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, 198O), 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 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).

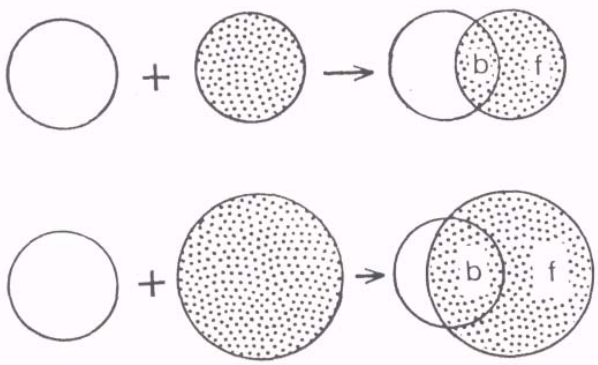
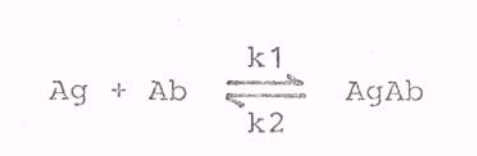


Fig. 1.1 The basic principle of a binding assay, using RIA as an example. If given amounts of antigen and antibody are allowed to react together an equilibrium antigen-antibody complex is formed (B) together with a portion of both antibody and free antigen (F). An increase in the amount of antigen (below)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

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:



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

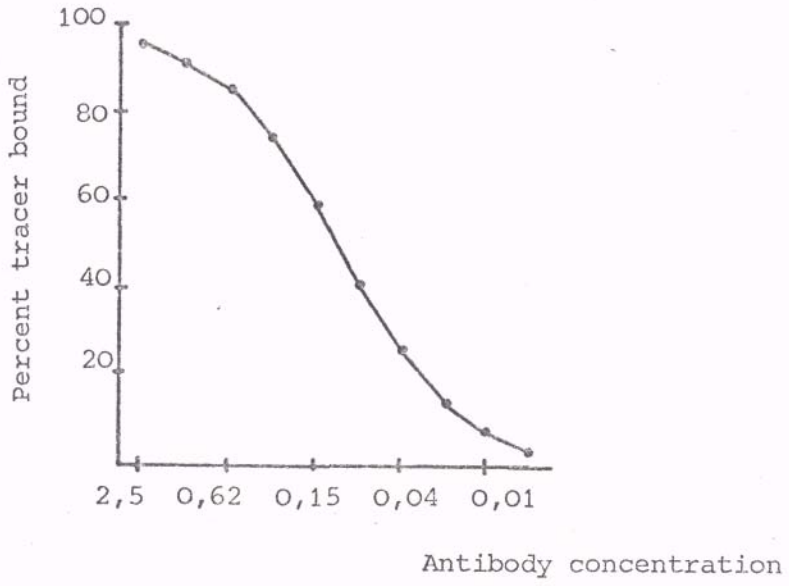


Fig. 1.2 Binder dilution curve. Serial dilutions of binder are incubated with fixed amount of tracer and the percentage of the latter plotted on the vertical axis (Chard, 1981).

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).

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).

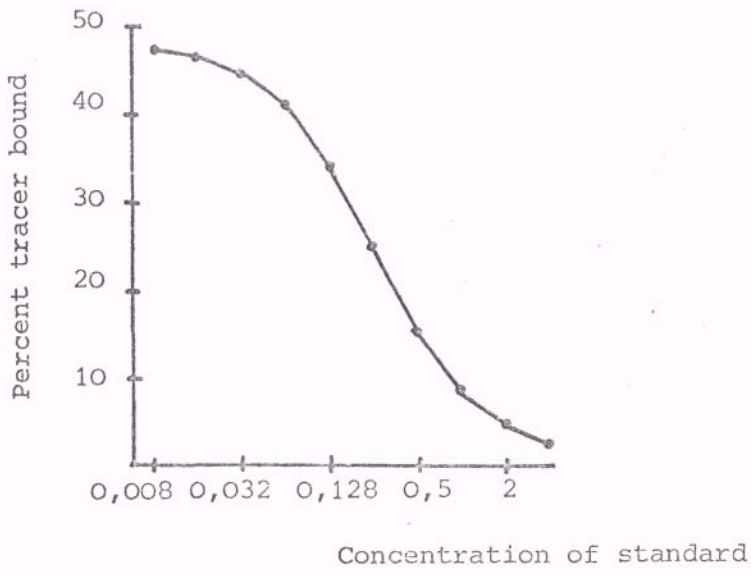


Fig. 1.3 A standard dose-response curve using fixed amounts of tracer and antibody (Chard, 1981).

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 imiminologicälly-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).

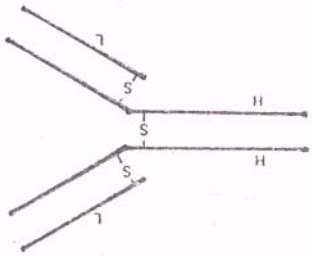


Fig. 1.4 The structure of the IgG molecule, consisting of two heavy chains (H) and two light chains (L) linked by disulphide bridges (S) (Chard, 1981).

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).

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[1]" 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.

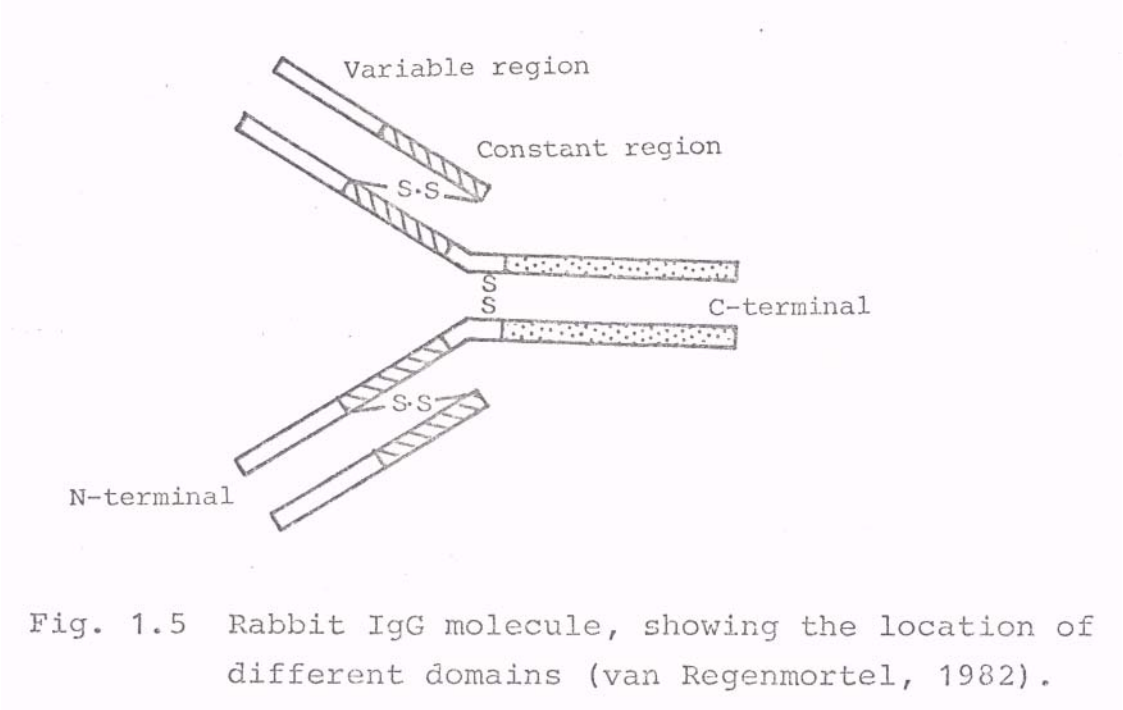


Fig. 1.5 Rabbit IgG molecule, showing the location of different domains (van Regenmortel, 1982).

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 cooperation (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 Boehrner, 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 (Hum & 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 gammaglobulins.

(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 anti-sera. 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 rnacrophage 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 (Hum & Landen, 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 prerequisite 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 (Banghara & 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 (Landori & 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 arid 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 60ym 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 radioimmunoassay (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 (Redding, 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 & Ràndle (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 gammaglobulins 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 calculated. 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 (USA) 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 aderiosine 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 anti- sera 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 (Gummings & 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 (Lethara & 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 investigators (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).