 1960. The postharvest biochemistry of tropical and subtropical fruits. In: CHICHESTER, C.O., MRAK, E.M. & STEWART, G.F. (eds.), Advances in Food Research. New York: Academic Press.

BANDURSKI, R.S. & SCHULTZE, A. 1977. Concentrations of indole-3-acetic acid and its derivatives in plants. Plant Physiol. 60: 211-213.

BANGERTH, F. 1978. The effect of a substituted amino acid on ethylene biosynthesis, respiration, ripening and preharvest drop of apple fruits. J. Amer. Soc. Hort. Sci. 103: 401.

_____ 1980. Funktion der abscisinsaure bei der reife von apfelfruchten. Gartenbauwiss 45: 224 (Cited by RHODES, 1980).

BANGHAM, D.R. & COTES, P.M. 1971. Reference standards for radioimmunoassay. In: KIRKHAM, K. & HUNTER, W. (eds.), Radioimmunoassay methods. Edinburgh: Churchill Livingstone.

_____ 1974. Radioimmunoassay and saturation analysis: standardization and standards. Br. Med. Bull. 30: 12-17.

BARMORE, C.R. 1977. Avocado fruit maturity. Proc. First Internat. Trop. Fruit Short Course. pp. 103-109. Gainsville: Florida Univ. Press.

BAUMINGER, S. & WILCHEK, M. 1980. The use of carbodiimides in the preparation of immunizing conjugates. Methods in Enzymol. 70: 151-159.

BERNIER, G.M. 1978. Antibodies and immunoglobulins: structure and function. In: BELLANTI, J.A. (ed.), Immunology. Philadelphia: Saunders Press.

BERSON, S.A. & YALOW, R.S. 1968. General principles of radioimmunoassay. Clin. Chim. Acta. 22: 51-69.

BINOUX, M.A. & ODELL, W.D. 1973. Use of dextran-coated charcoal to separate antibody-bound from free hormone: a critique. J. Clin. Endocr. Metab. 36: 303-310.

BLUMENFELD, A. & GAZIT, S. 1970. Cytokinin activity in avocado seeds during fruit development. Plant Physiol. 46: 331-333.

BLUMENFELD, A. & GAZIT, S. 1972. Gibberellin-like activity in the developing avocado fruit. Physiol. Plant.27: 116-120.

_____ 1974. Development of seeded and seedless avocado fruits. J. Amer. Soc. Hort. Sci. 99: 442-448.

BOWER, J.P., VAN LELYVELD, L.J. & NEL, M.E. 1982. The influence of 'Fuerte' fruit water potential on ripening. S. Afr. Avocado Grs. Assoc. Yrbk. 5: 36-38.

BREDELL, G.S. 1983. Voorwoord. S. Afr. Avocado Grs. Assoc. Yrbk. 6: 5.

BRENNER, M.L. 1981. Modern methods for plant growth substance analysis. Ann. Rev. Plant Physiol. 32: 511-538.

BRUINSMA, J. 1981. Hormonal regulation of senescence, ageing, fading and ripening. Acta Hort. 138: 141-163.

BUCHANAN, K.D. & McCARROL, A.M. 1971. Comparison of methods of separation of free and bound hormone in the radioimmunoassay of insulin and glucagin. In: KIRKHAM, K. & HUNTER, W. (eds.), Radioimmunoassay methods. Edinburgh: Churchill Livingstone.

BUCHANAN, K.D., McCARROL, A.M. & ARDILL, J. 1971. Separation of bound and free labelled antigen. In: KIRKHAM, K. & HUNTER, W. (eds.), Radioimmunoassay methods. Edingurgh: Churchill Livingstone.

CATT, K. & TREGEAR, C.W. 1967. Solid phase radioimmunoassay in antibody coated tubes. Science 158: 1570-1571.

CHASE, M.W. 1967. Production of antiserum. In: WILLIAMS, C.A. & CHASE, M.W. (eds.), Methods in immunology and immunochemistry. New York: Academic Press.

CHARD, T. 1971. The extraction and concentration of hormones from biological fluids. In: KIRKHAM, K. & HUNTER, W.M. (eds.), Radioimmunoassay methods. Edinburgh: Churchill Livingstone.

_____ 1978. An introduction to radioimmunoassay and related techniques. Amsterdam: North Holland Press.

CHARD, T. 1980. Ammonium sulphate and polyethylene glycol as reagents to separate antigen from antigen-antibody complexes. Methods in Enzymol. 70: 280-291.

_____ 1981. An introduction to radioimmunoassay and related techniques. Amsterdam: Elsevier Biomedical Press.

_____, KITAU, M.J. & LANDON, J. 1970. The development of a radioimmunoassay for oxytocin. J. Endocr. 46: 269-278.

_____, MARTIN, M. & LANDON, J. 1971. The separation of antibody bound from peptides using ammonium sulphate and ethanol. In: KIRKHAM, K. & HUNTER, W.M. (eds.), Radioimmunoassay methods. Edinburgh: Churchill Livingstone.

CHEN, W. 1983. Cytokinins of the developing mango fruit. Plant Physiol. 71: 356-361.

CIHA, A., BRENNER, M. & BRUN, W. 1977. Rapid separation and quantification of abscisic acid from plant tissues using HPLC. Plant Physiol. 59: 821-826.

CRANE, J.C. 1964. Growth substances in fruit setting and development. Ann. Rev. Plant Physiol. 15: 303-326.

CUMMINGS, K. & SCHROEDER, C.A. 1942. Anatomy of the avocado fruit. Calif. Avoc. Soc. Yrbk. 26: 56-64.

CUNNINGHAM, A.J. 1978. Understanding immunology. New York: Academic Press.

DAVENPORT, T.L. & MANNERS, M.M. 1982. Nucellar senescence and ethylene production as they relate to avocado fruitlet abscission. J. Exp. Bot. 33: 815-825.

DEN HOLLANDER, F.C. & SCHUURS, A. 1971. Separation of bound from free labelled antigen. In: KIRKHAM, K. & HUNTER, W.M. (eds.), Radioimmunoassay methods. Edinburgh: Churchill Livingstone.

DILLEY, D.R. 1969. Hormonal control of fruit ripening. HortScience 4: 111-114.

DREHER, T.W. & POOVAIAH, B.W. 1982. Changes in auxin content during development in strawberry fruit. J. Plant Growth Regul. 1: 267-276.

EHMANN, A. & BANDURSKI, R.S. 1974. The isolation of di-O-(indole-3-acetyl)-myo-inositol from mature kernels of *Zea mays*. Carbohydr. Res. 36: 1-12.

EKINS, R.P. 1974. Radioimmunoassay and saturation analysis. Br. Med. Bull. 30: 3-11.

ERICKSON, L.C. 1966. Seed coat thickness: a guide to avocado maturity. Calif. Citrogr. 51: 260-261.

ERLANGER, B.F. 1973. Principles and methods for the preparation of drug conjugates for immunological studies. Pharmac. Rev. 25: 271-301.

_____ & BEISER, S.M. 1964. Antibodies specific for ribonucleotides and their reaction with DNA. Proc. Natl. Acad. Sci. 52: 68-74.

ERNST, D., SCHAFER, W. & OESTERHELT, D. 1983. Isolation and quantitation of isopentenyladenosine in an anise cell culture by single-ion monitoring, radioimmunoassay and bioassay. Planta 159: 216-221.

FELDMAN, H. & RODBARD, D. 1971. Mathematical theory of radioimmunoassays. In: ODELL, W.D. & DAUGHADAY, W.H. (eds.), Principles of competitive binding assays. Philadelphia: Lippencott.

FEUNG, C.S., HAMILTON, R.H. & MUMMA, R.O. 1975. Indole-3-acetic acid: mass spectra and chromatographic properties of amino acid conjugates. J. Agric. Food Chem. 23: 1120-1124.

FREUND, J. 1947. Some aspects of active immunization. Ann. Rev. Microbiol. 1: 291-308.

_____ 1951. The effect of paraffin oil and mycobacteria on antibody formation and sensitization. Amer. J. Clin. Path. 21: 645-656.

FUCHS, S. & FUCHS, Y. 1969. Immunological assay for plant hormones using specific antibodies to indoleacetic acid and gibberellic acid. Biochim. Biophys. Acta 192: 528-530.

_____, HAIMOVICH, J. & FUCHS, Y. 1971. Immunological study of plant hormones. Detection and estimation by immunological assay. Europ. J. Biochem. 18: 384-390.

FUCHS, Y. & GERTMAN, E. 1974. Insoluble antibody column for isolation and quantitative determination of gibberellins. Plant Cell Physiol. 15: 629-633.

_____, MAYAK, S. & FUCHS, S. 1972. Detection and quantitative determination of abscisic acid by immunological assay. Planta 103: 117-125.

GARVEY, J.S., CREMER, N.E. & SUSSDORF, D.H. 1977. Methods in immunology. Massachusetts: Benjamin Press.

GAZIT, S. & BLUMENFELD, A. 1969. The role of plant growth regulators in the development, growth and maturation of the fruit. In: The Division of Subtropical Horticulture; The Volcani Institute of Agriculture Research, 1960-1969.

_____ 1970. Cytokinin and inhibitor activities in the avocado fruit mesocarp. Plant Physiol. 46: 334-336.

_____ 1972. Inhibitor and auxin activity in the avocado fruit. Physiol. Plant. 27: 77-82.

GENUTH, S., FROHMAN, L.A. & LEBOVITZ, H.E. 1965. A radioimmunoassay method for insulin I-125 and gel filtration. J. Clin. Endocr. Metab. 25: 1043-1049.

GIESE, J. & NIELSEN, M.D. 1971. Separation of bound and free labelled antigen. In: KIRKHAM, K. & HUNTER, W.M. (eds.), Radioimmunoassay methods. Edinburgh: Churchill Livingstone.

GOLDBACH, H. & GOLDBACH, E. 1977. Abscisic acid translocation and influence of water stress on grain abscisic acid content. J. Exp. Bot. 28: 1342-1350.

GOODFRIEND, T.L. 1968. Radioimmunoassay of brandykinin and angiotensin. In: MARGOULIES, M. (ed.), Proteins and polypeptide hormones. Internat. Congr. Series 161. Amsterdam.

GOODWIN, P.B. 1978. Phytohormones and fruit growth. In: LETHAM, D.S., GOODWIN, P.B. & HIGGINS, T.J. (eds.), Phytohormones and related compounds. Vol. 2. Amsterdam: Elsevier Biomedical Press.

GOTTLIEB, C.W., LAU, K.S., WASSERMAN, L.R. & HERBERT, V. 1965. Rapid charcoal assay for intrinsic factor (IF), gastric juice unsaturated B binding capacity, antibody to IF, and serum unsaturated B binding capacity. Blood 25: 875-884.

GOUSSARD, P.G. 1981. Effects of cytokinins on elongation, proliferation and total mass of shoots derived from shoot apices of grapevine cultured *in vitro*. Vitis 20: 228-234.

GRAEBE, J.E. & ROPERS, H.J. 1978. Gibberellins. In: LETHAM, D.S., GOODWIN, P.B. & HIGGINS, T.J. (eds.), Phytohormones and related compounds. Vol. 1. Amsterdam: Elsevier Biomedical Press.

GREENWOOD, F.C. 1971. Separation of bound from free labelled antigens. In: KIRKHAM, K. & HUNTER, W.M. (eds.), Radioimmunoassay methods. Edinburgh: Churchill Livingstone.

_____, HUNTER, W.M. & GLOVER, J.S. 1963. The preparation of I labelled human growth hormone of high specific activity. Biochem. J. 89: 114-123.

HABER, E., PAGE, L.B. & RICHARDS, L. 1965. Radioimmunoassay employing gel filtration. Anal. Biochem. 12: 163-172.

HACKER, B., VAN VUNAKIS, H. & LEVINE, L. 1970. Formation of an antibody with serologic specificity for N-(Δ-isopentenyl)adenosine. J. Immunol. 108: 1726-1728.

HALE, C.R. & COOMBE, B.G. 1974. Abscisic acid - an effect on the ripening of grapes. In: BIELSKI, R.L., FERGUSSON, A.R. & CREPWELL, M.M. (eds.), Mechanism of regulation of plant growth. Roy. Soc. New Zealand: Wellington.

HALES, C.N. & RANDLE, P.J. 1963. Immunoassay for insulin antibody precipitate. Biochem. J. 88: 137-146.

HATTON, T.T. & REEDER, W.F. 1969. Maintaining market quality of Florida avocados. Proc. Confer. Trop. and Subtrop. Fruits. London pp. 227-280.

HEDING, L.G. 1966. In: DONATO, L., MILHAUD, G. & SIRCHIS, J. (eds.), Labelled proteins in tracer studies. European Atomic Energy Commission, Brussels. (Cited by CHARD, 1981).

HERBERT, V., LAU, K.S., GOTTLIEB, C.W. & BLEICHER, S.J. 1965. Coated charcoal immunoassay of insulin. J. Clin. Endocr. Metab. 25: 1375-1384.

HILL, T.A. 1980. Endogenous plant growth substances. Studies in biology No. 40. London: Edward Arnold.

HOBART, M. 1976. Immunoglobulins as proteins. In: HOBART, M. & McCONNELL, I. (eds.), The immune system. Oxford: Blackwell Scientific.

HOBART, M. & McCONNELL, I. 1976. The Immune system. Oxford: Blackwell Scientific.

HODGSON, R.W. 1947. Bearing habits of the avocado. Calif. Avoc. Soc. Yrbk. pp. 35-39.

_____ & CAMERON, S.H. 1936. Temperature in relation to the alternate bearing behavior of the 'Fuerte' avocado variety. Proc. Amer. Soc. Hort. Sci. 33: 55-60.

HOOD, L.E. & McCONNELL, I. 1976. The immune system. Oxford: Blackwell Scientific.

_____, WEISSMAN, I.L. & WOOD, W.B. 1978. Immunology. San Francisco: Benjamin Cummings.

HORGAN, R. 1978. Analytical procedures for cytokinins. In: HILLMAN, J.R. (ed.), Isolation of plant growth substances. Cambridge: Cambridge Univ. Press.

_____ 1981. Modern methods for plant hormone analysis. In: REINHOLD, L., HARBORNE, J. & SWAIN, T. (eds.), Progress in phytochemistry. Vol. 7. London: Pergamon Press.

HSU, F.C. 1979. Abscisic acid accumulation in developing seeds of *Phaseolus vulgaris* L. Plant Physiol. 63: 552-556.

HUMAYAN, M.Z. & JACOB, T.M. 1973. Immunologic studies on nucleic acids and their components. Biochem. Biophys. Acta 331: 41-53.

HUNTER, W.M. 1971. The preparation and assessment of iodinated antigens. In: KIRKHAM, K. & HUNTER, W.M. (eds.), Radioimmunoassay methods. Edinburgh: Churchill Livingstone.

_____ & GREENWOOD, F.C. 1964. A radioimmuno-electrophoreticassay for human hormone. Biochem. J. 91: 43-56.

HURN, B.A. & LANDON, J. 1971. Antisera for radioimmunoassay. In: KIRKHAM, K. & HUNTER, W.M. (eds.), Radioimmuno-assay methods. Edinburgh: Churchill Livingstone.

JACKSON, A.J. 1978. Antigens and immunogenicity. In: BALLANTI, J. (ed.), Immunology. Philadelphia: Saunders Press.

JOLLES, P. & PARAF, A. 1973. Chemical and biological basis of adjuvants. London: Chapman & Hall.

KABAT, E.A. 1980. Basic principles of antigen-antibody reactions. Methods in Enzymol. 70: 3-49.

KENDE, H. 1965. Kinetin-like factors in the root exudate of sunflowers. Proc. Natl. Acad. Sci. 53: 1302-1307.

_____ 1982. Some concepts concerning the mode of action of plant hormones. In: MEUDT, W.J. (ed.), Strategies of plant reproduction. London: Allenheld.

KHAN, S.A., HUMAYAN, M.Z. & JACOB, T.M. 1977. A sensitive radioimmunoassay for isopentenyl adenosine. Anal. Biochem. 83: 632-635.

KING, R.W. 1976. Abscisic acid in developing wheat grains and its relationship to grain growth and maturation. Planta 132: 43-51.

KNIGHT, R. 1980. Origin and importance of tropical and subtropical fruit crops. In: NAGY, S. & SHAW, P.E. (eds.), Tropical and subtropical fruits. Westport: AVI Publishing.

KOSIYACHINDA, S. & YOUNG, R.E. 1975. Ethylene production in relation to the initiation of the respiratory climacteric in fruit. Plant Cell Physiol. 16: 595-602.

LAHAV, E. & KALMAR, D. 1983. Determination of the irrigation regimen for an avocado plantation in spring and autumn. Aust. J. Agric. Res. 34: 717-724.

LANDON, J. 1971. Separation of bound and free labelled antigen. In: KIRKHAM, K. & HUNTER, W.M. (eds.), Radioimmunoassay methods. Edinburgh: Churchill Livingstone.

_____ & MOFFAT, A.C. 1976. The radioimmunoassay of drugs. The Analyst 101: 225-243.

LANG, A. 1970. Gibberellins: structure and metabolism. Ann. Rev. Plant Physiol. 21: 537-570.

LEE, S. 1981. A review and background of the avocado maturity standard. Calif. Avocado Soc. Yrbk. 65: 101-109.

LETHAM, D.S. 1969. Regulators of cell division in plant tissues. VIII. The cytokinins of the apple fruit. Physiol. Plant. 22: 925-936.

_____ 1978. Cytokinins. In: LETHAM, D.S., GOODWIN, P.B. & HIGGINS, T.J. (eds.), Phytohormones and related compounds. Amsterdam: Elsevier Biomedical Press.

_____ & WILLIAMS, M.W. 1969. Regulators of cell division in plant tissues. Physiol. Plant. 22: 925-936.

_____ & PALNI, L.M. 1983. The biosynthesis and metabolism of cytokinins. Ann. Rev. Plant Physiol. 34: 163-197.

LEOPOLD, A.C. & KRIEDEMANN, P.E. 1975. Plant growth and development. 2nd ed. New York: McGraw Hill.

LIEBERMAN, M., BAKER, J.E. & SLOGGER, M. 1977. Influence of plant hormones on ethylene production in apple, tomato, and avocado slices during maturation and senescence. Plant Physiol. 60: 214-217.

LISHMAN, A.W. 1971. The influence of nutrition on reproduction in the ewe. Unpublished Ph D. thesis, University of Natal, Pietermaritzburg.

LOONEY, N.E., McGLASSON, W.B. & COOMBE, B.G. 1974. Control of fruit ripening in peach, *Prunus persica*: action of succinic acid-2,2-dimethylhydrazide and (2-chloroethyl)phosphonic acid. Aust. J. Plant Physiol. 1: 77-86.

LUCKWILL, L.C. 1981. Growth regulators in crop production. London: Edward Arnold Press.

MacMILLAN, J. & PRYCE, R.J. 1968. Phaseic acid, a putative relative of abscisic acid, from seeds of *Phaseolus multiflorans*. Chem. Comm. 124: 26.

MACVICAR, C.N., LOXTON, R.F., LAMBRECHTS, J.J.N., LE ROUX, J., DE VILLIERS, J.M., VERSTER, E., MERRYWEATHER, F.R., VAN ROOYEN, T.H. & HARMSE, H.J. 1977. Soil classification: a binomial system for South Africa. Science Bulletin 390. Department of Agriculture, Republic of South Africa.

MARTIN, G.C. & CAMPBELL, R.C. 1976. Hormonal bioassay of French prune (*Prunis domesticus* L.) seed and pericarp. J. Amer. Soc. Hort. Sci. 92: 284-289.

McONIE, A.J. & WOLSTENHOLME, B.N. 1982. Avocado fruit growth and maturity in two Natal localities. S. Afr. Avocado Grs. Assoc. Yrbk. 5: 74-77.

McDONALD, E.M., AKIYOSHI, D.E. & MORRIS, R.O. 1981. Combined high-performance liquid chromatography-radioimmunoassay for cytokinons. J. Chromatogr. 214: 101-109.

McGLASSON, W.B. & ADATO, I. 1976. Changes in the concentration of abscisic acid in fruits of normal and *Nr*, *rin* and *nor* mutant tomatoes during growth, maturation and senescence. Aust. J. Plant Physiol. 3: 809-817.

_____, WADE, N.L. & ADATO, I. 1978. Phytohormones and fruit ripening. In: LETHAM, D.S., GOODWIN, P.B. & HIGGINS, T.J. (eds.), Phytohormones and related compounds. Amsterdam: Elsevier Biomedical Press.

MIDGLEY, A.R. & HEPBURN, M.R. 1980. Use of the double-antibody method to separate anti-body bound from free ligand in radioimmunoassay. Methods in Enzymol. 70: 266-274.

MILBORROW, B.V. 1974. The chemistry and physiology of abscisic acid. Ann. Rev. Plant Physiol. 25: 259-307.

_____ 1978. Abscisic acid. In: LETHAM, D.S., GOODWIN, P.B. & HIGGINS, T.J. (eds.), Phytohormones and related compounds. Amsterdam: Elsevier Biomedical Press.

MORGAN, C.R. & LAZAROW, W.A. 1963. Diabetes 12: 15. (Cited by CHARD, 1981).

MORRIS, D.A. 1978. Germination inhibitors in developing seeds of *Phaseolus vulgaris* L. Z. Pflanzenphysiol. 86: 433-441.

MORRIS, R.O. 1977. Mass spectroscopic identification of cytokinins. Plant Physiol. 59: 1029-1033.

MUIR, R.M. & LAUTICAN, B.P. 1968. Purification and properties of the enzyme system forming indoleacetic acid. In: WIGHTMAN, F. & SETTERFIELD, G. (eds.), Biochemistry and physiology of plant growth substances. Ottawa: Runge Press.

NITSCH, J.P. 1950. Growth and morphogenesis of the strawberry as related to auxin. Amer. J. Bot. 37: 211-215.

ODELL, W.D. 1980. Use of charcoal to separate antibody complexes from free ligand in radioimmunoassay. Methods in Enzymol. 70: 374-379.

_____, WILBUR, J.F. & PAUL, W.E. 1965. Radioimmunoassay of thyrotropin in human serum. J. Clin. Endocr. Metab. 25: 1179-1188.

_____, ABRAHAM, G.A., SKOWSKY, W.R., HESCOX, M.A. & FISHER, D.A. 1971. Production of antisera for radioimmunoassays. In: ODELL, W.D. & DAUGHADAY, M.D. (eds.), Principles of competitive protein binding assays. Philadelphia: Lippincott.

ORSKOV, H. 1967. Wick chromatography for the immunoassay of insulin. Scand. J. Clin. Lab. Invest. 20: 297-304.

PARKER, W.C. 1971. Nature of immunological responses and antigen-antibody interaction. In: ODELL, W.D. & DAUGHADAY, W.H. (eds.), Principles of protein-binding assays. Philadelphia: Lippencott.

PEARSON, D. 1975. Seasonal English market variation in the composition of South African and Israeli avocados. J. Sci. Food Agr. 26: 207-213.

PENG, C.T. 1977. Sample preparation in liquid scintillation counting. Review 17. Amersham: Amersham International Ltd.

PENGELLY, W. & MEINS, F. 1977. A specific radioimmunoassay for nanogram quantities of the auxin indole-3-acetic acid. Planta 136: 173-180.

_____, BANDURSKI, R. & SCHULTZ, A. 1981. Validation of a radioimmunoassay for indole-3-acetic acid using gas chromatography - selected ion monitoring mass spectrometry. Plant Physiol. 68: 96-98.

PLAYFAIR, J.H., HURN, B.A. & SCHULSTER, D. 1974. Production of antibodies and binding reagents. Br. Med. Bull. 30: 24-31.

POHLIT, H., HAAS, W. & BOEHMER, H. 1979. Haptenation of variable biological carriers. In: LEFKOVITS, I. & PERNIS, B. (eds.), Immunological methods. New York: Academic Press.

POOL, R.M. & POWELL, L.E. 1972. The use of pellicular ion-exchange resins to separate plant cytokinins by high pressure liquid chromatography. Hortscience 7: 330.

RANADIVE, N.S. & SEHON, A.H. 1967(a). Antigenicity of 5-hydroxyindole-3-acetic acid, a derivative of serotonin. Can. J. Biochem. 45: 1681-1688.

_____ 1967(b). Antibodies to serotonin. Can. J. Biochem. 45: 1701-1710.

RATCLIFFE, J.G. 1974. Separation techniques in saturation analysis. Br. Med. Bull. 30: 32-37.

RAYNER, A.A. 1967. A first course in biometry for agricultural students. Pietermaritzburg: Univ. of Natal Press.

REEVE, D.R. & CROZIER, A. 1980. Quantitative analysis of plant hormones. In: MacMILLAN, J. (ed.), Hormonal regulation of development. Berlin: Springer-Verlag.

RHODES, M.J.C. 1980. Maturation and ripening of fruits. In: THIMANN, K. (ed.), Senescence in plants. Boca Raton: CRC Press.

RICHARDS, D. 1980. Root-shoot interactions: effect of cytokinin applied to the root and/or shoot of apple seedlings. Scientia Hort. 12: 143-152.

RIVIER, L. & PILET, P.E. 1974. Indolyl-3-acetic acid in cap and apex of maize roots: Identification and quantification by mass fragmentography. Planta 120: 107-112.

_____ 1980. Abscisic acid levels in the root tips of seven *Zea mays* varieties. Phytochemistry 20: 17-20.

ROBERTSON, B.L. 1971. Fruit growth of the 'Fuerte' avocado. Leaflet No. 58. Subtropical Fruit Series No. 10. Dept. Agriculture, South Africa.

RODBARD, D. 1971. Statistical aspects of radioimmunoassays. In: ODELL, W.D. & DAUGHADAY, W.H. (eds.), Principles of competitive binding assays. Philadelphia: Lippencott.

_____ & FRAZIER, G.R. 1975. Statistical analysis of radioligand assay data. Methods in Enzymol. 37: 3-22.

_____ & LEWALD, J.E. 1970. Computer analysis of radioligand assay and radioimmunoassay data. Acta Endocrinol. (Copenhagen) 64: Suppl. 147: 79-103.

_____, RAYFORD, P.C., COOPER, J.A. & ROSS, G.T. 1968. Statistical quality control of radioimmunoassays. J. Clin. Endocr. 28: 1412-1418.

ROSSELIN, G., ASSAM, R., YALOW, R.S. & BERSON, S.A. 1966. Separation of antibody-bound and unbound peptide hormones labelled with I-131 by talc powder and precipitated silica. Nature 212: 355-358.

SAGI, F. 1969. Silica gel or cellulose for thin-layer chromatography of indole-3-acetic acid. J. Chromatogr. 39: 334-335.

SCHROEDER, C.A. 1953. Growth and development of the 'Fuerte' avocado fruit. Proc. Amer. Soc. Hort. Sci. 61: 103-109.

SEDGLEY, M. 1977. The effect of temperature on floral behaviour, pollen tube growth and fruit set in avocado. J. Hort. Sci. 52: 135-141.

_____ 1980. Anatomical investigations of abscissed avocado flowers and fruitlets. Ann. Bot. 46: 771-777.

_____ & GRANT, W.J.R. 1982. Effect of low temperatures during flowering on floral cycle and pollen tube growth in nine avocado cultivars. Scientia Hort. 18: 207-213.

SELA, M. 1966. Studies with synthetic polypeptides. Adv. Immunol. 5: 29-35.

SKENE, K.G. & CARR, D.J. 1961. A quantitative study of gibberellin in seeds of *Phaseolus*. Aust. J. Biol. Sci. 14: 13-25.

SOULE, M.J. & HARDING, P.L. 1955. Relation of maturity of Florida avocados to physical characters. Proc. Fla. State Hort. Soc. 68: 303-308.

STOESSL, A. & VENIS, M.A. 1970. Determination of sub-microgram levels of indole-3-acetic acid: a new highly specific method. Anal. Biochem. 34: 344-351.

SWARTS, D.H. 1978. Na-oes probleme by avokados soos be-invloed deur die seisoen. S. Afr. Avocado Grs. Assoc. Yrbk. 2: 52.

_____ 1981. Fermometer ondersoeke by avokados. S. Afr. Avocado Grs. Assoc. Yrbk. 4: 42-46.

THOMPSON, A.G., HORGAN, R. & HEALD, J.K. 1975. A quantitative analysis of cytokinin using single-ion-current monitoring. Planta 124: 207-210.

THOMPSON, P.A. 1961. Evidence for a factor which prevents the development of parthenocarpic fruits in the strawberry. J. Exp. Bot. 12: 199-206.

TILLBERG, E. & PINFIELD, N.J. 1982. Changes in abscisic acid levels during and after ripening and germination of *Acer plantanoides* L. seeds. New Phytol. 92: 167-172.

TOMER, E. & GOTTREICH, M. 1978. Abnormalities in avocado (*Persea americana* Mill.) ovule development. Bot. Gaz. 139: 81-86.

TREWAVAS, A.J. 1981. How do plant growth substances work? Plant Cell Environ. 4: 203-228.

_____ 1982. Growth substance sensitivity: the limiting factor in plant development. Physiol. Plant. 55: 60-72.

TURK, J.L. & PARKER, D. 1977. Interaction of haptens with proteins and their immunogenicity. In: GLYNN, L. & STEWARD, M. (eds.), Immunochemistry: an advanced textbook. Chichester: Wiley & Sons.

TURNER, M.W. 1977. Structure and function of immunoglobulins. In: GLYNN, L. & STEWARD, M. (eds.), Immunochemistry: an advanced textbook. Chichester: Wiley & Sons.

UTIGER, R.D., PARKER, M.L. & DAUGHADAY, W.H. 1962. A radioimmunoassay for human growth hormone. J. Clin. Invest. 41: 254-261.

VAITUKAITIS, J.L. 1981. Production of antisera with small doses of immunogen: multiple intradermal injections. Methods in Enzymol. 73: 46-52.

_____, ROBBINS, J.B., NIESCHLAG, E. & ROSS, G.T. 1971. A method for producing specific antisera with small doses of immunogen. J. Clin. Endocr. 33: 988-991.

VALMAYOR, R.V. 1964. Cellular development of the avocado from bloom to maturity. Ph D. Thesis. Fruit Crops Dept. Univ. of Fla., Gainsville.

VAN DEN DOOL, B. & WOLSTENHOLME, B.N. 1983. Further studies on avocado fruit growth on maturity in inland Natal. S. Afr. Avocado Grs. Assoc. Yrbk. 6: 34-40.

VAN LELYVELD, L.J., DIXON, R.A. & NEL, M.E. 1983. Enzyme activities and the appearance of pulpspot in avocado fruit. S. Afr. Avocado Grs. Assoc. Yrbk. 6: 58-60.

VAN REGENMORTEL, M.H.V. 1982. Serology and immunochemistry of plant viruses. New York: Academic Press.

VAN STADEN, J. 1976. Extraction and recovery of cytokinin glucosides by means of a cation exchange resin. Physiol. Plant. 38: 240-242.

_____ 1980. Endogenous cytokinins in Bougainvillia 'San Diego Red'. The complex in mature leaves. Bot. Gaz. 141: 245-247.

_____, & DAVEY, J.E. 1979. The synthesis, transport and metabolism of endogenous cytokinins. Plant Cell Environ. 2: 93-106.

VAN VUNAKIS, H. 1980. Radioimmunoassays: an overview. Methods in Enzymol. 70: 201-209.

VREMAN, H.J. & CORSE, J. 1975. Recovery of cytokinins from cation exchange resins. Physiol. Plant. 35: 333-336.

WAKSAL, S. 1978. Immunomodulation. In: BELLANTI, J.A. (ed.), Immunology. Philadelphia: Saunders Press.

WALTON, D.C., DASHEK, W. & GALSON, E. 1979. A radioimmunoassay for abscisic acid. Planta 146: 139-145.

WANG, C.Y., WANG, S.Y. & MELLETHIN, M.J. 1972. Identification of abscisic acid in Bartlett pears and its relation to premature ripening. J. Agric. Food Chem. 20: 451-453.

WAREING, P.F. 1982. A plant physiological odyssey. Ann. Rev. Plant Physiol. 33: 1-26.

WEAVER, R.J. 1972. Plant growth substances in Agriculture. San Francisco: Freeman.

_____ & VAN OVERBEEK, J. 1963. Kinins stimulate grape growth. Calif. Agric. 17: 12.

WEILER, E.W. 1979. Radioimmunoassay for the determination of free and conjugated abscisic acid. Planta 144: 255-263.

_____ 1980(a). Radioimmunoassay for transzeatin and related cytokinins. Planta 149: 155-162.

WEILER, E.W. 1980(b). Radioimmunoassay for the differential and direct analysis of free and conjugated abscisic acid in plant extracts. Planta 148: 262-272.

_____ 1981. Radioimmunoassay for pmol-quantities of indole-3-acetic acid for use with highly stable (^{131}I)- and (^{3}H)-IAA derivatives as radiotracers. Planta 153: 319-325.

_____ 1982(a). Plant hormone immunoassay: mini-review. Physiol. Plant. 54: 230-234.

_____ 1982(b). An enzyme-immunoassay for cis(+)-abscisic acid. Physiol. Plant. 54: 510-514.

_____ & SPANIER, K. 1981. Phytohormones in the formation of crown gall tumors. Planta 153: 326-337.

_____ & WIECZOREK, U. 1981. Determination of fentomol quantities of gibberellic acid by radioimmunoassay. Planta 152: 159-167.

_____, JOURDAN, P.S. & CONRAD, W. 1981. Levels of indole-3-acetic acid in intact and decapitated coleoptiles as determined by a specific and highly sensitive solid phase enzyme immunoassay. Planta 153: 561-571.

WHENHAM, R.J. 1983. Evaluation of selective detectors for the rapid and sensitive gas chromatographic assay of cytokinins, and application to the analysis of cytokinins in plant extracts. Planta 157: 554-560.

WHITEHOUSE, M.W. 1977. The chemical nature of adjuvants. In: GLYNN, L.E. & STEWARD, M.W. (eds.), Immunochemistry: an advanced textbook. Chichester: Wiley & Sons.

WIDE, L. & PARATH, J. 1966. Radioimmunoassay of proteins with the use of Sephadex-coupled antibodies. Biochem. Biophys. Acta 130: 257-260.

WILLIAMS, P.D. 1984. Opening address, SAAGA Research Symposium 1983. S. Afr. Avocado Grs. Assoc. Yrbk. 7: 6.

WILTBANK, W.J. & KREZDORN, A.H. 1969. Determination of gibberellins in ovaries and young fruits of navel oranges and their correlation with fruit growth. J. Amer. Soc. Hort. Sci. 94: 195-201.

YALOW, R.S. & BERSON, S.A. 1960. Immunoassay for endogenous plasma insulin in man. J. Clin. Invest. 39: 1157-1175.

_____ 1971. Problems of validation of radioimmunoassays. In: ODELL, W.D. & DAUGHADAY, W.H. (eds.), Principles of competitive binding assays. Philadelphia: Lippencott.

ERATA

LUCKWILL, L.C. 1953. Studies of fruit development in relationship to plant hormones. J. Hort. Sci. 28: 14-24.

NITSCH, J.P. 1956. Methods for the investigation of natural auxins and growth inhibitors. In: WAIN, R.L. & WIGHTMAN, F. (eds.), The chemistry and mode of action of plant growth substances. London: Butterworth.