IMMUNOLOGICAL STUDIES ON THE REGULATION OF ENZYME LEVELS IN PLANTS

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ABBREVIATIONS

P.A.L. = phenylalanine-ammonia-lyase (E.C. 4.3.1.5)

A.A.O. = ascorbic acid oxidase (E.C. 1.10.3.3)

N.R.S. = normal rabbit serum (from non-immunized rabbits)

R1A, R2A = anti-potato P.A.L. serum from rabbits 1 and 2 (Section 2.9)

R1B, R2B = anti-potato P.A.L. serum from rabbits 1 and 2, partially purified by ammonium sulphate fractionation (Section 2.11.1)

R1C, R1C2 = anti-potato P.A.L. serum from rabbit 1, purified by D.E.A.E.-cellulose chromatography, run 1 and 2 (Section 2.11.2)

P.B.S. = phosphate buffer saline (50mM Na-phosphate buffer, pH 7.2, containing 0.15M NaCl.)

D/U = potato P.A.L. sample purified by D.E.A.E. Sephadex A-25 chromatography and ultrafiltration, steps 5 and 6 (Section 3.2.11A)

S/U I, S/U II = potato P.A.L. sample purified by Sepharose 6B chromatography and either one ultrafiltration step or two, steps 7, 8 and 9 (Section 3.2.11A)

E.D.T.A. = ethylenediaminetetra-acetate

S.D.S. = sodium dodecyl sulphate

SECTION 1 INTRODUCTION

The biochemical mechanisms co-ordinating the development and growth of higher plants, are at present still challenging the research efforts of plant biochemists. The development of higher plants, like other organisms, is determined by the genome and modified by environmental factors. A distinct feature of plants is their autotrophic ability; sunlight is perceived by the plant, and the radiant energy used to drive the anabolic and catabolic processes of the plant. The two main light-requiring processes are photosynthesis and photomorphogenesis. The latter term applies to all morphogenetic processes that are affected by the incident radiation, including those regulating the overall pattern of development. These processes are thought to operate via photoreceptor molecules distinct from the photosynthetic pigments (chlorophylls and carotenoids), and two photoreceptors have been recognized; phytochrome, a photochromic protein, particularly sensitive to light in the red and far-red region of the spectrum, and to a lesser extent to blue light (Hendricks and Borthwick,1965), and a specific blue-absorbing photoreceptor, less well characterised, and often referred to as cryptochrome (Briggs,1976; DeFabo,1980). Both have been implicated in the biochemical processes underlying the morphological changes occurring in response to certain light treatments (Hartmann,1966; Mitrakos,1972; Smith, 1974,1975; Mohr,1972,1980).

The earliest biochemical observations relating to photomorphogenesis were the light-dependent changes in the levels of particular enzymes involved in the development of the photosynthetic apparatus (Marcus,1960; Margulies,1965). Light-induced changes in the levels of certain metabolites have also been observed, such as the accumulation of phenylpropanoid compounds, anthocyanins (Siegelman and Hendricks,1957; Grill and Vince,1969), flavonoids (Bottomley et al.,1966), chlorogenic acid (Zucker,1963), and hydroxycinnamic acids (Engelsma and Meijer,1965), and the photo-oxidation and -reduction of ascorbic acid (Mapson and Swain,1966), and these have led

to studies on related enzymes. The first enzyme in the pathway to phenylpropanoid compounds is phenylalanine-ammonia-lyase (P.A.L.), and this enzyme is subject to photocontrol in many plants. Amongst the earliest plants used in these studies were potatoes (Zucker,1965), mustard (Durst and Mohr,1966) and gherkin (Engelsma,1967a,b). Studies on the photocontrol of ascorbic acid oxidase (A.A.O.) have been largely confined to mustard (VanPoucke et al.,1969; Drumm et al.,1972). Mustard seedlings (Sinapis alba) have been widely used for studies relating to photomorphogenesis (Mohr,1972), and the two enzymes mentioned above are of particular interest, as the mechanisms controlling the activity of these enzymes , in the cotyledons of mustard, may be different. These two enzymes were chosen for further studies,which will be described in this thesis.

As enzymes control the flux of metabolites through various pathways, it is reasonable to link developmental changes, especially differentiation, with the activities of specific enzymes. However the link between the primary photoresponse (as yet unidentified), and the changes in enzyme levels is still unclear. Separate experiments in micro-organisms on enzyme levels indicated that transcription of the genome was directly responsible; synthesis of inducible enzymes in the presence of their substrates, was the result of de-repression of the normally repressed transcription of the D.N.A. (Jacob and Monod,1961). The same mechanism could be applied to the co-ordinated induction of several enzymes of a particular pathway or metabolic sequence, where these enzymes were mapped close together on the bacterial genome. The portion of the genome coding for a group of enzymes and also responsible for it's expression was called an 'operon'. Having established that phytochrome was involved in photomorphogenic responses (Hartmann,1966), Mohr,(1966) advanced the theory of differential gene activation as a mode of action of phytochrome, with phytochrome acting as a signal to switch on and off, the transcription of regions of the genome, by direct interaction of phytochrome with the D.N.A. in the chromosomes. This theory has been the basis of many experiments aimed at determining the level of control, and whether changes in enzyme levels are the

result of de novo synthesis of the enzyme, arising from an increase in the availability of m.R.N.A. coding for each enzyme.

In the relatively simple, unicellular organism, where protein synthesis occurs rapidly (1-2min.) and the enzyme can be removed by dilution through cell division; and where the genetic material is readily accessible on a single chromosome, the model of Jacob and Monod can account for the changes observed. Multicellular organisms differ in many respects, apart from each individual cell possessing an intricate membrane system, distinct organelles, a separate nucleus and more than one chromosome, each cell functions as a part of the whole organism, becoming limited to a specific role, ie. differentiated, and dependent on the functions of the other cells. Plant cells range from rapidly dividing meristematic tissue with full genetic ability, to mature non-dividing tissues with only a limited ability, and it is difficult to generalise, and propose mechanisms of control applicable to all plant cells. Differentiation of the plant cell, may result in a range of regulatory mechanisms, which develop as the cells age, in a manner appropriate to the type of cell, and which allow intercellular control and co-ordination of development and growth. Plant growth hormones appear to have an important role in this respect, and many photomorphogenic responses are also sensitive to the level or balance of these substances (Stoddart,1976; Gregor,1974; Asahira and Masuda,1977; Craker and Wetherbee,1973; Bühler et al.,1978; Pilet and Takahashi,1979). Only those cells remaining in an undifferentiated state could be expected to behave in a manner similar to the bacterial cell; but the similarity is limited. Recent studies on yeast chromosomes, which are similar in structure and mode of replication to those of higher eucaryotes, show that the genes coding for enzymes within one biosynthetic pathway are not clustered (Petes,1980). Therefore co-ordinated changes in groups of enzymes in eucaryotes must be controlled in a manner different from the operon-regulator gene theory. There is also a lack of enzyme repression in plants, and it is more likely that feedback inhibition has an important role (Miflin,1973). Davis and Chapman,(1980) recently

proposed that proteolytic activity in cotyledons of germinated seeds involved in food mobilization, might be controlled via the products of protein degradation, ie. self-regulating. Many changes in enzyme levels in plants are transient, even where the stimulus persists, and are quantitative rather than qualitative changes, and therefore are not directly comparable to the inducible enzymes in procaryotes. Plant cells (in common with other eucaryotic cells), possess an intricate internal membrane system, which may allow separation of biochemical reactions, in the same way that certain metabolic processes are restricted to mitochondria, or chloroplasts, or other organelles. Indeed, work on phytochrome led many to doubt whether phytochrome itself interacted directly with the genome, and it was proposed that phytochrome was situated at or in the membrane, and operated by regulating the properties of the cell membrane (Hendricks and Borthwick,1967; Boeshore and Pratt,1980; Whitelam and Johnson,1981).

In animals, many hormone responses are mediated via 'secondary messengers' (Sutherland and Rall,1957; Birnbaumer et al.,1970; Greengard,1972), the hormones interact with receptors in the outer surface of the plasma membrane, which leads to release of a secondary messenger, inside the cell. A similar mechanism was postulated by Smith (1970), for the action of phytochrome, separating the primary photoresponse, a possible membrane effect, from the change in enzyme level, which could occur at the level of the genome or at some later stage, elsewhere in the cell. Membrane-bound phytochrome and phyochrome-mediated enzyme activity has been demonstrated in vitro (Penel et al.,1976). It is perhaps more likely that mechanisms operating in animal cells would be similar to those in plant cells, but so far attempts to compare the action of plant growth substances (Trewavas,1976a), with hormones has failed to add to our understanding of regulatory mechanisms in plants. The genetic totipotency of some somatic cells of plants indicates a greater flexibility in plants; differentiation in animal cells is usually irreversible.

The 'wounding' response of plant tissue, for example the excision of

storage organs, is characterised by an increase in metabolic activity (Kahl,1973,1978) and is an example of partial de-differentiation, leading "to a change of metabolism typical of a mature quiescent storage parenchyma to that typical of rapidly elongating cells, recently ceased dividing" (Bryant, 1976). The ability of tissue to respond, decreases as the tissue ages (Rosenstock and Kahl,1978); this may be due to changes in the regulatory mechanisms, as suggested earlier. The germination of seeds and subsequent development of the seedling, also represents a changing pattern of biochemical processes. Storage tissues and seedlings are the two main sources of plant tissue for photomorphological studies, and are obviously not ideal for biochemical studies. Although storage tissue is relatively homogeneous, many biochemical changes occur upon cutting, which provide the background to any light effects. In contrast, seedlings are heterogeneous and undergoing differentiation, but with many light effects directly involved in the morphogenesis.

The potential to possess particular enzymes is determined by the genome, but as Marcus (1971) pointed out, control may occur at several levels in eucaryotic cells, anywhere from the initial transcription of the D.N.A. (a relatively slow process) to the expressed activity of the enzyme. The nearer to the latter stage, the more rapid the response is likely to be, and may involve the presence of inactivating-activating systems, or modifications of the enzyme itself (Goatly and Smith,1974; Goatly et al.,1975; Salminen and Young,1975; Atkinson and Hammes,1975; Trewavas,1976b; Miflin,1977). Three main stages can be identified; transcriptional control, where synthesis of m.R.N.A. is limiting; translational control, where synthesis of protein (or enzyme) is limiting, and post-translational control, where activation, turnover or other modifications are limiting, the expression of enzyme activity. The potential sites for the photocontrol of gene expression are outlined by Smith et al.(1977). Intermediate points of control, such as the stability of m.R.N.A. (Marcus and Feeley,1964; Kafatos and Gelinas,1974; Payne,1976; Roberts and Lord,1979), or further processing of m.R.N.A. prior to protein

synthesis (Scragg et al.,1975; Dure and Harris,197 ; Mori et al.,1978), which may be described as post-transcriptional control, may also exist. There is no reason why control should not occur at several of these points, thereby allowing a greater degree of co-ordination within the cell.

Perhaps the underlying reason for the present lack of understanding of biochemical mechanisms in plants, lies in the difficulties encountered when using plant tissues, (extracts are often unstable, or artefacts may arise during or following,extraction.or excision of sections of tissue), and the nature of the techniques used and their limitations. As Bryant (1976) commented when reviewing the molecular aspects of differentiation, "the current emphasis on translation and post-translational controls may.....be a reflection of the availability of techniques rather than the relative importance of these processes in cell differentiation" (a large part of the review dealt with studies based on the use of inhibitors). Inhibitors have been widely used in studies on photomorphogenesis; inhibitors of R.N.A. synthesis (eg. actinomycin D) and processing (eg. cordycepin), and protein synthesis (eg. cycloheximide) have provided some evidence for the role of these processes in the photo-response. The value of inhibitor studies can be assessed by looking at the results of such experiments; many enzymes have been studied, but the following review will be restricted to experiments relating to the photocontrol of P.A.L. and A.A.O. levels (as these two enzymes are the topic of the research undertaken here).

Amongst the earliest relevant experiments were those concerned with P.A.L. levels in mustard seedlings; following exposure to far-red light (or brief periods of red light) P.A.L.-activity in the cotyledons increases dramatically over that in dark controls, this increase is partially inhibited by actinomycin D, which suggests that some R.N.A. synthesis is required for the response (Durst and Mohr,1966). Inhibitors of protein synthesis prevented the increase in P.A.L.-activity following a second light treatment (actinomycin D was again only partially effective), and from this it was concluded that the induction of P.A.L. was a rapid process involving de novo synthesis of

enzyme protein (Rissland and Mohr,1967). In contrast, the accumulation of ascorbic acid in mustard seedlings following a far-red light treatment, was not inhibited by actinomycin D (Bienger and Schopfer,1970), and therefore gene regulation was unlikely. More recent experiments using inhibitors of D.N.A.-dependent m.R.N.A. synthesis and processing, reduced the magnitude of the far-red induced increase in P.A.L. in mustard cotyledons (Acton et al., 1980), and from this it was deduced that the rate of light-mediated P.A.L. accumulation was affected by the light. The light induced changes of P.A.L.-activity in parsley cell suspension cultures were strongly inhibited by both actinomycin D and cycloheximide, implying that R.N.A. synthesis and cytoplasmic protein synthesis were necessary for the increase in P.A.L. (Hahlbrock and Ragg,1975). In cultures of Phaseolus vulgaris, actinomycin D was without effect on the induced P.A.L. levels (Dudley and Northcote, 1979). Cycloheximide failed to prevent the increase in P.A.L.-activity in callus cultures of Pinus elliotii (Lau et al.,1980). In these cases activation mechanisms may be present. Zucker (1968), used cycloheximide in studies on P.A.L. levels in potato tuber discs, fresh discs exposed to cycloheximide failed to give an increased P.A.L.-activity, and aged discs exposed to inhibitor (at a higher concentration) did not subsequently show a decline in P.A.L.-activity. This was taken as evidence for the role of de novo synthesis in the increase in P.A.L.-activity (enzyme synthesis) and also in the decline in P.A.L.-activity (synthesis of 'inactivating system'). Lamb (1977), carried out similar experiments using a range of inhibitors, including actinomycin D, cordycepin and cycloheximide, to pin-point the steps involved in the increase and decrease in P.A.L. levels in incubated potato tuber discs. Cycloheximide also increases the susceptibility of potato to infection (Zucker and El-Zayat,1968), by inhibiting P.A.L.-activity and the accumulation of chlorogenic acid, which is thought to inhibit the bacteria. In gherkin seedlings, cycloheximide again inhibits both the rise and fall of P.A.L.-activity, following a blue light treatment (Engelsma,1965,1967c). P.A.L.-activity also increased after a cold treatment,

but this increase was insensitive to cycloheximide. At lower temperatures (10°C) the sensitivity to light is reduced and P.A.L. levels do not decline (Engelsma,1970). These results indicate that protein synthesis is required for the light-induced increase and subsequent decline, but not for the cold-induced increase, therefore an inactivating system may be present, but not operating in cold treated tissue. The inactivating process required protein synthesis, but not the activation (Attridge and Smith,1973a; French and Smith, 1975), an inactivator has since been extracted from gherkin tissue (Billett et al.,1978). In radish cotyledons, the initial far-red stimulated increase in P.A.L. level was inhibited by actinomycin D, cycloheximide and chloramphenicol, but a second light treatment after a dark period was not affected by cycloheximide (Klein-Eude et al.,1974), also suggesting that an inactivating mechanism was involved. In leaf discs of sunflower, where P.A.L. levels were increased by culture on sucrose medium, transfer to water caused a loss of P.A.L.-activity, and a second transfer, back to a sucrose medium increased P.A.L. again. Cycloheximide inhibited the initial increase and decline, but stimulated the second increase (Creasy et al.,1974). An inactivating fraction was subsequently extracted from the leaves pre-treated with light and sucrose (Creasy,1976). Protein synthesis inhibitors stimulate anthocyanin synthesis in the skins of whole apples, but are inhibitory in apple skin discs (Chalmers and Faragher,1977). The presence of an inactivating system was invoked to explain these effects; low temperatures also stimulated the accumulation of P.A.L. and anthocyanins in the skin of whole apples; an inactivating system has been isolated, and in this case the inactivation was not reversible (Tan,1979,1980). In asparagus spears, the excision-promoted increase in P.A.L.-activity was prevented by cycloheximide, and also by the products of the phenylpropanoid pathway, especially cinnamic acid (Goldstein et al.,1972); a cold treatment also increased the P.A.L.-activity, for a limited period. Here again, both synthesis and inactivating mechanisms seem to be operative. Inhibitors have also been used to show that phytochrome was the only photoreceptor in the photocontrol of anthocyanin synthesis in a range

of plants; streptomycin inhibited the synthesis of chlorophylls and the development of the chloroplasts, but stimulated the synthesis of anthocyanin (Mancinelli et al.,1975), it was therefore unlikely that the photoresponse was mediated via photosynthesis.

Inhibitors have contributed to studies on photomorphogenesis, but only provide a limited amount of information. Where the specificity of the inhibitor is in doubt (Jacobson et al.,1974; Ellis and MacDonald,1970), or the exact site of action is unclear (Walbot et al.,1974), or the inhibitor has effects beyond it's site of action (Pestka,1971), the interpretation of the results is awkward; negative results are easier to interpret, provided the uptake of the inhibitor is not in doubt. More specific inhibitors have also been used; such as compounds carrying the aminooxy group, which are potent inhibitors of phenylalanine deamination and transamination; these include α-aminooxyacetic acid (A.O.A.) and L-α-amonooxy-β-phenylpropionic acid (A.O.P.P.) (Amrhein et al.,1976). These competitive inhibitors, especially A.O.P.P. causes the superinduction of P.A.L.-activity in gherkin tissues, by inhibiting P.A.L.-activity, which prevents the accumulation of cinnamic acid and the feedback inhibition of the enzyme by it's product (Amrhein and Gerhardt,1979; Billett and Smith,1980). Trans-cinnamic acid also inhibits P.A.L.-activity but unlike A.O.P.P. also affects the growth rate and soluble protein content, and is less specific in effect in plants (Noé et al.,1980). The N-benzyl-oxycarbonyl derivatives also inhibit anthocyanin formation in a range of plants, with little interference in development (Amrhein and Holländer,1979). The role and importance of feedback inhibition in regulating P.A.L. levels has therefore been established. In Avena coleoptile segments, p-D,L-fluoro-phenylalanine enhances elongation, and inhibits P.A.L.-activity in vitro; it was then found that trans-cinnamic acid also inhibited elongation, and the enhanced elongation attributed to a lowering of the level of inhibitory phenolics (Hopkins and Orkwisewski,1971). Another in vivo P.A.L. inhibitor, piclorame, was used to distinguish between the increase in P.A.L.-activity in Nicotiana tabacum, due to virus infection, and that dependent on the

photoperiod (Paynot et al.,1976). More general inhibitors have also found uses, N-(phosphonomethyl)glycine, is a plant growth inhibitor,(generally called glyphosate),which stimulates P.A.L.-activity in roots of maize preceeding the reduction in growth rate (Duke and Hoagland,1978). The depressed growth has been attributed to the enhanced levels of inhibitory phenolic compounds (Hoagland et al.,1978). Light enhanced the inhibitory effect of root-fed glyophosate in soybean seedlings, via a synergistic increase in P.A.L.-activity, whereas A.O.P.P. tended to alleviate the inhibition (Duke et al.,1979,1980) indicating that growth rate was affected by the level of phenolics, and the size of the aromatic amino acid pool. The pyridazinone inhibitors of chloroplast development were used to show that P.A.L. was photocontrolled in barley plastids (Blume and McClure,1978). Inhibitors of photosynthesis were used to show that the production of P.A.L. and flavonoids in illuminated strawberry leaf discs was dependent on the availability of sucrose (Creasy,1968).

There is particular interest in the rates of protein turnover (Huffaker and Peterson,1974), and how changes in the rates of synthesis and/or degradation may affect those enzymes where synthesis has been implicated. The techniques available for such studies form three groups, two involving labelling techniques, and the third involving immunological techniques; a combination of radio-isotopic labelling and immunology provides more conclusive results, but suffers from the drawback that the enzyme must be purified. For this reason, density-labelling techniques have been used in preference. Filner and Varner (1967), were the first to apply this technique to plant studies, and were able to show that the gibberellic acid-induced increase of α-amylase in the barley aleurone layer was due to synthesis of new enzyme molecules; label from $H_2{}^{18}O$ (incorporated into amino acids derived from hydrolysis of pre-existing seed protein) was incorporated into the enzyme. Density-labelling with heavy water, ^2H_2O, was used to show that de novo synthesis of P.A.L. was responsible for the far-red stimulated increase in enzyme in mustard cotyledons (Schopfer and Hock,1971). However, using

the same technique and the same tissue, with an improved procedure (Johnson et al.,1973), Attridge et al.(1974), failed to obtain an increase in density labelling of the enzyme, following far-red light treatment of the tissue, compared to a dark control. Using a relatively long labelling period (48h.), the bouyant density of the enzyme under far-red light, was lower than that of the control. The interpretation of the results was discussed, and together with evidence for a pool of inactive P.A.L. in dark-grown gherkin (Attridge and Smith,1973b), it was concluded that the light-mediated increase in P.A.L.-activity in mustard cotyledons was due to activation of existing enzyme. Similar experiments were also carried out for gherkin hypocotyl sections, to confirm the presence of inactive P.A.L. in dark-grown seedlings; the blue light- and cycloheximide-induced P.A.L.-activity was accompanied by a lower buoyant density, compared to the dark control (Attridge and Smith,1974). In an attempt to clarify the situation, Acton and Schopfer (1975), repeated the density labelling experiments for mustard seedlings, but finally concluded that the half-life of P.A.L. in mustard cotyledons was too short to allow a suitable labelling period together with a measurable increase in P.A.L.-activity under far-red light, but because some label was incorporated in both the control (dark) and far-red treatment, that de novo synthesis occurred, and not activation, as they failed to observe any lowering of the buoyant density in light-stimulated tissue. To overcome the lag-phase of far-red stimulated P.A.L., Tong and Schopfer (1976), used pre-irradiated mustard seedlings where P.A.L.-activity was readily measured, and as a dark control gave seedlings a brief red treatment followed by darkness. Their results showed that an increase in buoyant density occurred for far-red treated P.A.L., compared to the control, supporting their claims that the rate of enzyme synthesis was stimulated by the light. They also studied the kinetics of the density labelling (band shift and bandwidth changes) and concluded that the results were not due merely to an increase in the rate of labelling of the amino acid pool in the light (Johnson and Smith,1978). They also pointed out that their results did not eliminate the possibility

that an activation mechanism was operating, as the interpretation of density labelling experiments requires the establishment of the precise mechanism of enzyme turnover for the enzyme in question (Lamb and Rubery,1976a). The technique of density labelling involves the measurement of active enzyme only, any inactive enzyme molecules are inferential. When the effects of cinnamic acid on the increase in P.A.L.-activity following excision of hypocotyl segments of dark-grown gherkin seedlings, were studied using density-labelling Johnson et al.(1975) obtained results indicating that the rate of enzyme synthesis was lowered, in contrast to the results of their experiments on the light effect. In parsley cell suspension cultures, ^{15}N was used in labelling experiments, the phytochrome-mediated increase in P.A.L.-activity (which requires an initial U.V. irradiation before being obtained) was shown to be due to de novo synthesis. In the dark controls the labelling was only 50% of the far-red treated tissue, and an increase in bandwidth was observed after a 5h. labelling period, indicating the presence of a mixture of differently labelled P.A.L. molecules. Overall, the turnover rate was less than expected if any activation mechanisms were operating (Wellman and Schopfer,1975; Schopfer et al.,1976). The results of density labelling experiments with ^2H_2O for the photocontrol of P.A.L. in potato tuber discs (Sachar et al.,1972; Lamb and Merritt,1979), and in radish cotyledons (Fourcroy,1980) also suggested that de novo synthesis of enzyme was involved. The decrease in P.A.L.-activity in potato tuber discs was shown to result from a reduction in the rate of synthesis and an increased rate of degradation, or removal of active enzyme, in the absence of inactivation mechanisms (Lamb et al.,1979).

It is evident from the foregoing review that the question whether de novo synthesis or activation is involved, is still a contentious issue, especially with regard to the phytochrome-mediated P.A.L. levels in mustard cotyledons. Density-labelling experiments with A.A.O. in mustard cotyledons by Attridge (1974), suggested that phytochrome controls the rate of synthesis of the enzyme, in contrast to parallel experiments with P.A.L. in the same

tissue. The results were this time in agreement with similar experiments by Acton et al.,1974. The possibility therefore exists that the action of phytochrome upon the levels of P.A.L. and A.A.O. may be via different mechanisms within the same plant.

Where it is likely that de novo synthesis is involved in the light-induced increases in enzyme activity, it is worth looking for changes occurring at the level of transcription. As yet there are no techniques for isolating m.R.N.A. coding for specific enzymes. Initial experiments into phytochrome action at this level were aimed at measuring light-induced increases in the number of polysomes (Pine and O'Klein,1972; Smith,1976; Fourcroy et al,1979), or increases in ribonuclease activity (Acton,1974; Acton and Schopfer,1974), or even differences between the translation products of R.N.A. extracted from dark and light grown plants (Giles et al., 1977; Spiers and Greirson,1978). One way of identifying the presence of specific m.R.N.A.s is by comparing the molecular weights of the enzyme or enzyme subunits, with those obtained from in vitro translation, using extracted R.N.A. (usually by S.D.S.-gel electrophoresis). The problem here is finding an in vitro translation system which is capable of forming completed enzyme subunits. Of the three commonly used systems (rabbit reticulocyte, wheat germ or Escherichia coli), the rabbit reticulocyte system was successfully used by Ragg et al.(1977); radio-isotopically labelled P.A.L. subunits formed in vitro from extracted R.N.A. were identified by precipitation with a specific antiserum to the enzyme, and the immunoprecipitate analysed by S.D.S. gel electrophoresis. Polyribosomal R.N.A. for translation was isolated from parsley cell suspension cultures from U.V.light-induced cells. It was therefore apparent that de novo synthesis of the P.A.L. was induced by light (no P.A.L. subunits were obtained from R.N.A. extracted from dark controls). The rates of appearance of enzyme were consistent with the changes in the ability of extracted R.N.A. to translate for the enzyme in 7 day-old cultures, older cultures gave a slightly inconsistent pattern (Schröder,1977). Immunological techniques have also

been carried out on the increase in P.A.L.-activity in sweet potato tuber injured by cutting (Tanaka and Uritani,1976). Antibody specific for P.A.L. was used to measure the P.A.L. protein; the development of P.A.L.-activity was accompanied by an increase in P.A.L. protein, ie. de novo synthesis, and the decline in P.A.L.-activity was accompanied by a decline in P.A.L. protein. However using a combination of immunoprecipitation and radio-isotopic labelling to measure rates of enzyme turnover, there was no decrease in the rate of P.A.L. synthesis during the decline (Tanaka and Uritani,1977b), and it was suggested that inactivation occurred (Tanaka et al.,1977), but this was not operative until at least 10h. after cutting, as postulated for the decline in P.A.L. in incubated potato tuber discs (Zucker,1968). Experiments carried out by Blondel et al.(1973) on P.A.L. levels in radish cotyledons, used antibodies against P.A.L., and obtained results which indicated that a pool of inactive P.A.L. (recognized immunologically) was present in unstimulated tissue; these results have since been refuted, on the grounds that the purification technique for P.A.L. was not satisfactory (Faye, 1977). It now appears more likely that a mechanism similar to that described for sweet potatoes and potatoes, operates, ie. the increase in P.A.L. being due to synthesis, inactivation occurring later on (Huault and Klein-Eude,1978; Huault and Klein,1979).

Immunological techniques provide a valuable additional means of studying the problem of enzyme levels; with the advantage that inactive enzyme molecules (if present) may also be detected and measured. The methodology required is much more time consuming than previous techniques, and careful checks must be made throughout to eliminate errors. It is also necessary to have a reasonable understanding of the basis of the techniques, so the results can be interpreted.

The immunological response of vertebrates is elicited by the presence of foreign proteins or other macromolecules, and relies on the ability of special proteins, the immunoglobulins, to recognise invading macromolecules. (The recognition is based on the arrangement of atoms within the molecule,

(conformation) giving each molecule a distinctive 'shape', a property that is fundamental to all biochemical reactions.) The size of molecule that can be recognised is limited; the larger the molecule, the greater the potential for recognisable differences. The immunological response is based on the ability of specific immunoglobulin molecules to react with specific molecular configurations exposed on the surface of the invading macromolecule (Gill,1971). These regions of the invading macromolecule are called 'determinants', and have not been characterised yet (Blake,1975; White et al., 1978), but it has been estimated that there is one determinant/5,000 daltons (Crumpton,1974). Auto-antibody formation is suppressed by naturally acquired immunological tolerance (Cinader,1967). Once a foreign macromolecule has been identified by a specific antibody, many identical immunoglobulin molecules are produced by the plasma cells of the lymphoid system and circulated in the blood where they combine with and inactivate the invading macromolecules, by forming antibody-antigen immunocomplexes. It is thought that clonal selection allows a clone of specific antibody forming cells to proliferate, and thereby increase the amount of specific antibody (Rogers,1980; Yanchinski,1981) Different clones of plasma cells differ from each other in the formation of a single protein only. This means that for each determinant there is a population of antibody molecules which will react with only a particular determinant. The specificity of antibodies makes them ideal tools for measuring specific macromolecules.

Antibodies are widely used for studying molecules in animals, provided there is sufficient immunological difference between the molecules being investigated and those in the animal being used to raise antibodies. The problem of measuring small molecules (5,000 daltons) has been overcome by coupling the molecule to a large inert molecule (a 'hapten'). Smaller molecules are more likely to produce an homogeneous population of antibodies (Arnon,1971); with larger macromolecules, the presence of more than one determinant on the antigen, brings about an inevitable heterogeneity in the antibody population produced. Each clone of antibodies is specific for

a certain determinant, Cinader (1967) recognised three functional types of antibody, distinguished by their effect on enzyme activity (of the antigen) (1) inhibitory,(2) not inhibitory but competes with the sites of (1),(3) no effect. Stimulation of enzyme activity is thought to be associated with poor substrate, or with mutant enzymes; binding of the antibody to the antigen resulting a change in conformation which improves the enzyme activity (Arnon,1973).

Immunogenicity, the ability to induce antibody formation can only be determined by empirical means, and may depend upon the animal and/or species chosen, and the immunization schedule. Rabbits are most commonly employed, sheep are usually equally successful, and more suitable when large volumes of antiserum are required. Immunogenicity is augmented by simultaneous injection of antigen in saline solution and Freund's adjuvant ('incomplete' contains only an emulsifier in mineral oil,'complete' also contains a mycobacterial suspension), the combination is foreign to the animal, even if the antigen is similar to a native molecule, and usually initiates antibody production. The number of immunocompetent cells may be increased by periodic stimulation/injection (Gill,1971), therefore it is likely that serum from later bleeds will have a different avidity and specificity, if only small quantities of antiserum are required the batches of serum are kept separate and the most suitable batch used.

The only reliable test of antibodies is the primary binding test, ie. measuring the amount of antibody bound to antigen. This is not usually very easy to measure, unless the antibodies are labelled (Schröder and Schäfer,1980), and here the sensitivity of the test is limited by the degree of labelling of the antibody. Secondary tests include the precipitin reaction, agglutination and complement fixation, which are directly dependent on the amount of antibody-antigen complex formed. Tertiary tests include competition assays involving the immune elimination of antigen, either by immunoprecipitation and assay of the antigen at a range of antiserum or antigen concentrations (immunotitration), or by precipitation of antigen in

the presence of labelled antigen and a limited amount of antibody, followed by separation of the bound and unbound antibody, the amount of label in the bound fraction being inversely proportional to the amount of unlabelled antigen (radioimmunoassay, Haber and Poulsen,1974). The latter technique is particularly sensitive (ng. range) and is widely used for measuring hormone levels in animals (Felber,1974), and is an invaluable tool in clinical biochemistry. The use of specific antibodies for plant research has only recently gained support, partly because of the work involved (Daussant et al.,1977). The earlier studies with antibodies used the precipitin reaction as a measure of the antibody-antigen reaction. A combination of immunoprecipitation and radio-isotopic labelling was used by Kleinkopf et al.(1970), to show that the light-induced increase in ribulose diphosphate carboxylase in greening barley leaves was due to de novo synthesis of the enzyme. Cases where immunological techniques have been used for studying changes in P.A.L. levels have already been discussed. It is worthwhile considering the results of experiments with other enzymes, (no immunological studies with A.A.O. have been published yet), and comparing the results obtained, with those from experiments using other techniques. For example, immunological comparison of the amylase enzymes in germinating seedlings, showed that the increase in α-amylase activity in wheat seedlings, was due to de novo synthesis of at least two isoenzymes, (in agreement with results obtained by the density labelling technique, Filner and Varner,1967), whereas β-amylase was detected in ungerminated seeds, and was activated during germination (Daussant and Corvazier,1970; Daussant and Hill,1979). Kruger (1970) demonstrated that latent β-amylase could be activated in vitro, supporting evidence for the role of activation in increasing the level of β-amylase activity.

Antibodies were used to study sugar transport in sugar cane; plant tissue treated with rabbit anti-invertase serum was unable to transport sucrose. (Bowen and Hunter,1972). The interpretation of the results was based on the assumption that invertase activity was inhibited by the antiserum,

and that sucrose was normally hydrolysed by invertase prior to transport into the cell, across the plasma membrane, and reformed within the cell.

Studies on the effect of temperature on acid invertase in potatoes suggested that a specific inhibitor of the enzyme controlled invertase activity (Pressey and Shaw,1966; Pressey,1967). The effects of cycloheximide on the levels of acid-invertase and P.A.L. in sweet potato following wounding were compared; the increase in both enzymes was inhibited, but whereas the decrease in P.A.L. was prevented by cycloheximide, the decline in invertase was stimulated (Matsushita and Uritani,1975). The differential action of cycloheximide was explained by differences in the inactivating mechanisms for the two enzymes, invertase being inactivated immediately synthesis was complete, and P.A.L. inactivation occurring prior to the observed decline in activity. An inactivator was subsequently isolated from sweet potatoes specific for invertase (Matsushita and Uritani,1976). In radish, invertase (β-fructofuranosidase) was found to be under phytochrome control (Zouaghi and Rollin,1976), inhibitor studies showed that in roots, cycloheximide was inhibitory, but was not in hypocotyls. This was attributed to a transfer of cytosolic enzyme to cell wall-associated activity induced by far-red light in the hypocotyls. Immunological studies, based on immunotitration, double diffusion precipitin formation and radioimmunoassay, showed that β-fructofuranosidase was synthesised in ripening tomato fruit (Iki et al.,1978) but inactive enzyme was identified in senescent fruit (Nakagawa et al.,1980). This was in agreement with the results of earlier studies on the development of cell wall-bound activity in ripening and senescent tomato fruit (Iwatsubo et al.,1975; Iwatsubo et al.,1976). The activation of invertase activity in chicory root was shown to be stimulated by 2,4-dichlorophenoxyacetic acid; dual radio-isotopic labelling experiments suggested that de novo synthesis was not responsible for the large 2,4-D stimulated increase in activity, but a second isoenzyme was probably increased through de novo synthesis. (Gordon and Flood,1980). Therefore both synthesis and activation appear to be involved in increasing inveratse activity,

although different isoenzymes may be involved. An equally complex picture is revealed by studies on nitrate reductase (N.R.)-activity. Inhibitor studies suggested that protein synthesis was required for the disappearance of N.R. in barley leaves following removal of light (Travis et al.,1969), and the nitrate-induced increase in N.R.-activity required a light period (Travis et al.,1970), suggesting that inactivation occurred, the light treatment releasing active enzyme. Density labelling experiments showed that the nitrate-induced increase in cultured tobacco cells was accompanied by de novo synthesis of N.R. (Zielke and Filner,1971). The enzyme in peas, was subsequently shown to be mediated via phytochrome; enzyme activity was also seen to decline in the presence of nitrate (Jones and Sheard,1975). N.R. in mustard is also under phytochrome control; the nitrate induction was sensitive to cycloheximide, but the light induction of the enzyme was not (Johnson,1976), indicating the operation of at least two mechanisms in the control of N.R.-activity. A dual mechanism was also suggested for the N.R. response in green algae (Hipkin and Syrett,1977). A recent study showed that immunologically recognizable N.R. was present in Chlorella, but was not active, therefore although the enzyme had been newly synthesised, activity was not expressed immediately (Funkhauser et al.,1980). The increase in activity of a specific isoenzyme of cellulase in the abscission zone of leaves of Phaseolus vulgaris, was shown to be due to an increase in immunologically detectable isoenzyme, using the radioimmunoassay technique to measure the cellulase protein (Sexton et al.,1980). Antibodies were also used to detect cellulase in in vitro translation products formed using R.N.A. extracted from auxin-treated pea epicotyls, auxin treatment resulted in an increase in the cellulase m.R.N.A. and after a 24h. lag, an increase in cellulase activity (Verma et al.,1975). The radioimmunoassay technique was also used to measure the levels of two polygalacturonase isoenzymes during ripening of tomato fruit (Tucker et al.,1981), de novo synthesis of enzyme occurred, although in fully ripened tomatoes some inactive enzyme was detected.

This review has shown that immunological techniques can be used for studying enzyme levels in plants, and that isoenzymes and inactive enzyme molecules are often recognised by the antiserum raised against active enzyme. It is hoped to use similar techniques in this investigation into the photocontrol of P.A.L. and A.A.O. levels. By comparing changes in enzyme activity and changes in enzyme protein content, it should be possible to distinguish between increases in enzyme activity arising via de novo synthesis of the enzyme, and increases arising from activation of existing but inactive enzyme molecules. Where transient increases in enzyme activity occur it should also be possible to obtain information relating to the decline in enzyme activity. The two enzymes being studies are sufficiently large to possess many determinants, making it likely that iso-functional enzymes from different plant sources will cross-react with the antiserum raised against enzyme from a particular plant; whether the degree of cross-reactivity will be adequate to allow enzyme from other plants to be measured (ie. whether there will be a sufficient number of recognisable determinants on each enzyme), can not be predicted, and must be determined by experiment.

SECTION 2 MATERIALS AND METHODS

2.1 PLANT MATERIAL

2.1.1 Source of Potatoes: Potato tubers (Solanum tuberosum,L.), of several varieties, most frequently 'King Edward's'or'Desiree', were purchased from the local market and stored in darkness at 4°C. Tubers that had been stored in this way for more than 4 months or that showed signs of physical damage, sprouting or fungal infection were discarded.

2.1.2 Preparation and Incubation of Potato Tuber Discs: The method used for preparation of the potato discs was taken from Havir and Hanson (1970). The tubers were first washed in tap water, columns of tuber tissue were removed with a cork-borer (internal diameter 18mm), and then sliced into 1-2mm thick discs using a razor blade. The discs were rinsed in distilled water several times (until the rinsing solution was no longer cloudy), and blotted dry on filter paper before being weighed. The discs were incubated in transparent plastic petri-dishes, each containing 14 discs and 5ml. distilled water (sufficient to keep the discs moist without being completely submerged) and placed under white lighting (Section 2.3.1) in a constant temperature cabinet, between 18° and 25°C. For dark incubations, petri-dishes were covered in two layers of aluminium foil before placing in the light cabinet. Discs were incubated for up to 42h., discs from petri-dishes showing blackening or considerable softening of tissue were not used. The discs were blotted dry on filter paper and reweighed before extraction.

2.1.3 Source of Seeds: Mustard (Sinapis alba,L.), gherkin (Cucumis sativus,L.var.'Venlo Pickling') and pumpkin (Cucurbita pepo,L.var.'Gourd') seeds were obtained from Asmer Seeds Ltd.,Leicester. Damaged or irregular seeds were removed.

2.1.4 Preparation of Seeds: To prevent fungal contamination during the germination and growth of seedlings, mustard and pumpkin seeds were soaked in 10% Na-hypochlorite solution (for 5 and 10 min. respectively) and rinsed in distilled water twice; excess water was removed on filter paper. Untreated seeds of mustard and pumpkin were rapidly contaminated during

incubation at 25°C (conditions for germination), gherkin seeds did not require this treatment.

2.1.5 Germination and Growing Conditions: The prepared seeds were sprinkled on to 50ml. 1% agar ('Oxoid' Agar No. 1), approx. 0.5cm. depth in clear plastic lunch boxes with fitted lids (11.5 x 17.5cm. base area, 6.0cm. depth) and these were placed in a growth room at 25°C.

Mustard seeds were germinated in darkness (Section 2.3.5); after 36-48h. some boxes of seedlings were transferred to continuous far-red light (Section 2.3.3). Seedlings up to 72h.-old were harvested and extracted within ½h.

Gherkin seeds were germinated in darkness (Section 2.3.5); when seedlings were 3 days-old boxes of seedlings were either transferred to continuous blue light (Section 2.3.4) or white light (Section 2.3.1), or left in darkness, for up to 7h. (or 24h.). Tissue was harvested and extracted within 20min..

Pumpkin seeds were germinated under white light (Section 2.3.1) or in darkness (Section 2.3.5). At various stages of development boxes of dark-grown seedlings were transferred to the white light. Seedlings up to 120h. (5 days) -old were harvested (the tough seed coats were removed from seedling 48h.-old or less by squeezing across the widest part of the seed), and extracted within 20min..

2.1.6 Tobacco Cell Suspension Cultures: Cultures of tobacco cells (Nicotiana tabacum, L.) were initiated and maintained by D.Pearson (Phd. thesis 1979). Subculturing on to fresh medium was carried out at 7-21 day intervals, using sterile techniques. Cells from six stock cultures (in 250ml. conical flasks) were filtered through a sterilized gauze then pooled and transferred to fresh medium in a 4l. fermenter flask. (For details of culture medium see D.Pearson 1979) The main difference between the stock and experimental medium was in the hormone concentrations. The stock medium contained auxin (naphthalene acetic acid) at 2mg/l. and kinetin at 0.2mg/l., with sucrose at 30g/l. and caesin hydrolysate at 1g/l..

The experimental medium contained auxin at 0.2mg/l. and kinetin at 0.02mg/l., with sucrose at 30 or 50g/l., but no caesin. Cell suspension cultures were incubated for up to 14 days with a continuous filtered air-flow, both aerating and mixing the cells. Samples (between 100 and 200ml.) were taken after shaking the flasks to distribute the cells evenly; the cells were harvested by vacuum filtration on a Büchner funnel lined with 1 layer of 'Miracloth', and extracted immediately.

2.1.7 Source of Enzymes: Cucurbita A.A.O. was obtained from Boehringer Mannheim GmbH. Biochemica, Boehringer Corp. Ltd.(catalogue no.236314). Rhodotorula P.A.L. and the enzymes and proteins for calibration of gel filtration and polyacrylamide gel electrophoresis columns were obtained from Sigma Chemical Co.

2.2 BIOCHEMICALS

All biochemicals were analytical grade reagents, obtained from Sigma Chemical Co., B.D.H. Chemicals Ltd. or Fisons Scientific Apparatus Ltd. unless otherwise stated.

2.3 LIGHT SOURCES

2.3.1 White Light: An irradiation area of 50 x 100cm. was provided by three white fluorescent tubes, in a temperature-controlled environment. Plant material was placed 20-30cm. below the light source, and the fluence rate (400-800nm.) was 40-60μMol.$m^{-2}s^{-1}$.

2.3.2 Dim White Light: A single white fluorescent tube provided lighting in a temperature-controlled room; fermenter flasks and stock flasks were placed 1.5-2.0m. away, receiving a fluence rate (400-800nm.) of 0.7-0.8μMol. $m^{-2}s^{-1}$.

2.3.3 Far-Red Light: An irradiation area of 36 x 66cm. was provided by ten 150W. single coil, clear envelope, tungsten bulbs, above a transparent water-cooled plate (2cm. thick), 1 layer(3mm.) of'red 400' perspex, 1 layer (3mm.) of 'green 600' perspex and 1 layer(3mm., sandwiched between sheet glass) of'deep blue Cinemoid No.20'. (Cinemoid was obtained from Rank Strand, Brentford., perspex from I.C.I. Welwyn Garden City) The fluence rate (400-

800nm.) at the position of the seedlings, 5cm. below the filters, was 58μMol $m^{-2}s^{-1}$. Photosynthetically active radiation (P.A.R.) (400-700nm.), gave a fluence rate of 0.09 Mol$\mu m^{-2}s^{-1}$.. The far-red irradiation (peak at 730nm gave an estimated photostationary state ([Pfr]/[Ptot]), ϕc(calculated) of 0.005. (Holmes and Smith, 1975)

2.3.4 Blue Light: An irradiation area of 36 x 66cm. was provided by six 20W. cool-white daylight tubes (with black insulation tape masking the ends of the tubes to obscure emission of far-red light), above 1 layer 'deep blue Cinemoid No.20' and a 2cm.-thick layer of 1.5% copper sulphate solution. The fluence rate(400-800nm.), 5cm. below the filters was 1.51μMol. $m^{-2}s^{-1}$., and 1.50μMol. $m^{-2}s^{-1}$. for the photosynthetically active radiation. The photostationary state, ϕc of 0.45.

2.3.5 Green Safe Lights: A portable green safe light was provided by a manufacturer's luminair with a 20 or 40W. green fluorescent tube. Dark rooms were fitted with fluorescent tubes (masked at the ends to obscure emission of far-red light), filtered through two layers of 'primary green Cinemoid No.39', and surrounded by several layers of black plastic sheet (neutral density filter), giving a fluence rate (400-800nm.) of 0.004μMol. $m^{-2}s^{-1}$., and photosynthetically active radiation (400-700nm.) of 0.004μMol. $m^{-2}s^{-1}$.. The photostationary state was measured using dark-grown tissue, ϕm(measured) of 0.01.

2.3.6 Measurement of Light Sources: Fluence rates were measured with a scanning spectroradiometer calibrated between 400 and 800nm. (Gamma Scientific Incorp.,San Diego.) Photostationary state was either calculated ϕc (Holmes and Smith,1975) or measured ϕm. For the green safe lights, ϕm was determined by measuring samples of dark-grown pea hypocotyls placed in the cuvette, in a dual wavelength spectrophotometer (Perkin Elmer, Model 156) and calculating the phytochrome photoequilibrium (the ratio of absorbances at 660 and 720nm.,following brief far-red and red irradiations).

2.4 EXTRACTION PROCEDURES

All extractions were carried out at 4°C (cold room or ice-bucket). Upon

harvesting, seedlings were divided into cotyledon pairs, hypocotyls (including the hook region) and radicles, depending on the stage of development. The seed coat was removed from seedlings during the early stages. No hypocotyl region was distinguishable in mustard seedlings less than 40h.-old, or pumpkin seedlings less than 5 days-old. A minimum of 50 tissue sections were extracted together.

2.4.1 Extraction Procedures: Plant tissue was ground in a mortar and pestle with a suitable volume of extraction buffer (Section 2.4.2) for 1min.. The homogenate was filtered through 1 layer of 'Miracloth' and centrifuged in an Eppendorf Centrifuge 3200 for two 2min. spins.(Running speed 12,000rpm.=10,000 x g.) Volumes greater than 20ml. were centrifuged in an M.S.E.18 high speed centrifuge at 10,000 x g. for 15min. at 4°C.. The supernatant was collected, the pellet discarded; those samples containing large quantities of lipid (eg. from cotyledons) gave a surface layer of lipid material, which was discarded (together with the pellets) by using a Pasteur pipette to collect the supernatant. An additional centrifuge spin was carried out if lipid material mixed with the supernatant. All supernatant volumes were adjusted by the addition of extraction buffer to the supernatant so the volume was proportional to the number of tissue sections used. Samples were kept at 4°C before assaying, or frozen for storage.

2.4.2 Extraction Buffers and Volumes,P.A.L. Extractions:

[A] 75mM borate buffer, pH 8.8 containing 1mM E.D.T.A., 10mM D-iso-ascorbate and 5mM 2-mercaptoethanol.

Potato discs: 2ml/g. tissue, mustard tissue: 1ml/10 sections, gherkin tissue: 1.5ml/10 sections.

[B] 100mM Na-phosphate buffer, pH 6.5 or 7.2 containing either 5mM 2-mercaptoethanol or 1mM glutathione.

Mustard and gherkin tissue as [A].

[C] 75mM borate buffer, pH 8.8 containing 1mM E.D.T.A., 5mM Na-sulphite and 5mM 2-mercaptoethanol.

Tobacco cells: 2.5ml/g. harvested (moist) cells.

A.A.O. Extractions:

[D] 100mM citrate-phosphate buffer, pH 5.0.

Mustard tissue: 2.0ml/g. tissue, gherkin and pumpkin tissue: 2.5ml/g. tissue.

2.4.3 Desalting: Sephadex G-25 (medium) was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. The dry gel was added to buffer and allowed to swell overnight at room temperature. Swollen gel was stored at 4°C, but was allowed to warm up before pouring columns, at room temperature (reducing the extent of air bubbles formed). Columns were transferred to 4°C and equilibrated at that temperature with running buffer. The void and total column volumes were determined with samples of blue dextran and sodium nitrite (potassium dichromate was used with non-borate buffers). Fractions from the columns, up to 20ml. total volume were collected manually, otherwise an Ultrorac Fraction Collector (L.K.B., model 7000, fitted with an automatic valve, model 7017-2) was used. The protein-containing fractions were pooled and 'stabilizing' reagents added immediately(where necessary).

2.4.4 Desalting Buffers and Columns, P.A.L. Extracts:

[X] Sephadex G-25 column: 1.5cm. diameter x 16.0cm. height. equilibrated in 50mM borate buffer, pH 8.8.
Potato extract samples (2.0-5.0ml.) were desalted, and 2.0ml. fractions collected at a flow rate of 1.0ml/min.

[Y] Sephadex G-25 columns in 10ml. plastic syringe barrels (up to the 10ml. mark) equilibrated in either 50mM borate buffer, pH 8.8 or 100mM Na-phosphate buffer, pH 7.2.
Mustard and gherkin extract samples (1.0ml.) were desalted, the void volume (3.0ml) was discarded and the next 2.5ml collected. D-isoascorbate was added to desalted mustard samples, to 10mM.

A.A.O. Extracts:

[Z] Sephadex G-25 columns in 10ml. plastic syringe barrels (as [Y]) equilibrated in 100mM citrate phosphate buffer, pH 5.0.
Mustard extract samples (1.0ml.) were desalted as for [Y]. No

additional reagents were added.

2.5 ENZYME ASSAYS

2.5.1 Radioisotope Assay for P.A.L.-Activity: The assay was based on that of Kuokol and Conn, 1961, but using tritiated L-phenylalanine (Attridge, Johnson and Smith, 1974) All assays were carried out in duplicate and the mean value used. The reaction mixture contained 0.2ml. enzyme sample and 0.2ml. 75mM borate buffer, pH 8.8, and was incubated at 37°C. The reaction was initiated by adding 0.2ml. 6mM L-phenylalanine containing 0.05μCi L-[4-^{3}H] -phenylalanine (from The Radiochemical Centre, Amersham.) and incubated for 30-120min., then stopped by adding 1.5ml. solution, containing 1.0ml. 20% (w/v) trichloro-acetic acid and 0.5ml. 0.1% (w/v) trans-cinnamic acid in 0.05M KOH. Cinnamic acid was then extracted from the reaction mixture by mixing with 3.0ml. toluene for 10-20s., then centrifuging in a bench centrifuge for 5min.. A 2.0ml. aliquot of the toluene (upper) phase was mixed with 8ml. scintillation fluid (5g. 2,5-diphenyloxazole/l. toluene). Radioactivity was counted in a Beckmann scintillation counter, model L-3133P or LS-100. A control assay was carried out with buffer instead of enzyme sample to obtain a blank value (which, due to the slight solubility of phenylalanine in toluene was higher than the level of background radioactivity). A quench correction curve was determined using a set of standard carbon tetrachloride-quenched tritium-labelled toluene samples (from Amersham-Searle, no. 180050, 1975) This assay has several advantages over the conventional spectrophotometric assay (Section 2.5.4), being suitable for measuring low levels of P.A.L.-activity, in many samples together, with relatively crude enzyme preparations. The sensitivity of the assay was increased by using L-phenylalanine at a higher specific radioactivity (0.2μCi.L-[4-^{3}H] phenylalanine/0.2ml. L-phenylalanine). Samples with particularly low P.A.L.-activity (such as extracts from etiolated mustard and gherkin tissue or from freshly sliced potato tuber tissue),were assayed by mixing 0.4ml. 75mM borate buffer pH 8.8 with 0.4ml. enzyme sample, and adding 0.2ml. 10mM L-phenylalanine to initiate the reaction.

2.5.2 Linearity of the Radioisotope P.A.L. Assay: Potato P.A.L. samples from both dark- and light-incubated potato tuber discs were desalted (on separate Sephadex G-25 columns) and assayed for certain periods, between 30 and 180min. long. The results are shown in Figure 2.1, the assay was linear for at least 180min.. Using the more sensitive form of P.A.L. assay, samples of P.A.L from dark-grown and from far-red treated mustard seedlings were assayed (see Figure 2.2). The assay was linear for at least 150min. with far-red treated mustard cotyledon extracts, but with extracts from dark-grown seedlings the assay was only linear for 90min.. Short assay periods (30 min.) gave apparently high P.A.L. activity, and were not subsequently used. P.A.L. from dark-grown mustard was less stable in supernatant extracts than from far-red treated seedlings, the latter also contained higher levels of P.A.L. activity. Mixing experiments were carried out for both dark- and light-treated tissue (mustard, gherkin and potato) extracts; there was no evidence for inhibitors or activators in the extracts, although the stability of P.A.L. in dark- and light-treated tissue extracts was slightly different over longer periods of time (such as 24h.)

2.5.3 Assay of P.A.L. in Gel Slices: P.A.L.-activity was located on polyacrylamide rod gels and in agarose gel after electrophoresis by direct assay of the gel slices. The reaction mixture contained three 1mm-polyacrylamide rod gel slices or one 0.5 x 1.0cm sections of agarose gel, with 0.3ml. 75mM borate buffer, and incubated for 10min. at 37°C, before initiating the reaction, by adding 0.2ml. 6mM L-[^{3}H] phenylalanine. The reaction was stopped after 60min., and the cinnamic acid formed measured as in Section 2.5.1.

2.5.4 Spectrophotometric Assay for P.A.L.-Activity: The assay was based on that used by Zucker, 1965. The continuous assay was carried in quartz cuvettes in a temperature-controlled U.V. Spectrophotometer (Unicam, model SP800A). The reaction mixture contained 0.4ml. enzyme sample, 1.0ml. 50mM or 100mM borate buffer pH 8.8 and distilled water to 2.8ml., incubated at 37°C. A second cuvette acting as a blank, contained enzyme sample but

Figure 2.1

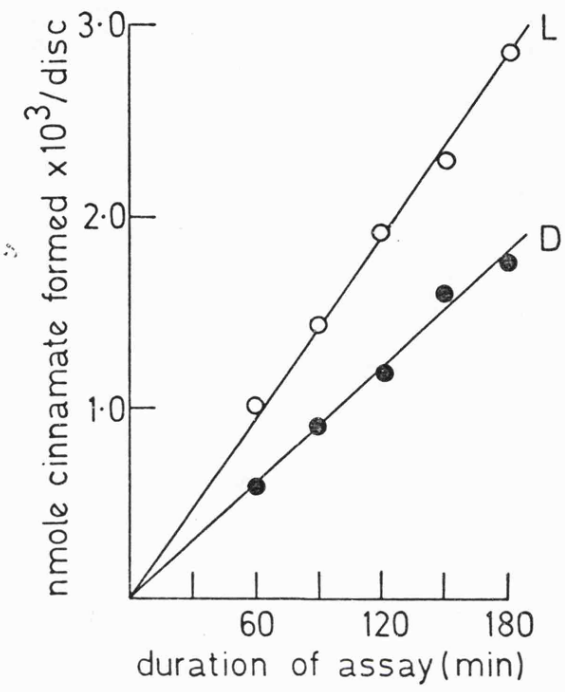
Graph to show the linearity of the radioisotope P.A.L. assay using potato P.A.L. extracts.

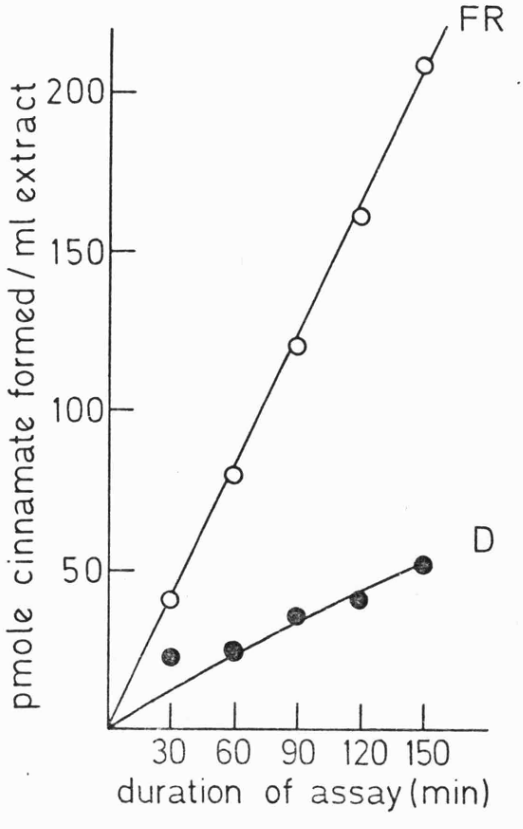
Potato tuber discs were incubated for 20h. in darkness (D) or in white light (L); supernatant extracts were desalted and assayed as described in Section 2.5.1 for a range of assay periods, between 60 and 180min..

Figure 2.2

Graph to show the linearity of the radioisotope P.A.L. assay using mustard cotyledon extracts.

Dark-grown mustard seedlings 40h.-old were either transferred to far-red light (FR) or left in darkness (D) for a further 24h.; supernatant extracts of cotyledons were assayed as described in Section 2.5.1 (using the more sensitive form of the assay), for a range of assay periods, between 30 and 150min..


nmole cinnamate formed x10³/disc
3·0
2·0
1·0
L
D
60
120
180
duration of assay (min)


pmole cinnamate formed / ml extract
200
150
100
50
FR
D
30
60
90
120
150
duration of assay (min)

no substrate was added. The increase in absorbance of cinnamate at 290nm. was followed over a 5-10min. period after initiating the reaction by adding 0.2ml. 100mM L-phenylalanine solution. The initial reaction rate was used to calculate the enzyme activity, using an extinction coefficient for cinnamate of ε(290nm.)=10,000. The reaction rate was linear for 0-5min., depending on the amount of enzyme activity. Slightly higher P.A.L.-activities were obtained using the lower buffer concentration (50mM). This assay was less sensitive than the radioisotope assay, and was not suitable for assaying mustard and gherkin cotyledon samples (which contained large quantities of protein, that interfers with absorbance readings). The stopped spectrophotometric assay of Kuokol and Conn, 1961 is more suitable for such samples, but also takes much longer to complete. The continuous spectrophotometric assay was compared with the radioisotope assay using potato P.A.L. samples, a good correlation was found between the two methods, although the latter gave consistently higher results (approx. 1.5 times) and was particularly convenient for assaying samples of P.A.L. also containing antiserum, (and therefore of high protein content).

2.5.5 Spectrophotometric Assay for A.A.O. Activity: The assay was taken from Drumm et al., 1972. The assay was carried out in two 3ml. quartz cuvettes (one acting as a blank) in a temperature-controlled U.V. spectrophotometer (Unicam SP800A) at 37°C. The reaction mixture contained 10-200µl. enzyme sample or two 1mm.-thick polyacrylamide rod gel slices, and 2.5ml. 100mM citrate-phosphate buffer pH 5.0. The reaction was initiated by adding 50µl. 5mM L-ascorbate solution, mixed thoroughly and the decrease in absorbance of ascorbate at 265nm. followed for 2-10min. The reaction was linear for up to 10min., depending on the amount of enzyme activity. A blank assay was also required, in the absence of added enzyme, ascorbate was slowly lost (0.005 O.D. units/min./2.5ml. reaction mixture.) Desalted enzyme samples gave the same rate of enzyme activity as supernatant extracts and improved the assay for low activity samples, by reducing the extent of background signal noise on the chart recorder, which was too high for accurate

assays when large sample volumes were required (ie. for low activity). A.A.O. activity was calculated using an extinction coefficient for ascorbate of ϵ(265nm.)=15,100,(1.0 O.D. unit = 165 nmoles ascorbate in 2.5ml.); the extinction coefficient was pH-dependent, and adjustments were necessary for calculating enzyme activities at other pHs.(ie. other than pH 5.0).

2.5.6 Units of Enzyme Activity: Enzyme activity was calculated as nmoles or pmoles of substrate used or product formed/min., and expressed in terms of protein content or number of tissue sections. (The latter was used for P.A.L. activity of seedling extracts, where the protein content changes during the experimental period, and has been justified by Mohr, 1972.) The correct form of expressing enzyme activity is the S.I. unit, katal (1 katal= 1mol. product formed/s.) but the older convention of enzyme activity units (1 Unit = 1μmol. product formed /min.) was preferred here, because the two enzymes being studied have been measured for at least 20yrs., and comparison of results is therefore simplified (conversion to katals is staightforward should this be required).

2.6 PROTEIN DETERMINATIONS

Aliquots of enzyme extract were diluted to 1.0ml. with distilled water and mixed with an equal volume of 20% trichloroacetic acid. The mixture was incubated at 37°C for 15min., centrifuged in a bench centrifuge for 10min. and the supernatant discarded. The precipitated protein was allowed to drain and re-dissolved in 1ml. 1M NaOH. The protein content was then measured using the method of Lowry et al.,(1951),using Folin's reagent diluted 1:1 with distilled water. A protein calibration curve was prepared using bovine serum albumin as a standard. The standard assay was biphasic, linear between 0 and 80μg. and also between 80 and 150μg. protein (B.S.A.). Dilute protein solutions (not adequately precipitated by trichloroacetic acid) were assayed as described by Potty, 1969; using a method which allows for the presence of phenolic compounds, which normally interfer with protein estimates made using an unmodified Lowry-type assay. For a recent review of methods of protein determination see Robinson,(1979).

2.7 PURIFICATION PROCEDURES FOR P.A.L.

2.7.1 Polyvinylpyrrolidone, P.V.P.: The presence of phenolic compounds in plant tissues affects extractability and stability; the formation of tannins, enhanced by disrupting plant cells, is often inhibited by the inclusion of polymeric protective agents in the extraction buffer. P.V.P. was washed before use by boiling in 10% HCl for 10min., then allowing to cool and settle for 15min..The supernatant was decanted and the P.V.P. washed with distilled water until chloride free (silver nitrate test was used to detect the presence of chloride ions),and finally rinsed in acetone, and allowed to dry (in a fume cupboard). The prepared P.V.P. was stored in dry form at 4°C. Before use the P.V.P. was allowed to soak in the extraction buffer overnight, 1g/10g fr.wt. tissue to be extracted. The P.V.P. was removed from the enzyme extract during the subsequent filtration and centrifugation.

2.7.2 Protamine Sulphate: Highly basic proteins, such as protamine sulphate can be used to precipitate acidic or negatively charged compounds from plant extracts. Protamine sulphate (grade III, from herrings, Sigma Chem. Co.) was used as a 2% solution in 100mM Na-acetate buffer,pH 5.0. This was added to the supernatant from the extraction, with stirring, 10ml/10g tissue extracted, and the pH lowered to 5.0 by adding 1M acetic acid. The solution immediately became cloudy, and after stirring for 15min. was centrifuged in an M.S.E.18 high speed centrifuge at 10.000 x g. for 15min. (at 4°C). The supernatant was decanted and 1M NaOH added to adjust the pH to 8.8. Unfortunately protamine sulphate was found to have little effect on the stability or in the purification (by precipitation of proteins) and some P.A.L. activity (5-10%) was lost from the supernatant during this step.

2.7.3 Dowex Resins: Dowex resins were washed repeatedly (5-6 times) in deionized water before use, then equilibrated in buffer overnight, at 4°C. The resin was collected, immediately before the extraction, in a Buchner funnel, lined with 2 layers of Whatman No.1 filter paper. The resin was then added to fresh buffer. In preliminary tests to select a suitable form

of resin, the dowex resin was added directly to the extraction buffer, and removed by filtration. Subsequently, the dowex resin was equilibrated in 150mM K-phosphate buffer pH 6.5 and added to the supernatant extract as a separate step. The mixture was lowered to pH 6.5 by adding 1M HCl, filtered and then returned to pH 8.8. The dowex resin was washed for re-use in 2M HCl or 2M NaOH, distilled water and 50% acetone; but the adsorption capacity was less than that of unused resin.

2.7.4 Salt Fractionation: Ammonium sulphate(especially low in heavy metals for enzyme work) $(NH_4)_2SO_4$ and tri-sodium citrate, were obtained from Fisons Ltd.. Solid salt was added slowly to the enzyme preparation, and the pH of the solution was followed. Ammonium sulphate tended to lower the pH, while tri-sodium citrate tended to increase the pH; drop-wise addition of 1M NaOH or 1M HCl was added to maintain the desired pH. The fractionation was usually carried out in two steps, the initial salt addition was ceased when the solution began to become cloudy, and the mixture centrifuged in an M.S.E. 18 or M.S.E. 21 high speed centrifuge at 10,000 x g. for 15min. (at 4°C). The material precipitated at the first step usually contained little enzyme activity. The supernatant was treated again by adding sufficient salt to precipitate the enzyme and centrifuged again. The fractionation could be carried out in 3 or 4 steps, using a smaller salt increment at each step. The precipitate was dissolved in buffer, either the extraction buffer or the desalting buffer, and the volume of the enzyme preparation concentrated 10-20 times (compared to the initial volume). Samples were stored under liquid nitrogen, with only a 10% loss of enzyme activity after 6 months storage. Samples stored in a deep freeze were less stable, 40-50% loss of enzyme activity occurred after 6months storage.

2.7.5 Calcium Phosphate Gel: Calcium phosphate (or 'Brushite') was prepared according to the method of Siegelman et al.(1965). Before use the gel suspension or slurry was washed several times in distilled water. The gel was used in a batch-wise procedure as described by Havir and Hanson,1970. A salt-concentrated enzyme preparation was adjusted to pH 5.5 with 1M acetic

acid and calcium phosphate added (approx. 20ml. gel suspension/2ml. enzyme preparation). The mixture was stirred for 15min., then centrifuged in a bench centrifuge for 5min.. The supernatant was treated in the same way with a second batch of calcium phosphate gel. The two batches of gel were then eluted with 10-20ml. volumes of 50mM borate buffer pH 8.8, followed by several similar volumes of 200mM borate buffer pH 8.8. The bulk of P.A.L. activity was present in the first two washes of each batch of gel. The washes containing P.A.L.-activity were concentrated by ammonium sulphate fractionation (The ultrafiltration cell could have been used to concentrate the washes, and probably would have given a better recovery of enzyme, but was not available at the time the experiments with calcium phosphate gel were carried out, see Section 2.7.10)

2.7.6 Cellulose Phosphate Gel: Cellulose phosphate gel was used in the same way that phosphocellulose gel (the same type of gel chemically, but from a different supplier) was used by Tanaka and Uritani,(1977a).The gel was equilibrated in 50mM K-phosphate buffer, pH 6.1 and poured into a column 2.8cm. diameter x 7.0cm. height. The column was washed through with at least two column volumes of pH 6.1 buffer until a constant pH eluent was obtained. A salt-concentrated enzyme preparation (6-10ml.) was adjusted to pH 6.1 by adding 1M acetic acid. (The sample was not desalted at this pH because losses of P.A.L activity occurred and the final recovery of P.A.L. activity from the cellulose column was unnecessarily reduced) The sample was loaded on to the column, and non-binding protein washed through with pH 6.1 buffer, P.A.L.-activity was retained by the gel. A pH gradient was used to elute the enzyme; a linear pH gradient was not possible (such gradients are very difficult to generate with limited volumes of buffer solutions), instead the running buffer (pH 6.1) was exchanged for a higher pH buffer, 50mM K-phosphate pH 8.0, which gave a steep increase in pH. The P.A.L.-activity was eluted between pH 6.5 and 7.5. Fractions of 4.0ml. were collected from the column and 0.4ml. aliquots assayed for P.A.L.-activity (Section 2.5.1) Those fractions containing P.A.L-activity were concentrated as described in

Section 2.7.5 and the same comments apply.

2.7.7 Diethylaminoethyl Cellulose Gel: DEAE-cellulose gel (DE 52) was obtained from Whatman Ltd., Maidstone, Kent. Pre-swollen gel was prepared as described in the Whatman information leaflet, I.L.2 'Advanced Ion Exchange Celluloses', and equilibrated in 50mM Tris-HCl buffer, pH 8.0(4°C). A column was poured (with the same dimensions as Section 2.7.6) and a salt-concentrated enzyme sample desalted on a Sephadex G-25 column in 50mM Tris-HCl buffer pH 8.0(4°C) before loading on to the DEAE-cellulose column. Non-binding protein was washed through the column with one column volume, and bound protein using a 0-0.3M NaCl gradient. The gradient was set up between 50 and 300mM NaCl solutions in buffer (100ml.:100ml.); 4.0ml. fractions were collected and 0.4ml. aliquots assayed for P.A.L.-activity. P.A.L.-activity was eluted from the column between 100 and 200mM NaCl, although the elution was partly due to an increase in pH that occurred as the salt concentration increased.

2.7.8 Diethylaminoethyl-Sephadex-A25 Gel: DEAE-Sephadex gel was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. The gel required little pre-treatment (unlike the cellulose gel, see Pharmacia pamphlet on 'Sephadex Ion-Exchangers'). The gel was equilibrated with 50mM Tris-HCl buffer, pH8.0 (4°C). In preliminary experiments a small column, 2.0cm. diameter x 5.0cm. height was used, a larger column was used for the actual purification. A desalted enzyme preparation (same buffer used as the column running buffer) was loaded on to the column and non-binding protein washed through. Bound protein and P.A.L.-activity was eluted using a 0-300mM NaCl gradient, as described in Section 2.7.7, or using a gradient set up between 50mM and 500mM Tris-HCl buffer pH 8.0. Recoveries of P.A.L-activity were improved by using 1mM E.D.T.A. and 0.5-1.0mM glutathione in both buffers. The Tris buffer gradient did not cause the same pH increase as the NaCl gradient, improving the degree of purification achieved, by allowing a sequential elution of bound protein with a narrow peak of P.A.L-activity.

2.7.9 Gel Filtration: Sepharose 6B-100 was obtained from Sigma Chemical

Co.. Pre-swollen gel was washed (to remove azide) and equilibrated in 50mM borate buffer pH 8.8. The gel was poured in to a column 3.2cm. diameter x 90.0cm. height (Wright Scientific Ltd. Kenley, Surrey). A continuous flow of buffer from the outlet tube was maintained as the column was poured to allow even sedimentation. The buffer reservoir was placed 1m. above the column (2l. 50mM borate buffer pH 8.8) and the column run until a constant bed height was obtained. The column was then calibrated for molecular weight (MW) determinations (Gruber and Marrink, 1969) using 10ml. sample volumes of standard proteins (see Figure 2.3A). The elution volume of each protein was converted to its Kav. value, $Kav. = \frac{Vs - Vo}{Vt}$ (Vs = sample elution volume, Vo = void volume (blue dextran elution volume) and Vt = total column volume (nitrite elution volume) and this was plotted against log.MW. (see Figure 2.3B). The calibration curve was re-calculated whenever it was necessary to repour the column (ie. when the flow rate fell below 0.8ml./min.) Kav. values of the standard proteins were consistent each time, although the column volume varied slightly. Samples were always prepared in borate buffer pH 8.8 before loading on to the column to avoid the effects of changing pH on the elution volume (see Lin and Castell, 1978). MWs. of sample proteins were calculated by reference to the calibration curve.

2.7.10 Ultrafiltration: An ultrafiltration cell, model 52 (60ml. capacity) and 'Diaflo' ultrafiltration membranes, type XM100A were obtained from Amicon Ltd., Woking, Surrey GU21 1UR. The membranes were washed with one cell volume of distilled water in the ultrafiltration cell before use. The ultrafiltration cell was connected to a nitrogen gas cylinder and operated at a pressure of 10-15 lbf/in^2, with continuous stirring. One cell volume of enzyme sample was concentrated 6-10 times within 1.5-2.0h. The pressure was released, and the outlet tube squeezed several times, forcing the flow back across the membrane, in the opposite direction, in order to reduce losses due to adhesion to the membrane. Samples were removed from the cell using a Pasteur pipette (the tip of which had been smoothed in a bunsen flame, to avoid damage to the membrane). The membrane was rinsed in distilled

Figure 2.3

Calibration of the Sepharose 6B column for molecular weight (MW) determinations.

The Sepharose 6B column (3.2cm.diameter x 90.0cm height) was equilibrated in 50mM borate buffer, pH 8.8. Samples of 10ml. volume were passed through the column using this buffer.

A. Elution profile of standard globular proteins.

The protein content of the fractions from each run (all proteins run separately) was measured by reading the absorbance at 280nm.

Thyroglobin (bovine type I) MW = 650,000

Ferritin (horse spleen) MW = 465,000

Catalase (bovine liver) MW = 244,000

Aldolase (rabbit liver) MW = 158,000

Lipoxidase (soybean) MW = 102,000

The void volume of the column was estimated by passing a sample of blue dextran through the column (absorbance at 640nm. measured). The total column volume was estimated by passing sodium nitrite through the column (nitrite assayed and absorbance at 540nm. measured).

B. Calibration Curve for MW determinations.

The elution volume (fraction number) of each of the standard proteins depends on their MW.. Kav. values, (see explanation in text, Section 2.7.9) were calculated for each protein and plotted against log. MW.

Sepharose 6B column
A
Blue dextran
Thyroglobin
Ferritin
Catalase
Aldolase
Lipoxidase
Nitrite
1·5
1·0
0·5
OD280 reading
OD640 or OD540 reading
(blue dextran) (nitrite assay)
10
20
30
40
50
60
70
fraction number
B
Lipoxidase
Aldolase
Catalase
Ferritin
Thyroglobin
Kav.
0·6
0·5
0·4
0·3
0·2
5·0
5·2
5·4
5·6
5·8
6·0
log (mw)

water and stored in 10% ethanol at 4°C while not in use. After 5 or 6 runs a new membrane was required, as the membrane became clogged, and the flow rate through the membrane was reduced. A second ultrafiltration cell, model 32 (10ml. capacity) was used (again with XM100A type membrane) to concentrate partially purified samples to smaller volumes (1.0-3.0ml.).

2.7.11 Polyacrylamide Gel Electrophoresis: Polyacrylamide rod gels (7%) were prepared by mixing together 10.5ml. 20% acrylamide solution, containing 0.5% bisacrylamide, 3.75ml. 3M Tris-HCl buffer pH 8.5(4°C), 15.5ml. distilled water, 0.25ml. freshly prepared 10% ammonium persulphate and 0.02ml. T.E.M.E.D. (N,N,N',N', tetramethylethylenediamine). The mixed solution was poured immediately into perspex rods (0.5cm. internal diameter x 9.0cm. height), sealed at the bottom with 'Parafilm', and then carefully overlaid with distilled water (approx. 1ml.). The gel set within 15-20min. at room temperature. The 'Parafilm' seal was removed as soon as the gel had set,and the rod gels placed in the electrophoresis apparatus. Six rod gels were suspended between two buffer reservoirs (each 600ml. capacity) with platinum electrodes. The running buffer contained 50mM Tris, 380mM glycine and 4mM 2-mercaptoethanol, pH 8.8. Samples (25-100µl.) were mixed with 40% sucrose solution and loaded on to the rod gels in situ with a 'Finn' pipette. Bromophenol blue marker (5-10µl. in buffer) was added to samples which were to be stained for protein (not those which were retained for their enzyme activity) and electrophoresis carried out at a constant current, 1mA/rod gel for 10-20min.; then at 4-6mA/rod gel for 2-3h. (Anode at the bottom of the rod gels) The gels were removed from the perspex rods, and either stained for protein or frozen in dry ice (solid CO_2), sliced and stored or assayed for P.A.L.-activity. For protein staining, the gels were submerged in Coomassie blue stain (1.25g. Coomassie blue, 454ml. 50% aqueous methanol, 46ml. glacial acetic acid and 50g. trichloroacetic acid) for 3-6h. and then destained (37.5ml. glacial acetic acid, 25ml.methanol and 437.5ml. distilled water),until protein-stained bands were visible. For enzyme assays the frozen gel was sliced in a 'Mickle ' gel slicer, and three 1mm-thick slices incubated

together in 0.3ml. 75mM borate buffer pH 8.8 at 37°C for 10min., and then assayed as described in Section 2.5.3. Frozen gel sections were kept in a deep freeze until required (not more that 4 weeks).

2.7.12 Sucrose Density Gradient Centrifugation: Sucrose gradients were prepared in 16ml. cellulose nitrate tubes, 1.1-1.2ml. enzyme sample (desalted salt-concentrated emzyme preparation, in borate buffer, pH 8.8) was placed on top of the sucrose gradient and the tubes carefully balanced (a few drops of distilled water were added where necessary) and then centrifuged in a Beckmann ultracentrifuge model L2-65B, using a SW-27 rotor with small buckets, at 27,000rpm. for 25h.. The gradient was fractionated by placing a long needle to the bottom of each tube and pumping out the solution, beginning with the most dense fractions (1.4ml. fractions). A 5-15% gradient was used, the separation of P.A.L.-activity from the bulk of protein was not improved by using a 5-20% gradient. In the absence of stabilizing agents a 50% recovery of P.A.L.-activity was obtained.

2.8 PURIFICATION PROCEDURES FOR A.A.O

2.8.1 Salt Fractionation: Ammonium sulphate, $(NH_4)_2SO_4$ (as in Section 2.7.4 was added to an enzyme preparation to 30% saturation, centrifuged at 10,000 x g. for 15min. in an M.S.E. 18 high speed centrifuge, and then further salt added to the supernatant to 60% saturation and centrifuged again. The precipitate was dissolved in 100mM citrate-phosphate buffer pH 5.0.

2.8.2 Sephadex G-150 Gel: The gel was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden., and was equilibrated in 50mM Na-phosphate buffer pH 7.0. The gel was poured into a column, 1.25cm. diameter x 21.0cm. height. Samples (1.0-2.0ml.) were passed through the column; 1.2ml. fractions were collected. The column was calibrated for MW determinations using 1.2ml. sample volumes (Figure 2.4A). The calibration was plotted as elution volume or fraction number against log. MW (Figure 2.4B), and was linear for this size column over the fractionation range. Identical columns were poured and used for radioisotopically labelled Cucurbita A.A.O., then discarded as radioactive waste. Samples were always run in Na-phosphate buffer, pH 7.0-7.2.

Figure 2.4

Calibration of the Sephadex G-150 column for molecular weight determinations.

The Sephadex G-150 column (1.25cm. diameter x 21.0cm. height) was equilibrated in 50mM Na-phosphate buffer, pH 7.0. Samples of 1.2ml. volume were passed through the column using this buffer.

A. Elution profile of standard proteins.

The protein content of the fractions from each run (all proteins run separately) was measured by reading the absorbance at 280nm.

Catalase (bovine liver) MW = 244,000

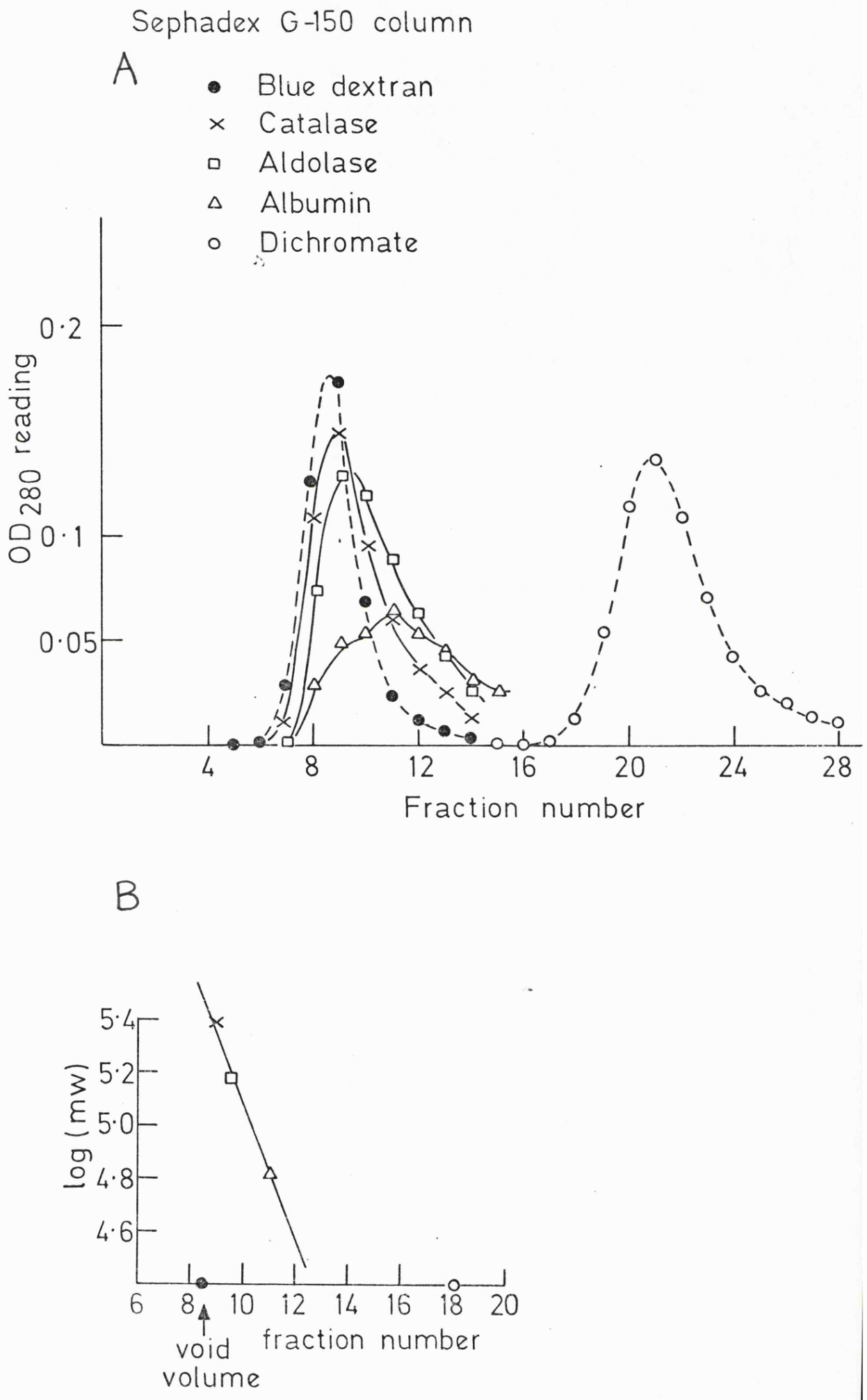
Aldolase (rabbit muscle) MW = 158,000

Albumin(bovine plasma) MW = 66,000

The void volume of the column was estimated by passing a sample of blue dextran through the column (absorbance at 640nm. measured). The total column volume was estimated by passing potassium dichromate through the column (absorbance at 420nm. measured).

B. Calibration Curve for MW determinations.

The fraction number corresponding to the elution volume of each of the standard proteins was plotted against log. MW.


Sephadex G-150 column
A
Blue dextran
Catalase
Aldolase
Albumin
Dichromate
OD 280 reading
0·2
0·1
0·05
4
8
12
16
20
24
28
Fraction number
B
log (mw)
5·4
5·2
5·0
4·8
4·6
6
8
10
12
14
16
18
20
fraction number
void volume

2.8.3 Lyophilisation: Samples of up to 2.0ml. volume were placed in open glass tubes and placed in the chamber of the freeze-drier (Edwards, High Vacuum Ltd., model EF2). The lyophilisation (or vacuum freeze-drying) was complete within 1-2days., and the dry powder samples were kept in sealed tubes in the deep freeze (not more than 4 weeks). Samples were re-dissolved in 100mM citrate-phosphate buffer pH 5.0, being concentrated by up to 4 times the original sample volume.

2.8.4 Polyacrylamide SDS Gel Electrophoresis: The purity of the commercially obtained Cucurbita A.A.O. was checked using electrophoresis. Denaturing slab gels (23.0 x 16.0cm.area, 0.2cm. thick) were prepared using a discontinuous buffer system as described by Chua and Benmoun, 1975. The Cucurbita A.A.O. sample was prepared by mixing 100µg. in 100µl. 80mM Tris-H_3BO_4 buffer pH 6.1, containing 5% 2-mercaptoethanol and 3% SDS (sodium dodecyl sulphate) and 20% sucrose, this was then heated on a boiling water-bath for 30s.(to denature the protein). Aliquots, 30µl.and 70µl. were run on separate slab gel channels,with marker proteins (especially for SDS-gel electrophoresis from Sigma Chemical Co.) in neighbouring channels. Electrophoresis was performed at a constant current, 17.5mA for 6-7h. The slab gel was then stained in Coomassie blue stain (0.1% Coomassie blue in 7% acetic acid and 40% methanol) for 4-6h., then destained (7% acetic acid and 30% methanol) for 2-3 days.

2.8.5 Polyacrylamide Gel Electrophoresis: Non-denaturing polyacrylamide rod gels were prepared as described in Section 2.7.11, using Na-phosphate buffer pH 7.5. Electrophoresis was carried out at a constant current for 1.5h. with Cucurbita A.A.O. or salt-concentrated mustard cotyledon samples. The gels were removed and either stained (as in Section 2.7.11) or assayed for A.A.O-activity (Section 2.5.5)

2.9 IMMUNIZATION PROCEDURE: P.A.L. (May-July 1979)

Two rabbits were provided and kept by the Animal House, Medical School , Leicester. All injections and bleeds were carried out by qualified staff under the supervision of Dr. D.Morton. Frozen polyacrylamide gel sections

from the P.A.L.purification (see Section3.2.9) were thawed and homogenized with 2.0ml. Freund's complete adjuvant (provided by Dr.D.Morton) in an Ultra-turrax homogenizer.(Eight gel sections were used). Half of the homogenate (equivalent to 90µg. P.A.L. protein) was injected into each of the rabbits, at one or two sites on the inside of the hind leg. Three further injections were given at 2-weekly intervals. Trial bleeds (5ml.) were taken from the ear-vein after the third injection , and then at 2-weekly intervals. At the end of the third month, 40-50ml. blood was taken from each rabbit(several 10ml. bleeds taken over a 2 week period).The blood was allowed to clot at room temperature, kept at 4°C overnight, then centrifuged in an Eppendorf centrifuge 3200, in 1.5ml. aliquots, until no further material was precipitated. The serum collected was frozen and kept in the deep freeze, several 100µl. aliquots were kept separately for immediate antibody tests. (Serum was labelled R1A or R2A, from rabbit R1 and R2 respectively.) Normal rabbit serum, NRS. was collected from non-immunized rabbits

2.10 IMMUNIZATION PROCEDURE: A.A.0 (July 1978-80)

Before proceeding with the immunization , one rabbit was injected with a sample of the A.A.0. lyophilisate to check for adverse side effects. For this 200µg.A.A.0.lyophilisate was dissolved in 1.0ml. saline and 2.0ml. Freund's complete adjuvant (provided by Dr.B.Crighton), 1.0ml. was injected (70-80µg. A.A.0.lyophilisate) into the rabbit at six subcutaneous sites along the back. After 2 weeks, the only noticeable effect was blistering at the sites of injection (which often occurs following immunization injections). Two more rabbits were immunized: 400µg. A.A.0.lyophilisate was dissolved in 0.5ml. saline and 2.0ml.Freund's complete adjuvant, 1.0ml (170µg. A.A.0.lyophilisate) was injected into each rabbit, as before. The rabbits were kept in the Animal House, Nottingham School of Agriculture, Sutton Bonington., and all injections and bleeds were carried out by a qualified technician (Martin Stainer). Booster injections (170µg A.A.0. lyophilisate) were given at 3 month intervals, using freshly dissolved Cucurbita A.A.0. Trial bleeds (5ml.) were taken 10-14 days after the

immunization or booster injections, then at weekly intervals for 4 weeks. The blood was allowed to clot at room temperature, then kept at 4°C overnight and the serum collected by centrifugation in a bench centrifuge. Serum was then divided into 100µl. aliquots and kept in the deep freeze. Normal rabbit serum, NRS. was taken from non-immunized rabbits, and normal guinea pig serum, NGS,, from a non-immunized guinea-pig.

2.11 PURIFICATION OF ANTI-P.A.L. SERUM

2.11.1 Ammonium Sulphate Fractionation: Serum from rabbits R1 and R2 was kept separate throughout. Serum was thawed and an equal volume of saturated (100%) $(NH_4)_2 SO_4$ solution added. This was stirred for 15min., then centrifuged in an M.S.E.21 high speed centrifuge (8 x 50ml. rotor) at 10,000 x g. for 15 min.. The supernatant was decanted and discarded, the precipitate was resuspended in 1.75M $(NH_4)_2 SO_4$, and then centrifuged again, as above. The precipitate was treated in this manner several times, until the supernatant discarded was almost clear (this procedure removes most of the haemoglobin still present in the serum.) The final precipitate was resuspended in 10mM K-phosphate buffer pH 8.0 (the serum was concentrated slightly by making up to a volume less than the original), and dialysed against the same buffer overnight.(2 changes of buffer) The serum was then centrifuged again(some material was precipitated) and dialysed against 20mM Na-phosphate buffer pH 7.2, containing 0.15M NaCl. for several days. The dialysed serum was divided into 8ml. aliquots for further purification or 100µl. aliquots for antibody tests, and kept in the deep freeze. This was labelled R1B or R2B, for each rabbit.

2.11.2 DEAE-Cellulose Chromatography: An 8ml. aliquot from the previous step was thawed and passed through a Sephadex G-25 column (2.0cm. diameter x 16.0cm. height),equilibrated in 50mM Tris-HCl buffer pH 7.6(room temperature) and then applied to a DEAE-cellulose (Whatman, DE52) column (4.8cm. diameter x 3.0cm. height) in the same buffer; 10ml. fractions were collected. The protein content of the eluted fractions was followed by measuring the absorbance at 280nm. in a Beckman spectrophotometer model 24; when no more

protein was eluted with the running buffer, a gradient was set up, with an increasing salt concentration: 0-0.3M NaCl in 125:125ml. buffer, to elute the remaining γ-globulins. The fractions were tested for γ-globulins using goat antibody to rabbit γ-globulins and to whole rabbit serum, on double diffusion plates, using 15μl. aliquots (see Section 2.12.2). Those fractions containing γ-globulins only, were pooled, and concentrated by ultrafiltration. The purified γ-globulins were divided into 250 or 500μl. aliquots, frozen and kept in the deep freeze(and labelled R1C, R2C, and subsequent purifications $R1C_2$, etc..).

2.12 IMMUNOLOGICAL TECHNIQUES

2.12.1 Simple Diffusion in Tubes: The technique used was taken from Chase and Williams,(1968). Pyrex tubes with an internal diameter of 10mm. were coated inside with a layer of 0.4% agar (Ionagar 'Oxoid' No.2) and dried. Equal volumes of antiserum and melted agar (0.6ml. each) were placed in a tube in a water-bath at 45°C and mixed. The agar-serum was poured into the precoated tubes to a height of 10mm., and allowed to cool. A series of tubes were prepared, containing serum from different bleeds or NRS. Antigen solution (100μg. Cucurbita A.A.O. lyophilisate in 4.0ml. 50mM Na-phosphate buffer pH 7.2, containing 0.15M NaCl) was layered on top of the agar-serum to a height of 5mm. or more, and the tubes sealed with 'Parafilm' and kept at room temperature. The tubes were examined several hours later and also the following day. This method provides results within 1 day, and is suitable for initial antibody tests. In the presence of antibody and antigen a precipitin reaction occurs, forming an opaque meniscus-shaped disc just below the surface of the agar-serum layer, as the antigen diffuses into the agar.

2.12.2 Double Diffusion Plates: The method of Ouchterlony(1949), was used; 1% agarose (from B.D.H. Chemicals Ltd., especially for electrophoresis) was dissolved in 50mM Na-phosphate buffer pH7.2, containing 0.15M NaCl. Glass plates were precoated with a thin layer of the agarose, dried and then the agarose was poured on to the plates on a horizontal surface, with 2.0ml. for

microscope slides (2.6 x 7.6 cm), 8.0ml. for glass plates (5.0 x 10.0cm.). The gel was allowed to cool and set. Cork borers (sizes 1-5) were used to cut wells out of the agarose layer (2-6mm diameter). Aliquots of antiserum and enzyme sample (up to 100µl.) were placed in the wells and allowed to diffuse, in a moist atmoshpere, at 4°C., for 1-2 days. Precipitin lines formed between those wells containing antibody and antigen respectively, these were often visible as white arcs between wells, but were also easily stained for protein with Coomassie blue stain. Double diffusion plates were washed pressed and dried before staining as described by Mayer and Walker(1978). Protein stain contained 5g. Coomassie blue in 900ml. mathanol, 200ml. glacial acetic acid and 900ml. distilled water. Destain was the same minus the blue stain. (Since the beginning of these experiments Coomassie blue has been discontinued and is now no longer available commercially. Instead Page Blue G-90 was obtained from B.D.H.Chemicals, this stain was not as successful, but was the only alternative on the market at the time; B.D.H.are presently working on several alternatives to the Coomassie blue stain which should be available in the future. For staining with the Page Blue G-90, 10g. were used in place of 5g. Coomassie blue).

2.12.3 Immunoelectrophoresis: Glass plates (5.0 x 10.0cm) were prepared as in the previous section, with 1% agarose in 20mM Svendsen's buffer pH 8.7 for immunoelectrophoresis (Grabar, 1964). Wells were cut into the agarose gel with cork borers (sizes 1-3, 2-4mm diameter) towards one end of the plates and placed on the water-cooled plate of the flat-bed electrophoresis system, and filter paper wicks placed on the edges of the plates, between the agarose gel and the buffer reservoirs. The buffer was Svendsen's and contained 5mM 2-mercaptoethanol. Enzyme samples were placed in the wells and electrophoresis was performed with a constant current, 10mA/plate, for 2.5-3h. Three identical plates were usually run together. Following electrophoresis one plate was immersed in picric acid solution (7g. picric acid dissolved in 500ml. water at 60°C, filtered and 100ml. glacial acetic acid added), for 10min., then rinsed in ethanol, washed and dried down and

stained for protein as in the previous section (the picric acid step is required to fix the plate,ie. prevent the proteins from diffusing from the agarose during staining). A trough was removed from the second plate, 2mm wide,from between the wells, parallel to the direction of electrophoresis, and filled with antiserum (50-250µl.); the plate was incubated in a moist atmosphere at 4°C for 2-3 days, then stained for protein. The third plate was used to locate P.A,L.-activity. The gel was cut into long strips parallel to the direction of electrophoresis, including the sample well, and then cut along it's length into 0.5cm. wide sections, which were placed in 0.3ml. 75mM borate buffer pH 8.8 at 37°C for 10min. and assayed as in Section 2.5.3. The first plate gave the position of the proteins in the original enzyme sample, and was used to check that electrophoresis had taken place. The second plate was used to locate P.A.L. protein immunologically and to check for possible isoenzymes.

2.12.4 Immunotitration: Aliquots of enzyme sample (up to 400µl.) were mixed with a series of antiserum samples of increasing volume (0-50µl.) in 20 or 50mM Na-phosphate buffer pH 7.2, containing 0.15M NaCl, (to a final volume of 880µl.) in Eppendorf micro-testtubes (1.5ml. capacity). Controls were also set up using normal rabbit serum, NRS. or saline-buffer The tubes were incubated, usually overnight, (16-20h.) at 4°C. Aliquots of the mixture were then re-assayed for P.A.L.-activity (as in Section 2.5.1). Alternatively the tubes were centrifuged in the Eppendorf centrifuge 3200 for two 2min. spins (12,000rpm), aliquots of the supernatant were then re-assayed for P.A.L.-activity. The immunoprecipitates were washed by resuspending in 500µl. phosphate-saline buffer(PBS.),and centrifuging twice, this was repeated and finally the precipitates were dissolved in 200µl. 100mM glycine-NaOH buffer pH 9.5 for the P.A.L. assay. (A higher pH buffer was more successful in dissolving the precipitate, but the recovery of P.A.L. activity was poor or negligible.) Second antibody, goat anti-rabbit γ-globulin was added to the incubated tubes,100µl. GARGG, obtained from Calbiochem-Behring Corp. catalogue no. 539844, 125 units dissolved in 6.25ml.

phosphate-buffer-saline (PBS)) per tube, and incubated for a further 6h. at 4°C, and then centrifuged and treated as above. Use of a second antibody was made to check for complete immunoprecipitation of P.A.L.(antigen)-antibody complexes. This was not used in the final immunotitration experiments because at higher anti-P.A.L.serum concentrations, addition of GARGG did not give complete precipitation. This may have been due to the preferred precipitation of rabbit antiserum to which the P.A.L.antigen was not bound at these concentrations, a large pellet of material was always obtained when GARGG was added.

2.13 RADIOIMMUNOASSAY FOR A.A.O.

2.13.1 Iodination of Cucurbita A.A.O.: The iodination was based on that of Greenwood, Hunter and Glower,(1963). 200µg. A.A.O. lyophilisate was dissolved in 200µl. 50mM Na-phosphate buffer pH 7.2. A 25µl. aliquot (25µg.A.A.O. lyophilisate = 2.5µg protein)was mixed with 20µl. [^{125}I]-sodium iodide (1.0mCi.) in 500mM buffer and Chloramine T (from Fisons S.A. Ltd. AR) 2mg/ml. for 15s., them stopped by the addition of 75µl. Na-metabisulphite (2.4mg/ml.). 1% potassium iodide (0.5ml.) was added to the reaction mixture, and this was passed immediately through a Sephadex G-50 column (se Section2.13.2). This method gave a very low yield of [^{125}I]-labelled A.A.O. (0.2% label in the protein peak eluted from the column). An alternative method, based on the modification described by Redshaw and Lynch,(1974), was found to give much better yields, probably due to the milder oxidation conditions (see Bolton,(1977),for methods of radioiodination). A 25µl. aliquot of A.A.O. lyophilisate was mixed with 1.0 or 0.5 mCi. [^{125}I]-sodium iodide in 500mM Na-phosphate buffer amd 20µl. Na-hypochlorite (from B.D.H.) 2µl/2ml. for 20-80s.(40s. gave the optimum results) and the reaction was stopped as described above. [^{125}I]-sodium iodide was obtained from Amersham Radiochemical Centre. The actual iodination was carried out by Dr.B.Crighton and all subsequent steps were carried out in the radioisotope laboratory, observing the appropriate regulations for handling [^{125}I].

2.13.2 Separation of [^{125}I]-labelled A.A.O. and Free Iodide: The iodination

mixture was passed through a Sephadex G-50 column (1.25cm.diameter x 15.0cm height) with 50mM Na-phosphate buffer pH 7.2. The glass column was fitted with a glass-wool filter and disposable plastic tip; these and the gel were discarded as radioactive waste after use, and the glass column decontaminated Pre-treatment of the Sephadex G-50 column was required to reduce loses due to non-specific binding to the gel or glass. 3ml. 5% egg albumin was passed through the column, followed by 20ml. buffer and 20ml. 1% KI in buffer Fractions were collected into a rack of disposable plastic tubes, the void volume,(four tubes of 1.5ml.eluent each determined from the elution volume of the egg albumin during preparation of the column) was discarded,then forty 20-drop fractions were collected. Radioactivity was measured in a Bioscint γ-counter with a lead shield in the well, 10s. counts were sufficient. Two peaks of radioactivity were eluted from the column, the first containing [^{125}I]-labelled A.A.0 and the second containing free [^{125}I] iodide (the second peak was usually largest). The specific radioactivity was expressed in terms of (μCi. radioactivity used - μCi. radioactivity in the second peak) or (μCi. in the first peak) / μg.protein used. Using freshly dissolved Cucurbita A.A.0 and freshly purchased [^{125}I]-sodium iodide, the specific radioactivity was 30-70μCi/μg. A.A.0.protein.

2.13.3 Purification of [^{125}I]-labelled Cucurbita A.A.0.:Cellulose Column:

CF11 cellulose powder was obtained from Whatman Ltd. The powder was poured on to 10mM Na-phosphate buffer pH 7.2, the slurry was poured into a column (1.25cm. diameter x 10.0-15.0cm.height) with a glass-wool filter and disposable tip. The iodinated Cucurbita A.A.0. sample (1.0-2.0ml.), from the Sephadex G-50 column was placed on the column, and washed through with 10mM Na-phosphate buffer pH 7.2, until no further radioactivity was eluted. The radioactive material eluted at this stage represents the 'damaged' material. Fractions of 1.0-1.5ml. were collected manually. The 'undamaged' labelled A.A.0. was eluted with 1% egg albumin in buffer. Higher concentrations of protein were tried in order to improve the yield of labelled A.A.0, such as 2.5% egg albumin and 2% bovine serum albumin, but at these concentrations

the flow rate was considerably reduced and occassionally the column was completely blocked. Yields of 2-10% radioactive A.A.O. were obtained from this step(as a% of the radioactivity loaded onto the column). The labelled A.A.O. obtained was used withim a week.

Sephadex G-150 Column:

A column identical to that in Section 2.8.2 was prepared in 50mM Na-phosphate buffer pH 7.2, and an iodinated Cucurbita A.A.O sample (1.0-1.5ml.) from the Sephadex G-50 column was placed on the column. Fractions of 1.2ml. were collected. Two peaks of radioactivity were eluted. The first peak, which corresponded to the elution volume of unlabelled Cucurbita A.A.O, containing the 'undamaged' material and the second peak, of lower MW material, which eluted close to total column volume, representing 'damaged' material. Purified [^{125}I]-labelled Cucurbita A.A.O from the first peak was diluted with buffer to give 5,-20,000 cpm./100µl. in the Gamma Set 500 γ-counter, and used for the radioimmunoassay. The yield from this column was 60-80% radioactive A.A.O. Samples were frozen or kept at 4°C, but stored samples were not suitable for the radioimmunoassay after several days or more. Freshly iodinated and purified Cucurbita A.A.O. was used for radioimmunoassay.

2.13.4 Test Binding Assays: All assays were carried out at 4°C in 2.5ml. plastic disposable tubes (in duplicate or triplicate). Each tube contained 100µl. antiserum (at dilutions from 1:1 to 1:20,000), 100µl. Na-phosphate buffer pH 7.2 and saline (0.15M NaCl) and 100µl. [^{125}I]-labelled Cucurbita A.A.O., diluted to give 5-20,000cpm (in the Gamma Set500 γ-counter), incubated for 24h at 4°C. In the first binding assays carried out 200µl. ethanol was then added, left 2h. and 500µl. phosphate-buffer-saline added before centrifuging, but non-specific precipitation occurred, so that there was no effect of antiserum over the control, NRS. Instead a second antibody was employed to precipitate the antigen-antibody complexes, 100µl. RD17 (a commercial preparation of donkey anti-rabbit γ-globulins, from Wellcome Reagents Ltd.) diluted 1:40 with phosphate-buffer-saline was added and the mixture incubated for a further 20h. Again before centrifuging

500µl.phosphate-buffer-saline was added (mainly to obtain a more easily manageable volume following centrifugation), the mixture was centrifuged in an M.S.E. centrifuge at 3,000rpm for 30min. The supernatant was decanted into a sink for radioactive waste and the precipitate remaining in the tube counted in the Gamma Set 500 model GS-100 γ-counter (ICN Pharmaceuticals N.V., Tracerlab Instruments Division, Belgium). The results were expressed in terms of % binding = $\frac{\text{(counts in ppt.) - (counts in NRS ppt.)}}{\text{(total counts used/tube)}}$

(ppt.=precipitate in tubes, counts = cpm from Gamma Set counter)

2.13.5 Standard Curve: A series of dilutions of Cucurbita A.A.0. were made using the same freshly dissolved A.A.0. as used for the iodination. 100µl. aliquots were mixed with 100µl. antiserum (at a pre-determined dilution, from the test binding assays) and 100µl.phosphate-buffer-saline were incubated at 4°C for 24h., then 100µl. [^{125}I]-labelled Cucurbita A.A.0. was added (5-20,000cpm/100µl.) and incubated for a further 24h.. Then 100µl. RD17 (second antibody) was added at 1:40 dilution, this was left another 24h.; 500µl. phosphate-buffer-saline was added and the mixture centrifuged at 3,000rpm for 30min. The immunoprecipitates were collected and counted in the tubes in the Gamma Set γ-counter. The results were expressed in terms of % binding of labelled protein to antiserum at each protein dilution (see Section 2.13.4). A linear plot was obtained using a logit.b plot against protein concentration (A.A.0 protein),

$$\text{logit.b} = \log_e\left(\frac{\%\ \text{binding}}{100 - \%\ \text{binding}}\right)$$

2.13.6 Unknown Samples: Tissue extracts of mustard, gherkin and pumpkin were tried in the radioimmunoassay, 100µl. aliquots of the extracts and dilutions thereof, were mixed with 100µl. phosphate-buffer-saline and 100µl. antiserum, 1:1000 or 1:5000 dilution (as determined from the test binding assays) and incubated for 1-4 days at 4°C, (there was little improvement in the sensitivity of the assay by increasing this incubation period beyond 2 days), 100µl. [^{125}I]-labelled Cucurbita A.A.0. (5-20,000cpm) was added and incubated for 24h.and then 100µl. 1:40 dilution RD17, incubated for 24h. and then 500µl. phosphate-buffer-saline added, the mixture centrifuged and

the radioactivity of the immunoprecipitates counted (1-2min. counts). % binding curves against extract dilution were drawn. The degree of cross reaction of the antiserum with A.A.O. from mustard or gherkin cotyledin extracts was poor, giving only minimal inhibition of binding by labelled A.A.O., the cross reaction with puppkin extracts was good. The results of these assays are presented in the next main section.

SECTION 3 RESULTS: P.A.L.

3.1 EXTRACTION PROCEDURES

3.1.1 Introduction: The enzymological and immunological methods used in these investigations rely completely on *in vitro* determinations of enzyme levels (in terms of the catalytic and the antigenic activities of the enzyme). These measurements were carried out using extracts of plant tissue, which were prepared by grinding the tissue in a suitable extraction buffer, followed by filtration and centrifugation of the homogenate, to give a solution containing the soluble enzymes under investigation. The composition of the extraction buffer was therefore extremely important, affecting both the amount of soluble protein and the stability of the proteins in the extract. Proteins are generally more soluble at higher pHs, and the stability is often improved by including additional reagents in the extraction medium, particularly with tissues containing phenolic compounds. However the suitability of the extraction buffer depends largely on the enzymes being studied and the tissue from which they are extracted. A series of trial extractions were carried out using different buffers, pHs and additional reagents such as metal chelators, anti-oxidants, thiol compounds, polymeric adsorbents and sugars, in order to select a suitable extraction buffer (see Rhodes,1977) for the P.A.L. extractions. The following points were considered:(1) Efficiency of extraction - The extraction of P.A.L. should be complete, ie. all the soluble P.A.L. present in the tissue should be present in the extract or the amount of P.A.L. extracted should be directly proportional to the amount of P.A.L. in the tissue, regardless of the growing conditions or light treatments received by the tissue. (2) Specific activity of the Extract - The extraction should be optimized for maximum extraction of P.A.L. and minimum extraction of other proteins. By attempting to reduce the amount of contaminating protein in the original extract, the stability of the extracts may be improved, and the number of steps required for the purification of P.A.L. kept to a minimum. (3) Stability of the Extract - In order to be able to measure P.A.L. levels in the extracts

accurately, the P.A.L. should be stable for a minimum period, determined by the length of time taken to carry out the extraction and the length of the assays to be used. (Or longer if the enzyme is to be purified.) There are many factors which may contribute to the stability, or lack of stability, and these include other enzymes, inhibitors, activators, substrates, products, co-factors, phenolic compounds, lipids or other molecules which may be present in the extract. The greatest concern of the plant biochemist is the effect of disrupting the cell and mixing of its contents, and particularly the release of substances retained within the vacuole, following rupture of the tonoplast. In an aqueous extract phenolic compounds are susceptible to oxidation, and this may lead to the formation of quinones and tannins, or tannin-like compounds, which will polymerise with the proteins present in the extract, leading to an irreversible loss of enzyme activity (or enzyme protein.) (4) Interpretation - The greatest problem encountered with a biochemical approach is the interpretation of results obtained in vitro and the extrapolation of any interpretation to the situation occurring in the whole plant. In these particular investigations the changes in the activities of one enzyme are measured, but it must be remembered that there are other biochemical changes occurring, and this may affect the extraction of tissue during the experimental period used.

3.1.2 Extraction of P.A.L. from Potato Tuber Discs: P.A.L. has been extracted and purified from potato tuber discs (Havir and Hanson, 1970), but not all varieties of potato were suitable for the procedure used. Also, the amount of P.A.L. and the response to light treatments is dependent on the temperature at which the potatoes have been stored (Rhodes and Wooltorton, 1978) and the duration of storage, the maturity of the tubers when harvested, the season and growing conditions (Hyodo, 1976). For these reasons trial extractions were carried out using the most readily available potato varieties, before proceeding with the purification procedures.

The highest levels of P.A.L.-activity were obtained from discs of tuber from either King Edward's or Desirée varieties which had been incubated

for 18-26h. The paler tissue varieties contained less P.A.L., but the pattern of changes in P.A.L.-activity were the same. The interval between slicing and the peak of P.A.L.-activity was dependent om the temperature at which the incubations were carried out, occurring earlier at 25°C and later at 18°C. (This was demonstrated by Lamb and Rubery,1976b) The amount of soluble protein extracted from the discs did not change appreciably during the incubation period (Figure 3.1). The trial extractions were carried out using discs containing easily measurable levels of P.A.L.-activity; discs of potato tuber were incubated for 16h. or more, when the highest levels of P.A.L.-activity were present. A borate buffer extraction medium was used, (Havir and Hanson,1970) and a range of thiol compounds and anti-oxidants compared (Table 3.1A). The extraction was not improved by the presence of any of the reagents tried; several reagents were inhibitory, L-cysteine, D,L-dithiothreitol and Na-metabisulphite. Of the other reagents tried (Table 3.1B), E.D.T.A. was without significant effect, sucrose also gave results comparable to the control. The polymeric adsorbents (see Lam and Shaw,1970; Loomis,1974; Gray,1978),were either without significant effect, such as polyethyleneglycol, or gave a reasonable specific activity but a reduced total amount of P.A.L., such as 'Amberlite' and 'Dowex' resins. The latter two are not usually included in extraction buffers, but this provided a useful way of assessing their potential value for subsequent treatment of the extracts. The 'Dowex' resin gave a particularly high specific activity, and although the total yield of P.A.L. was reduced; this was probably due to the incomplete removal of buffer from the 'Dowex' resin, this also applies to the 'Amberlite' extraction. Although the extraction per se was not improved, the stability was dependent on the presence of additional reagents; those which did not reduce the yields of P.A.L. were likely to be most suitable. Various other buffers and pHs were also tried, as shown in Table 3.2. Polyvinylpyrrolidone (P.V.P.) had been included in the extraction buffer in the earlier experiments, but as the results show

Figure 3.1

Time Course of the development of P.A.L.-activity in incubated potato tuber discs.

Potato tuber discs were incubated in darkness (closed symbols) or in white light (open symbols).

3.1A P.A.L.-activity (●,○) and protein content (■,□) were measured and are given in terms of tissue weight.

3.1B Specific activity of P.A.L. (▲,△), given in terms of protein content.

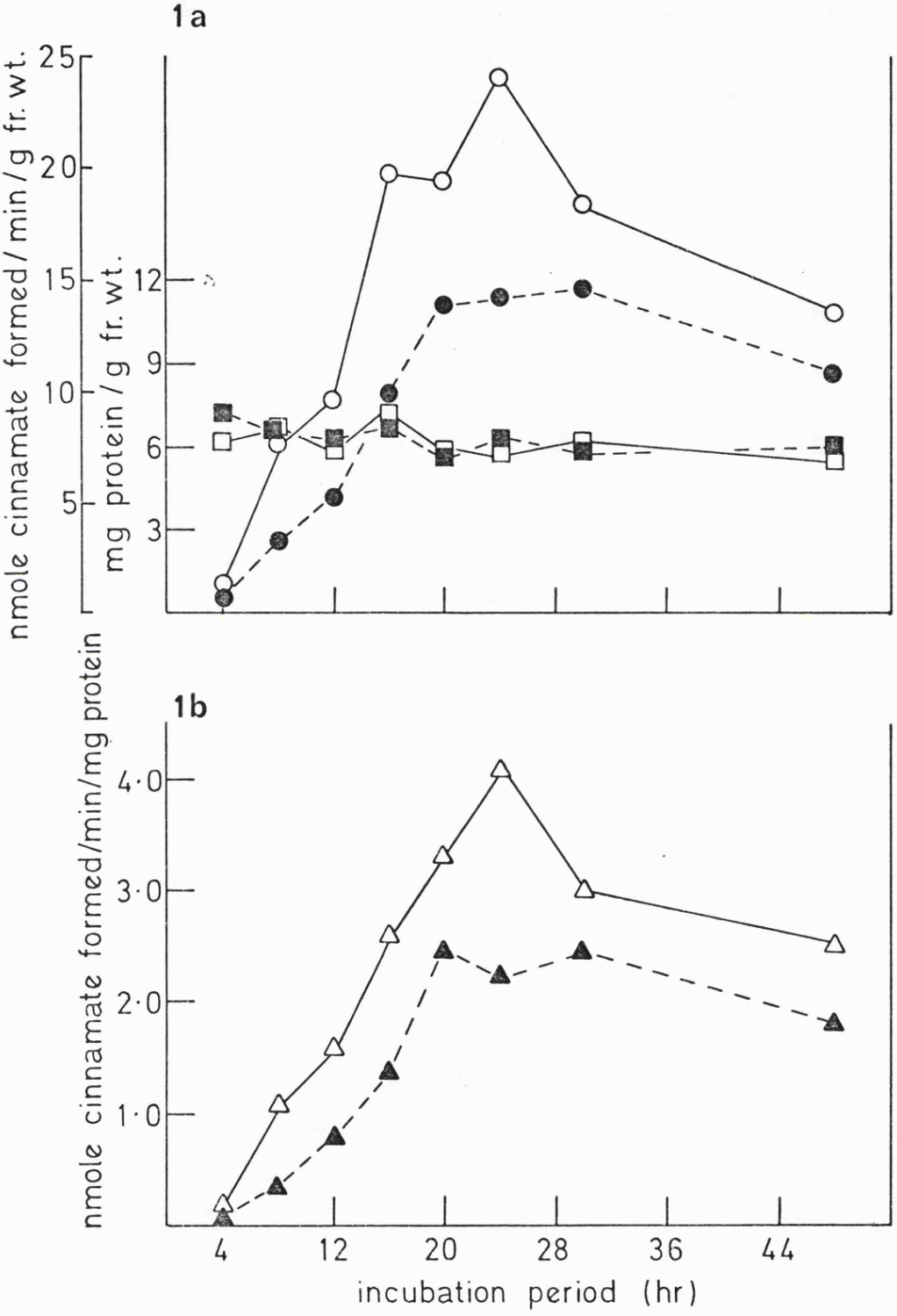

1a
nmole cinnamate formed / min / g fr. wt.
mg protein / g fr. wt.
25
20
15
10
5
12
9
6
3
1b
nmole cinnamate formed/min/mg protein
4·0
3·0
2·0
1·0
4
12
20
28
36
44
incubation period (hr)

Table 3.1

P.A.L.-activity in extracts of light-incubated potato tuber discs.

Effect of additional reagents in the buffer during extraction.

Potato tuber discs were incubated for 22h. in white light. The extraction (5g. discs/10ml. buffer) was carried out using 100mM borate buffer, pH 8.8, containing 1gP.V.P/10ml. and one of the following:

3.1A Effect of anti-oxidants and thiol reagents.

Additional reagent	P.A.L.-activity:nmoles cinnamate formed/mim. /g. incubated tissue	/mg. protein(T.C.A.ppt[d])
Control (none)	14.2	2.7
1mM mercaptoethanol	10.0	1.9
10mM mercaptoethanol	12.1	2.6
1mM glutathione	14.3	2.6
10mM glutathione	14.2	2.4
10mM dithiothreitol	11.4	2.4
10mM L-cysteine	10.4	2.2
20mM D-isoascorbate(Na)	12.7	2.7
10mM metabisulphite(Na)	8.5	2.2

3.1B Effect of other reagents.

Additional reagent	P.A.L.-activity:nmoles cinnamate formed/min. /g. incubated tissue	/mg. protein(T.C.A.ppt[d])
1mM E.D.T.A.	13.2	2.6
1% polyethyleneglycol	13.0	2.6
1g. 'Amberlite'	11.0	2.7
2g. 'Dowex-1' (no P.V.P)	5.8	4.4
0.1M sucrose	14.0	2.6

Table 3.2

P.A.L.-activity in extracts of light-incubated potato tuber discs.

Use of a range of different extraction buffers.

Potato tuber discs were incubated for 22h. in white light. The extraction (5g. tissue/6ml. buffer) was carried out using the buffers described below:

Extraction buffer	P.A.L.-activity nmoles cinnamate formed/min. /g. tissue	/mg. protein	Protein content mg.protein /g. tissue
100mM borate buffer,pH 8.8 + 0.6g.P.V.P./6ml.	14.2	2.7	5.3
100mM borate buffer,pH 8.0 + 0.6g.P.V.P./6ml.	9.0	2.2	4.1
100mM borate buffer,pH 8.8 + 10mM mercaptoethanol	16.0	2.9	5.6
100mM borate buffer,pH 8.8 + 10mM mercaptoethanol + 0.6g.P.V.P./6ml.	15.0	2.9	5.2
100mM borate buffer,pH 8.8 + 10mM mercaptoethanol + 20mM D-isoascorbate	17.7	3.2	5.4
As above, but using 3 volumes (18ml.)buffer.	14.0	1.9	7.4
200mM borate buffer,pH 8.8 + 10mM mercaptoethanol	13.6	2.5	5.4
100mM Tris-HCl buffer,pH 7.4 + 10mM mercaptoethanol	12.4	2.5	5.0
100mM Tris-HCl buffer,pH 8.0 + 10mM mercaptoethanol	13.1	2.6	5.0
100mM Tris-HCl buffer,pH 8.0 + 10mM mercaptoethanol + 20mM D-isoascorbate	15.0	2.9	5.2
100mM Tris-HCl buffer,pH 8.4 + 1mM mercaptoethanol + 20mM D-isoascorbate	17.8	2.6	6.7
100mM Tris-HCl buffer,pH 8.0 + 1mM mercaptoethanol + 20mM D-isoascor ate	15.8	2.9	5.5
100mM K-phosphate buffer,pH 8.0 + 1mM mercaptoethanol + 20mM D-isoascorbate	16.8	3.1	5.4
100mM K-phosphate buffer,pH 6.1 + 1mM mercaptoethanol + 20mM D-isoascorbate	4.3	1.2	3.6

it had little effect, probably due to the pH used (Loomis,1974; Gray,1978), and was excluded from subsequent extractions. Buffer in the pH range 8.0-8.8, gave the highest yields of P.A.L., and the highest specific activities. Increasing the ratio of extraction buffer to tissue did not increase the extraction of P.A.L., although the yield of soluble protein was increased. A buffer:tissue ratio of 1:1 (v/w) gave the highest yields and specific activity for P.A.L..A higher buffer concentration tended to reduce the yields; which was consistent with the observation that higher buffer concentrations in the assay also reduced the measurable enzyme activity. The presence of D-isoascorbate was found as effective as mercaptoethanol, and a combination of the two was slightly more effective than either alone. On the basis of these results the extraction buffer selected consisted of either 100mM (later reduced to 75mM) borate buffer, pH 8.8, or 100mM Tris-HCl buffer, pH 8.4(4°C) containing 1mM mercaptoethanol and 20mM D-isoascorbate.

3.1.3 Stability of P.A.L.-activity in Potato Tuber Disc Extracts: There were several ways in which the stability of extracts could be followed, (1) A range of extraction buffers could be used, and the samples assayed after various intervals. (2) A single extraction buffer could be used, the extract desalted, and additional reagents added to samples of the desalted extract and assayed after various intervals. (3) The effect of additional reagents in the assay mixture itself could be compared, using desalted extracts. Using the first method, the stability was greatest in those extracts giving the highest yields. Using the second method (Table 3.3), similar results were obtained; mercaptoethanol, D-isoascorbate and glutathione were more effective than L-cysteine, D,L-dithiothreitol or the control. The P.A.L.-activity recovered after desalting was always less than the amount present in the original extract, recoveries were between 70 and 90%. The % values given in Table 3.3 refer to the amount of P.A.L.-activity as a % of that in the original extract, so values between 70 and 80% are as good as could be expected. Recoveries of P.A.L. in the salt-precipitated samples were usually >90%. the losses therefore occurred during or after desalting.

Table 3.3

Stabilization of P.A.L.-activity.

A salt-concentrated P.A.L. sample was desalted in 75mM borate buffer, pH 8.8. Aliquots of desalted sample were mixed with thiol compounds or anti-oxidants (as below) and kept at 4°C. The samples were assayed after 24 and 48h.

Added reagent	% P.A.L.-activity remaining after- (as a % of the activity in the original extract) 24h.	48h.
Control (none)	49.4	41.6
1mM mercaptoethanol	76.5	59.7
10mM mercaptoethanol	77.3	64.7
1mM glutathione	64.1	68.9
10mM glutathione	80.0	68.9
1mM D,L-dithiothreitol	60.8	44.8
10mM D,L-dithiothreitol	52.5	41.6
5mM L-cysteine	57.6	46.5
20mM D-isoascorbate	56.4	56.1
1mM mercaptoethanol + 10mM D-isoascorbate	69.3	70.9
5mM L-cysteine + 10mM D-isoascorbate	51.3	49.3

Using the third method (Table 3.4), P.A.L.-activities higher than the control were obtained in the presence of P.E.G.(polyethyleneglycol), E.D.T.A. (ethylenediaminetetra-amine), D-isoascorbate, sucrose and possibly Ficoll. (E.D.T.A. was subsequently included in the extraction buffer, although P.E.G. could have been used instead.) Sorbitol and mannitol were almost completely inhibitory at 0.1M, in agreement with the results of Kopp et al.1977. Trans-cinnamic acid was also inhibitory (Lamb and Rubery,1976b). The denaturing reagent S.L.S.(sodium lauryl sulphate), similar in effect to S.D.S.(sodium dodecyl sulphate), was also almost completely inhibitory at 0.05%, the concentration normally used to cause breakdown of enzymes into their subunits. The thiol compounds were slightly inhibitory, L-cysteine showed the greatest degree of inhibition as expected from previous results. Fructose also inhibited enzyme activity, in contrast to the effect of sucrose.

3.1.4 Selection of Extraction Buffer: The results from the proceeding sections were used to select the extraction buffer: 75mM borate buffer, pH 8.8, containing 1mM E.D.T.A., 5mM mercaptoethanol and 10mM D-isoascorbate. P.A.L.-activity in such extracts was stable for 2-3 days at 4°C, desalting was not necessary for enhanced stability.

3.1.5 Storage of Potato P.A.L. Extracts: Extracts used in the assays (enzyme assay and immunological assay) were kept at 4°C for 1 or 2 days without significant losses of P.A.L.-activity. Freezing and thawing of extracts resulted in losses of P.A.L.-activity, although storage under liquid nitrogen (-160°C), instead of in a deep freeze (-20°C), was less damaging. Extracts which had been frozen were not used in the immunological assays, but were suitable for the purification, as they were salt-concentrated prior to storage. More purified samples were more stable, particularly when stored under liquid nitrogen.

3.1.6 Effect of Light on P.A.L.-activities in Potato Tuber Discs: Incubation of discs in white light usually gave an increase in P.A.L. levels compared to a continuous dark incubation. Some batches of potato failed to respond to the presence of white light. The reasons for this are not known, but a

Table 3.4

P.A.L.-activity measured in the presence of additional reagents.

A salt-concentrated P.A.L• sample was desalted in 75mM borate buffer, pH 8.8. P.A.L.-activity was assayed as described in Section 2.5.1, but the following reagents were included in the assay mixture.

Added reagent	% P.A.L.-activity as a % of the control
Control (none)	=100
1% P.E.G. 600	115
2% P.E.G. 600	112
1% Ficoll	103
1mM E.D.T.A.	112
5mM E.D.T.A.	107
5mM mercaptoethanol	95
10mM D-isoascorbate	104
5mM glutathione	82
5mM L-cysteine	76
0.1M sucrose	105
0.1M fructose	30
0.1M sorbitol	2
0.1M mannitol	3
0.5mM trans-cinnamic acid	50
0.05% S.L.S.	2

P.E.G.= polyethyleneglycol
E.D.T.A.=ethylenediaminetetra-amine
S.L.S.= sodium lauryl sulphate

similar failure to respond was reported in tuber discs of certain varieties of Helianthus tuberosum for the blue light stimulated increase in P.A.L.-activity (Nitsch and Nitsch,1966). Possibly the treatment and storage of these potatoes (before being sold on the market) was responsible. Potato discs that did not respond to light were not used for the experiments. Blue light was also effective in increasing P.A.L.-activity over that obtained in darkness, a lower irradiance might have been resposible for the difference when compared to P.A.L.-activity in white light (Table 3.5).Far-red light was without effect at the irradiance level tried.

3.1.7 Effect of an Initial Dark Treatment on P.A.L.-activities in Light-Incubated Potato Tuber Discs: When potato discs were incubated in darkness for 4h., and then transferred to white light, P.A.L.-activities were slightly higher than with continuous white light (Table 3.5). If the dark period was extended to 6h., P.A.L. levels were slightly reduced compared to continuous white light. It was also observed that if a dark period (8-10h.) interupted the white light treatment 8-10h. after cutting, P.A.L. levels were no less than with an uninterupted light treatment. The light sensitive step appears to be operative 4-10h. after cutting.

3.1.8 Effect of Submersion of Potato Tuber Discs During Incubation: Experiments were carried out on the effect of completely submerging the incubating discs during incubation, following the results of Shirsat and Nair,1976.(Submersion was shown to inhibit the increase in P.A.L.-activity, and this was thought to be due to a reduction in the availability of oxygen.) Table 3.6 shows the results that were obtained when discs normally incubated on a layer of water (necessary to maintain the humidity) were completely submerged in water. P.A.L.-activities were 2-fold higher in the normal incubations. More significantly (with respect to these investigations), the light effect was still obtained. This suggests that the wounding effect(ie. slicing and dark incubation), and not the subsequent light effect was affected by submersion of the discs. The lower levels of P.A.L.-activity in the submerged discs may be due to a reduction in the rate of change of

Table 3.5

P.A.L.-activity in extracts of light-incubated potato tuber discs. Effect of an initial dark period.

Potato tuber discs were incubated at 25°C under the following light programs, and P.A.L.-activity in extracts made at the end of the light period measured.

Incubation treatment (h.)		P.A.L.-activity
Dark	White light	nmoles cinnamate formed/min /g. incubated tissue
0	17	11.2
0	19	13.6
0	21	16.0
0	23	19.2
2	17	14.0
4	17	17.6
6	17	16.3
2	17 (far-red light)	8.6
0	19 (blue light)	11.8
19	0	8.8

Table 3.6

P.A.L.-activity in extracts of light-incubated potato tuber discs. Effect of submersion of the discs during incubation.

Potato tuber discs were incubated in petri dishes, 10 discs/dish, with either 4.0ml. (normal) or 20.0ml. (submerged) distilled water, in darkness or white light for 20h. and P.A.L.-activity in the extracts measured.

Incubation treatment	P.A.L.-activity nmoles cinnamate formed/min. /g. incubated tissue
Darkness	13.8
White light	19.6
Darkness + submerged	5.9
White light + submerged	14.0

P.A.L.-activity during the incubation.

3.1.9 Extraction of P.A.L. from Mustard and Gherkin Seedlings: The amount of P.A.L.-activity in these tissues is much lower than in potato tuber discs, and the amount of protein much higher. Comparison of P.A.L.-activity and protein content in extracts of etiolated and light-stimulated seedlings is given in Table 3.7. These tissues are more heterogeneous than the potato tuber (mainly parenchyma tissue), the cotyledons especially contain large amounts of lipid and storage protein. During germination of mustard, reserve materials in the cotyledons are utilized, storage proteins are used during the first few days, and then the fats are used between the third and fifth days after imbibition (Gould and Rees,1965). A similar pattern of biochemical changes probably occurs in gherkin during germination (Mayer and Shain,1974). The cotyledons not only act as storage organs, but become photosynthetically active following exposure to light (Mohr and Schopfer,1977; Murphy and Stumpf,1979). It is perhaps not surprising to find that P.A.L.-activity in seedling extracts is less stable than from the relatively homogeneous potato tuber tissue. Extractions were carried out at pH 8.8 (as for potato P.A.L.) and at pH 6.5 or 7.2 (a more physiological pH) in order to find conditions suitable for extracting P.A.L. efficiently and giving a reasonably stable preparation (suitable for carrying out enzyme and immunological assays). Extracts from these tissues are usually desalted before assaying, so P.A.L.-activities were measured in both the crude supernatant extracts and desalted samples. The tissues of particular interest in these investigations were mustard cotyledons and gherkin hypocotyls, so trial extractions were largely carried out on these tissues.

A. Mustard Seedling Extractions: Using mustard cotyledons, a higher buffer pH was found to give the highest P.A.L.-activities in the extracts of far-red treated seedlings (Table 3.8), although there was no apparent difference between P.A.L.-activities from the dark-grown seedlings.(This may be due to differences in the composition of the extracts from dark- and far-red treated seedlings.) When extracts were desalted at the lower pH (pH 7.2) P.A.L.-

Table 3.7

P.A.L.-activity and protein content of tissue extracts.

Extractions were carried out using 75mM borate buffer,pH 8.8 containing 1mM E.D.T.A., 10mM D-isoascorbate and 5mM mercaptoethanol. P.A.L.-activity and protein content were measured in the crude supernatant extracts.

Tissue	Treatment	P.A.L.-activity nmoles cinnamate formed /min./g.fr.wt. tissue	Protein content mg.protein /g.fr.wt.tissue
Potato tuber discs	24h.darkness	14.1	6.5
	24h.white light	24.0	6.6
Mustard cotyledons	60h.darkness	2.4	57
	36h.darkness + 24h.far-red	20.4	55
Gherkin hypocotyls	3 days darkness	1.2	5.2
	3 days darkness + 2.5h.white light	3.2	5.1

Number of tissue sections equivalent to 1g.fr.wt.(fresh weight)tissue

Potato tuber discs 2.2 discs(freshly sliced)

Mustard cotyledons 90 cotyledon pairs(60h.-old seedlings)

Gherkin hypocotyls 15 hypocotyl sections(3 day-old seedlings)

Table 3.8

P.A.L.-activity in extracts of mustard cotyledons.

P.A.L. was extracted from dark-grown or far-red treated mustard cotyledons, using the buffers as described in the Table.

Extraction buffers:	100mM Na-phosphate buffer, pH 6.5 + 1mM glutathione	75mM borate buffer, pH 8.8 + 4mM mercaptoethanol
Light treatment	P.A.L.-activity pmoles cinnamate formed/min./cotyledon pair	
36h. dark	23	26
60h. dark	29	28
36h.dark + 24h. far-red	240	320
48h.dark + 24h. far-red	130	170

Table 3.9

P.A.L.-activity in extracts of mustard cotyledons.

Effect of desalting on the assayable P.A.L.-activity.

P.A.L. was extracted from dark-grown or far-red treated mustard cotyledons, and then desalted, using the buffers as described in the Table. P.A.L.-activity was assayed after desalting and again after keeping the desalted samples at 4°C for 24h.

Extraction buffer	Desalting buffer	Light treatment	*% P.A.L.-activity 0h.	24h.
100mM Na-phosphate buffer, pH 7.2 + 1mM glutathione.	50mM Na-phosphate buffer, pH 7.2.	60h. dark	23	0
		38h. dark + 22h. far-red	76	32
100mM Na-phosphate buffer, pH 7.2 + 4mM mercaptoethanol.	100mM Na-phosphate buffer, pH 7.2 + 0.5mM glutathione.	60h. dark	52	0
		36h. dark + 24h. far-red	85	62
——"——	50mM borate buffer, pH 8.8 (D-iso ascorbate added to 10mM after desalting)	36h. dark + 24h. far-red	85	80
75mM borate buffer, pH 8.8 + 1mM E.D.T.A. + 10mM D-isoascorbate + 5mM mercaptoethanol		60h. dark	93	90
		36h. dark + 24h. far-red	98	95

*% P.A.L.-activity as a % of the activity in the original extract, after desalting (0h.) and after a further 24h.

activity was rapidly lost, especially from dark-grown seedlings (Table 3.9). P.A.L.-activities were assayed after desalting and again after keeping the desalted samples at 4°C for 1 day, and are given in the table as a % of the original P.A.L.-activity (in the extract before desalting). P.A.L.-activity in extracts of cotyledons from dark-grown seedlings was particularly unstable at pH 7.2, even in the presence of glutathione, (unlike P.A.L. extracted from peas, where glutathione prevents the loss of stability during dialysis, and dark extracts were more stable than light extracts, Attridge and Smith,1973b). Samples desalted at pH 8.8 were stable for at least 24h provided D-isoascorbate (10mM) was added immediately after desalting was completed. Various additional reagents were tested and D-isoascorbate found to be more effective than mercaptoethanol or glutathione, as shown in Table 3.10, where the results of assays carried out in the presence of these reagents are compared. The most unexpected finding was that desalting did not enhance the P.A.L. levels obtained, as it is generally believed that the presence of phenolic compounds inhibits enzyme activity, and desalting is carried out routinely to avoid this. P.A.L. levels in mustard radicles and hypocotyls were not affected to the same extent by the far-red treatment as cotyledons (Table 3.11), and would therefore appear to be regulated in a different manner (as suggested by Dittes et al.1971).The most suitable buffer pH for both the extraction and desalting was pH 8.8, although P.A.L.-activities from stimulated and non-stimulated tissue showed slightly different stabilities. Table 3.12A shows the P.A.L.-activity remaining in crude supernatant extracts after keeping at 4°C for 1 or 3 days. As mentioned earlier, P.A.L.-activity from cotyledons is relatively stable under these conditions (but much less stable after desalting), whereas P.A.L.-activity from radicles and hypocotyls was unstable, particularly in extracts from older (64h) seedlings. This difference may be due to differences in the nature and levels of phenolic compounds or other compounds (these extracts were not desalted.) The difference was less noticeable when extracts were desalted and D-isoascorbate or mercaptoethanol added (Table 3.12B).

Table 3.10

P.A.L.-activity in extracts of mustard cotyledons.

Effect of additional reagents in the assay mixture.

Mustard cotyledons were taken from seedlings which had received a light treatment of 36h. dark + 24h. far-red, and were extracted in the potato extraction buffer (see Section 2.4.2, [A]), then desalted in either Na-phosphate buffer, pH 7.2 or borate buffer, pH 8.8, and assayed for P.A.L.-activity as described in Section 2.5.1, but the following reagents were included in the assay mixture.

pH of desalting buffer:	7.2	8.8
Added reagent	% P.A.L.-activity as a % of the control	
Control (none)	=100	=100
1mM glutathione	104	99.
4mM mercaptoethanol	106	103
1mM E.D.T.A.	108	98
10mM D-isoascorbate	120	112

Table 3.11

P.A.L.-activity in extracts of mustard seedlings.

Extractions were carried out using the potato extraction buffer (see Section 2.4.2,[A]). P.A.L.-activity was measured in the crude supernatant extracts.

Light treatment	Section of seedling.	P.A.L.-activity pmoles cinnamate formed/min. / tissue section.
36h. dark	cotyledon pair	36
	radicle	72
60h. dark	cotyledon pair	32
	hypocotyl	30
	radicle	235
36h. dark + 24h. far-red	cotyledon pair	320
	hypocotyl	60
	radicle	354

Table 3.12

Stability of P.A.L. in mustard seedling extracts.

3.12A Crude supernatant extracts.

Extractions were carried out using the potato extration buffer (see Section 2.4.2, [A]). P.A.L.-activity was assayed after keeping the samples at 4°C for 24h. and 72h.

Light treatment	Section of seedling.	P.A.L.-activity remaining after- (as a % of the activity in the original extract) 24h.	72h.
40h. dark	cotyledon pair	103	87
	radicle	78	52
64h. dark	cotyledon pair	93	90
	radicle + hypocotyl	64	32
40h. dark + 24h. far-red	cotyledon pair	87	64
	radicle + hypocotyl	53	28

3.12B Desalted extracts.

The samples were prepared as above, then desalted in 50mM borate buffer, pH 8.8 and additional reagents added immediately after desalting. P.A.L.-activity was assayed after keeping the samples at 4°C for 24h.

Light treatment	Section of seedling.	% P.A.L.-activity, with added reagent (as a % of the activity in the original extract) Control (none)	D-isoascorbate	mercaptoethanol
40h. dark	cotyledon pair	--	69	54
	radicle	--	87	76
64h. dark	cotyledon pair	--	71	47
	radicle + hypocotyl	--	80	76
40h. dark + 24h. far-red	cotyledon pair	73	86	78
	radicle + hypocotyl	64	69	64

B. Gherkin Seedling Extractions: Using hypocotyl sections, the P.A.L.-activity extracted at pH 7.2 was only half that obtained at pH 8.8 (Table 3.13). When samples were extracted at pH 7.2 and desalted at the same pH, P.A.L.-activities were reduced, but to a greater extent for extracts from dark-grown seedlings (as shown by the numbers in square brackets in Figure 3.2). The effect of the light treatment was apparently higher in the desalted extracts, but this was due to a large proportion of the P.A.L.-activity in the dark-hypocotyl extracts being lost following desalting. When extractions were carried out at pH 8.8 (Figure 3.3), this effect was largely eliminated, with slightly higher P.A.L.-activities following desalting, provided D-isoascorbate was added. D-isoascorbate was more effective than mercapto-ethanol or glutathione (see Table 3.14). P.A.L. levels in gherkin cotyledons were also increased by a white (or blue) light treatment, but unlike the situation occurring in the hypocotyls, P.A.L. levels did not fall after 3h. light treatment, but instead remained at the stimulated level (Figure 3.4), and were still high after 24h. continuous blue light (Table 3.15). Following desalting at pH 8.8 P.A.L.-activities were unchanged (Figure 3.4B). The desalted samples were also particularly stable, as shown in Table 3.16, where the effect of additional reagents on the P.A.L.-activity in extracts of hypocotyls and cotyledons which have been desalted is compared. No additional reagents were required to maintain the P.A.L.-activity of desalted cotyledon extracts.

C. Summary: The lower pHs (6.5-7.2) were tried in these extractions because phenolic oxidation is inhibited at lower pHs (Rhodes,1977), however the higher pH (8.8) was found to be most suitable for all P.A.L. extractions. Mustard cotyledon and gherkin hypocotyl P.A.L.-activities were more stable in the crude supernatant extracts than in the desalted samples, although D-isoascorbate improved the stability (probably by inhibiting oxidation). With gherkin (and far-red treated mustard cotyledon) P.A.L., not only the stability was dependent on the pH of the extracting buffer, but the actual

Table 3.13

P.A.L.-activity in extracts of gherkin hypocotyls.

P.A.L. was extracted from dark- or white light-treated gherkin hypocotyls, using the buffers as described in the Table.Seedlings were 3 days old.

Extraction buffers:	100mM Na-phosphate buffer, pH 7.2 + 1mM glutathione	75mM borate buffer, pH 8.8 + 4mM mercaptoethanol
Light treatment	P.A.L.-activity pmoles cinnamate formed/min./hypocotyl	
0h. dark	49	83
5h. dark	55	90
1.5h. white light	94	165
3h. white light	142	227
5h. white light	89	162

Table 3.14

P.A.L.-activity in extracts of gherkin hypocotyls.

Effect of additional reagents in the assay mixture.

Gherkin hypocotyls were taken from 3 day-old seedlings which had received a 3h. white light treatment, and were extracted in the potato extraction buffer (see Section 2.4.2,[A]), then desalted in either 100mM Na-phosphate buffer, pH 7.2 or 50mM borate buffer, pH 8.8, and assayed for P.A.L.-activity as described in Section 2.5.1, but the following reagents were included in the assay mixture.

pH of desalting buffer:	7.2	8.8
Added reagent	% P.A.L.-activity as a % of the control	
Control (none)	=100	=100
1mM glutathione	109	98
4mM mercaptoethanol	110	104
1mM E.D.T.A.	103	100
10mM D-isoascorbate	118	123

Figure 3.2

P.A.L.-activity in extracts of gherkin hypocotyls.

Gherkin seedlings, 3 days-old, were given a white light treatment (solid line) or left in continuous darkness (dashed line). Hypocotyl sections were taken from the seedlings, between 0 and 6h. after the beginning of the treatment period, and extracted in 100mM Na-phosphate buffer, pH 7.2 containing 0.5mM glutathione. P.A.L.-activity was measured in the crude supernatant extracts (A) and in desalted samples (B) (desalted in the same buffer used for the extractions.

Figure 3.3

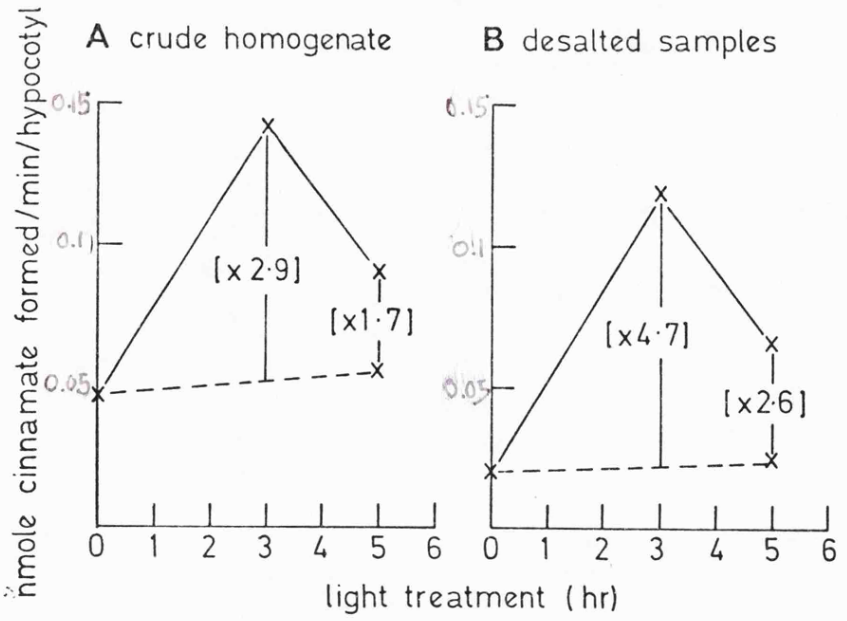
P.A.L.-activity in extracts of gherkin hypocotyls.

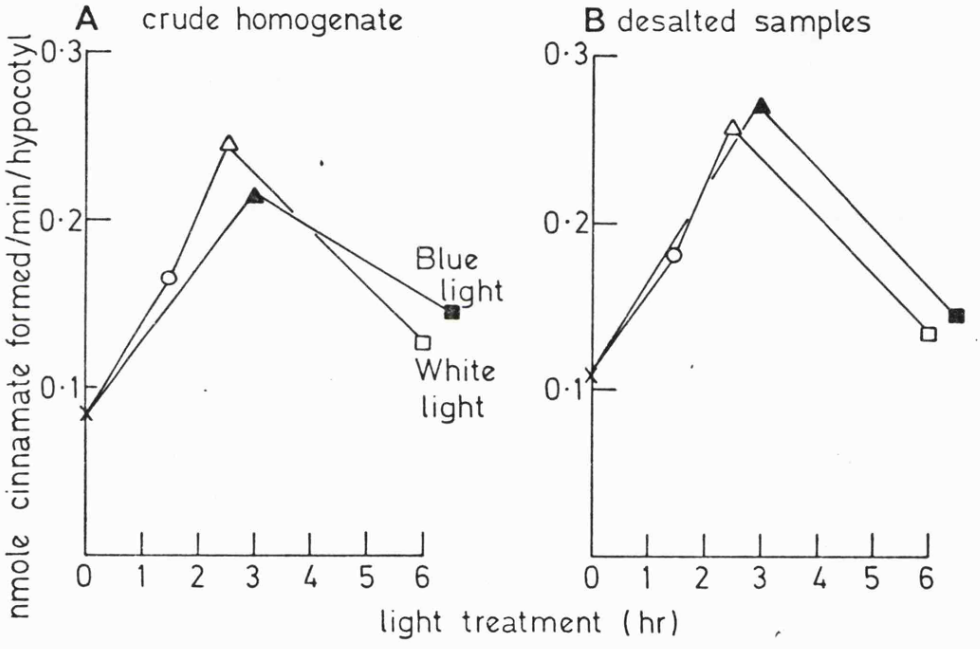
Gherkin seedlings, 3 days-old, were given a white (open symbols) or a blue (closed symbols) light treatment. Hypocotyl sections were taken from the seedlings, between 0 and 7h. after the beginning of the light treatment, and extracted in the potato extraction buffer (Section 2.4.2. [A]). P.A.L.-activity was measured in the crude supernatant extracts (A) and in desalted samples (B),desalted in 50mM borate buffer, pH 8.8, and D-isoascorbate added (to 10mM) immediately after desalting.

Figure 3.4

P.A.L.-activity in extracts of gherkin cotyledons.

Gherkin seedlings, 3 days-old, were given a white (open symbols) or a blue (closed symbols) light treatment. Cotyledon pairs were removed from seedlings, between 0 and7h. after the beginning of the light treatment, and extracted in the potato extraction buffer (Section 2.4.2 [A]). P.A.L.-activity was measured in the crude supernatant extracts (A) and in desalted samples (B), desalted in 50mM borate buffer, pH 8.8.


A crude homogenate
B desalted samples
nmole cinnamate formed/min/hypocotyl
0.15
0.1
0.05
[x 2·9]
[x1·7]
[x4·7]
[x2·6]
0 1 2 3 4 5 6
light treatment (hr)


A crude homogenate
B desalted samples
nmole cinnamate formed/min/hypocotyl
0·3
0·2
0·1
Blue light
White light
0 1 2 3 4 5 6
light treatment (hr)

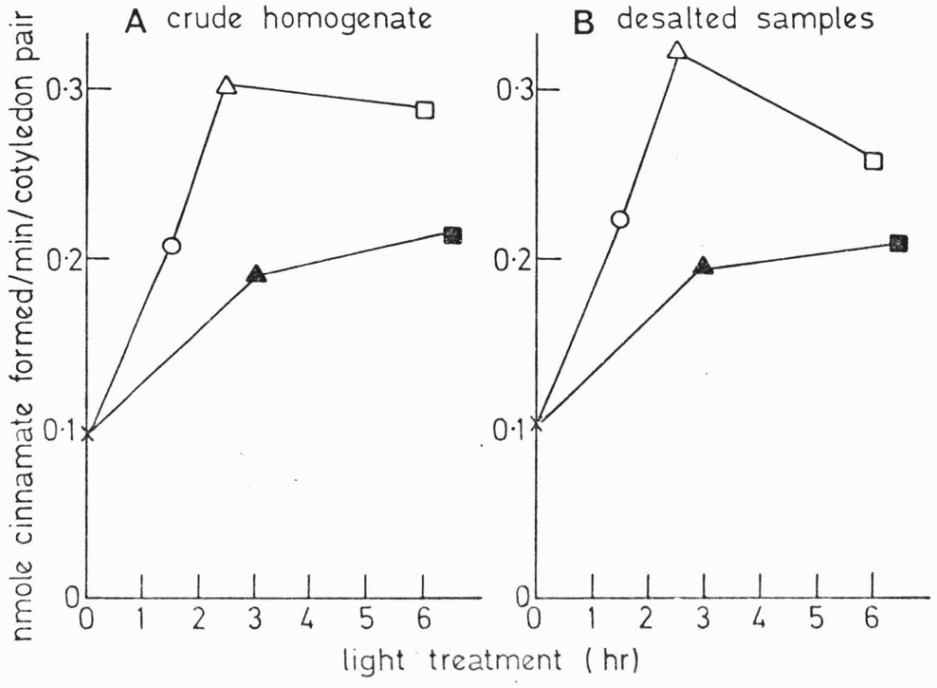

A crude homogenate
B desalted samples
nmole cinnamate formed/min/cotyledon pair
0·3
0·2
0·1
0
0 1 2 3 4 5 6
light treatment (hr)

Table 3.15

P.A.L.-activity in extracts of gherkin seedlings.

P.A.L. was extracted from 3 day-old gherkin seedlings using 100mM Na-phosphate buffer, pH 7.2 containing 1mM glutathione. P.A.L.-activity was measured in the crude supernatant extracts.

Light treatment	P.A.L.-activity pmoles cinnamate formed/min. /hypocotyl	/cotyledon pair
0h. dark	58	98
4h. blue light	115	199
24h. blue light	48	248
24h. dark	52	75

Table 3.16

P.A.L.-activity in extracts of gherkin seedlings.

Effect of adding D-isoascorbate to desalted samples to improve stability.

P.A.L. was extracted from 3 day-old gherkin seedlings which had received a range of light treatments, as described in the Table, using the potato extraction buffer (se Section 2.4.2,[A]), then desalted in 50mM borate buffer, pH 8.8.P.A.L.-activity was assayed after keeping the samples at 4°C for 24h., D-isoascorbate was added (to 10mM) to those samples indicated in the Table, +, immediately after desalting.

% P.A.L.-activity remaining after 24h. (as a % activity immediately after desalting)					
Light treatment	Hypocotyl extract +	-	Light treatment	Cotyledon extract +	-
0h. dark	99	30	0h. dark	90	101
1h. white light	73	19	2.5h. white light	86	75
2h. white light	69	30			
3h. white light	71	35	6h. white light	115	108
4h. white light	97	62	6.5h. white light	121	104
5h. white light	83	45			

levels of P.A.L. extracted were also dependent on the buffer pH. Neither mercaptoethanol or glutathione were effective in maintaining P.A.L.-activities in the desalted extracts compared to the control (no reagents added). Subsequently extractions were carried out in the same buffer as selected for the potato P.A.L. extractions, which included D-isoascorbate, and desalting was only carried out to check the results obtained with crude supernatant extracts.

3.2 PURIFICATION OF P.A.L. FROM POTATO TUBERS

3.2.1 Introduction: The procedure for purification of P.A.L. from potato tubers was initially based on that of Havir and Hanson,1970. The various steps involved were tried, along with several alternatives, and the results of these attempts are presented in the following sub-sections. The final procedure used for the purification is presented at the end of this section.

3.2.2 Extraction: The extraction procedure was selected as a result of trial extractions given in Section 3.1.3. When the extraction was scaled up for the purification, an 'Ato-mix' blender was used instead of a mortar and pestle, to cope with the larger quantities of tissue. P.A.L. yields were reduced by between 20 and 40%, depending upon the particular batch of potatoes used, compared to the trial extractions. This was probably due to the frothing of the crude homogenate, and some heating may have occurred, with the blender (even though the extractions were carried out in the cold room (4°C). The larger volumes of homogenate were also more difficult to filter, 'Miracloth' was not strong enough, so several layers of muslin were used to filter the homogenate before centrifugation. These factors contributed to the reduced yields, and lower specific activity of P.A.L. in the extracts for purification.

3.2.3 Removal of Phenolic Compounds from the Crude Supernatant Extract:

A. Polyvinylpyrrolidone (P.V.P.): P.V.P. was found to have little effect on either the efficiency of the extraction (see Table 3.17) or the stability of the extracts (see Section 3.1.2). The most likely reason for this was that P.V.P. is generally more effective at acidic pHs, whereas the extraction

Table 3.17

Purification of P.A.L. from potato tuber discs.

Effect of P.V.P. and Dowex resins.

P.V.P. or Dowex resin 1x2,chloride form (equilibrated in buffer beforehand) was added to a P.A.L. supernatant extract from 14 potato tuber discs (incubated for 20h. in white light) and stirred for 15min. at the pH indicated in the Table, then filtered through 1 layer of 'Miracloth'. All procedures were carried out in a cold room (4°C). P.A.L.-activity in the treated extracts was then assayed again (after returning the extracts to pH 8.8).

Addition	pH	P.A.L.-activity nmoles cinnamate formed/min. /g.incubated tissue.	Specific activity /mg.protein
Control (none)	8.8	21.0	2.6
1g.P.V.P.	8.0	19.0	3.2
2g.P.V.P.	8.0	17.4	3.1
5g.Dowex resin	6.5	20.4	5.3
——"——	6.75	19.4	5.5
——"——	7.0	17.8	3.9
——"——	7.25	17.4	3.6
——"——	7.5	16.9	3.5

was carried out at pH 8.8, and so P.V.P. was not used.

B. Protamine sulphate: This step was taken from the purification procedure of Havir and Hanson,1970. Although some material was precipitated by treatment with protamine sulphate at pH 5.5, P.A.L.-activity tended to start precipitating out at this pH. The low pH required for this step was not favourable towards the stability of P.A.L. so an alternative treatment was sought.

C. Dowex resins: As an alternative various Dowex resins were tried, following the result obtained when Dowex 1x2 was included in the extraction buffer(see Section 3.1.2). Both basic and acidic forms of the Dowex resin were tried at a range of pHs between 6.5 and 8.0 (Table 3.18). In most cases there was an increase in the specific activity following treatment of the crude supernatant extracts with the Dowex resins, but this was particularly obvious with the strongly basic Dowex resin. The recovery of P.A.L.-activity was greatest at pH 7.0, and was also proportional to the amount of resin used. At lower pHs the recovery of P.A.L. was increased (Table 3.17), and a 2-fold purification was achieved. This step also resulted in the extract losing much of the yellow coloration; this was taken as an indication of the removal of coloured phenolic compounds. Dowex resin (1x2, chloride form) was therefore used as the first step in the purification procedure.

3.2.4 Salt Fractionation: Crude supernatant extracts were initially concentrated by ammonium sulphate precipitation, but recoveries of P.A.L. were poor (especially when large volumes were being dealt with). The amount of ammonium sulphate necessary to precipitate all the P.A.L -activity, also precipitated most of the protein (Table 3.19). If the concentration was carried out in only one step, using ammonium sulphate at a final concentration of 60-70% saturation (for a solution at 0°C), almost 50% P.A.L.-activity was lost. The loses of P.A.L.-activity were also apparent in the P.A.L. purification procedures with mustard cotyledons (Gupta and Acton,1979), yeast, Rhodotorula glutinis (Hodgins,1971) and Sporobolomyces pararoseus (Parkhurst and Hodgins,1971) although not from potato tubers (Havir and Hanson,

Table 3.18

Purification of P.A.L. from potato tuber discs.

Effect of Dowex resins.

Dowex resin (equilibrated in buffer beforehand) was added to a P.A.L. supernatant extract from 14 potato tuber discs (incubated for 20h. in white light) and stirred for 15min. at the pH indicated in the Table, then filtered through 1 layer of 'Miracloth'. All procedures were carried out at 4°C. P.A.L.-activity in the treated extracts was then assayed immediately and again after keeping the samples at 4°C for 24h.

Dowex resin	Volume*[1]	pH*[2]	P.A.L.-activity nmoles cinnamate formed/min. /g.incubated tissue.	/mg.protein.	%P.A.L.activity remaining after 24h.*[3]
Control(none)	-	-	20.7	2.8	89
1x2 (strongly	$\frac{1}{3}$	7.0	19.5	3.9	76
—"— basic)	1	7.0	12.0	5.8	48
—"—	1	7.5	11.4	3.8	56
—"—	1	8.0	2.0	0.9	11
2x8 (moderately	$\frac{1}{2}$	7.0	20.9	3.1	91
—"— basic)	1	7.0	21.1	3.5	92
—"—	1	7.5	19.4	3.0	82
—"—	1	8.0	17.0	2.7	71
50x2 (strongly	1	6.5	12.3	2.9	88
—"— acidic)	1	7.0	18.9	3.4	76
—"—	1	7.5	17.1	3.5	93
—"—	$\frac{1}{3}$	7.5	19.7	2.7	89

*[1] 1 volume = 3g. Dowex resin/14 incubated discs

*[2] pH 6.5-7.0:100mM K-phosphate buffer
pH 7.5-8.0:100mM Tris-HCl buffer

*[3] as a % activity in the treated extract assayed immediately after filtering.

Table 3.19

Purification of P.A.L. from potato tuber discs.

Effect of salt-fractionation with ammonium sulphate, $(NH_4)_2SO_4$.

Ammonium sulphate (in solid form) was added to a P.A.L. supernatant extract from potato tuber discs (incubated for 20h. in white light) in a step-wise manner, and the fractionation carried out as described in Section 2.7.4, at either pH 8.4 or 5.6.

Fractionation range (% saturation with $(NH_4)_2SO_4$ at 0°C)	P.A.L.-activity nmoles cinnamate formed /min./ml.sample	Protein content mg.protein /ml.sample
Initial sample	80	36
At pH 8.4		
1(i) 0-35 (ppt.)	12	9
(ii) 35-55 (ppt.)	56	21
(iii) 55-75 (ppt.)	2	6
2(i) 0-60 (ppt.)	48	30
At pH 5.6		
1(i) 0-35 (ppt.)	26	9
1(ii) 35-55 (ppt.)	44	17
1(iii) 55-75 (ppt.)	1	5
2(i) 0-60 (ppt.)	52	28

(ppt. = precipitate collected by centrifugation after addition of salt.)

1970) or from the fungi, Ustilago hordei (Subba Rao et al. 1967) or from sweet potatoes (Tanaka and Uritani,1977a). No alternative 'salting-out' compounds have been found, with the exception of tri-sodium citrate (Hodgins,1971) and di-potassium phosphate (Parkhurst and Hodgins,1971). The second of these two, when tried with potato supernatant extracts was not particularly successful, but recoveries following tri-sodium citrate fractionation (25-35g./100ml. extract, Hodgins,1971) were reasonable. Table 3.20 shows the results obtained when tri-sodium citrate was used instead of ammonium sulphate; both fractionations were carried out between pH 8.4 and 9.0. The recoveries of P.A.L. were between 90 and 100% with tri-sodium citrate, and a 2-fold purification was also achieved.

3.2.5 Ion-Exchange Chromotography:

A. Calcium Phosphate Gel: This step was taken from Havir and Hanson,1970. The batchwise method was found to result in considerable dilution of the initial P.A.L. extract, with little purification, although the bulk of P.A.L.-activity was recovered in three washes (Table 3.21). Recovery of the P.A.L.-activity using the batchwise method was good, ie.>50%, but when calcium phosphate was used in a column, recoveries were poor,<30%. The major limitation to this step was the problem of concentrating the sample. When these experiments were carried out only ammonium sulphate was available; since then tri-sodium citrate and ultrafiltration have been used, and it seems likely that this would no longer be a problem.

B. Cellulose Phosphate Gel: This step was taken from the purification procedure for P.A.L. from sweet potatoes (Tanaka and Uritani,1977a). The same procedure was followed, and the results are shown in Figure 3.5. A purification of 2-3-fold was obtained with P.A.L. recoveries of 40-50%. It was necessary to load the P.A.L. samples at pH 6.1, so the salt-concentrated sample was lowered to this pH by adding 1M acetic acid. Attempts to prepare the sample by desalting in buffer at this pH, even in the presence of additional reagents, such as 1mM glutathione or 5mM mercaptoethanol, were not successful as some P.A.L.-activity was lost during the desalting, reducing

Table 3.20

Purification of P.A.L. from potato tuber discs.

Comparison of salt-fractionation with ammonium sulphate and tri-sodium citrate.

A P.A.L. supernatant extract from potato tuber discs (incubated for 20h. in white light) was fractionated with either ammonium sulphate or tri-sodium citrate, as described in Section 2.7.4, at pH 8.4.

Fractionation range	% P.A.L.-activity in precipitate*	% protein in precipitate*
Initial sample	=100	=100
g.tri-sodium citrate/100ml.		
1(i) 0-22.4	2	5
1(ii) 22.4-26.3	30	13
1(iii) 26.3-39.2	62	30
2(ii) 22.0-40.0	95	50
% saturation with $(NH_4)_2SO_4$ (at 0°C)		
1(i) 0-15	0	0.5
1(ii) 15-30	2	3
1(iii) 30-45	50	47
1(iv) 45-65	10	5

* as a % activity or protein in the initial sample.

Table 3.21

Purification of P.A.L. from potato tuber discs.

Calcium phosphate gel: batchwise method.

A salt-concentrated P.A.L. extract from potato tuber discs was treated with calcium phosphate gel using the batchwise method, as described in Section 2.7.5. P.A.L.-activity in the washes was measured.

	% P.A.L.-activity*	% protein*
Initial extract	=100	=100
1st batch of gel: 1st wash	11	15
2nd wash	1	10
2nd batch of gel: 1st wash	30	21
2nd wash	18	16
3rd wash	7	10
4th wash	1	9
Remaining supernatant	7	20

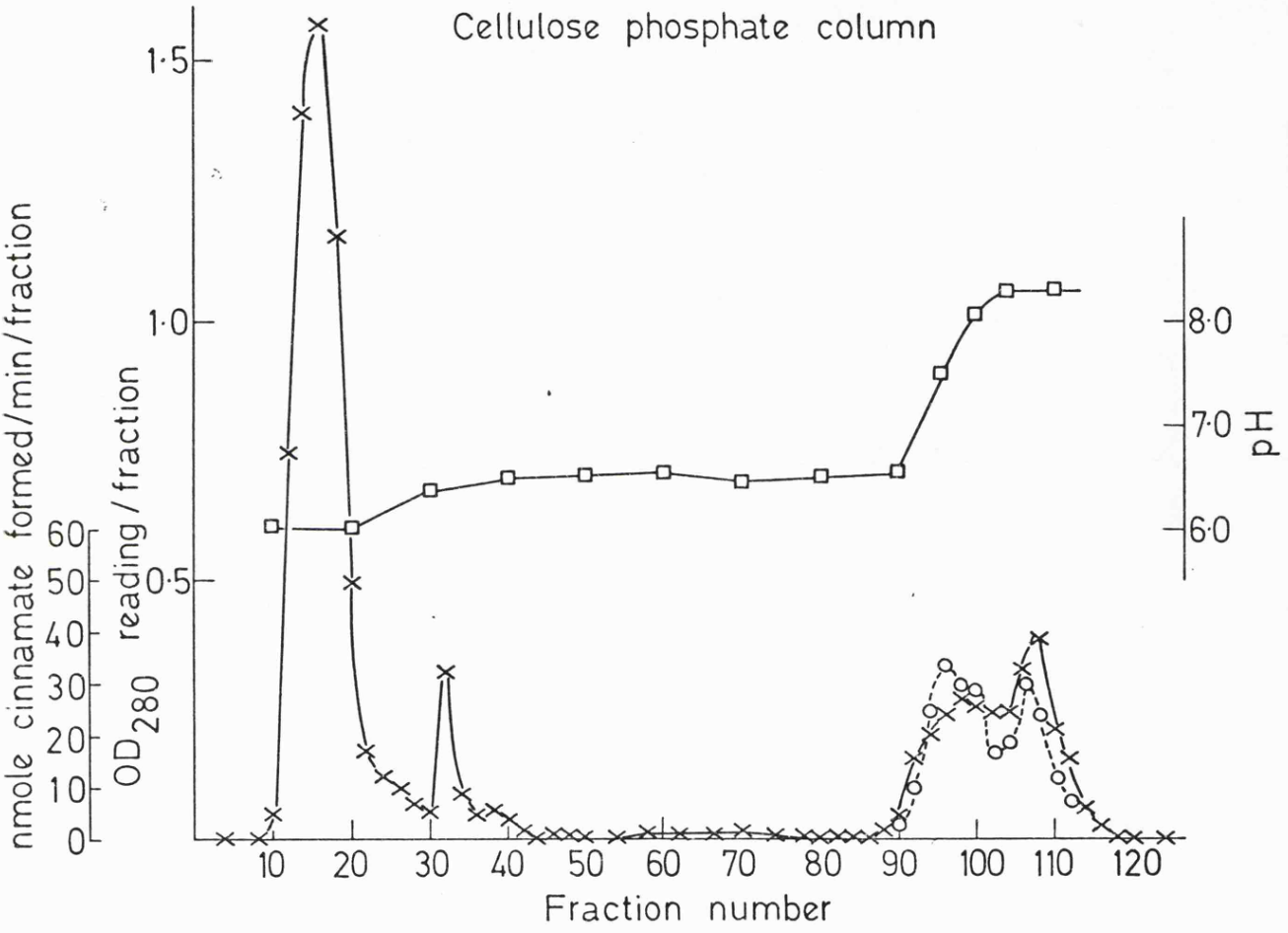
* as a % activity or protein in the initial extract.

Figure 3.5

Purification of P.A.L. from potato tuber discs.

Cellulose phosphate column.

A salt-concentrated potato P.A.L. sample (from 100g. discs incubated in white light for 20h.) was adjusted to pH 6.1 (with 1M acetic acid) and loaded ot to a cellulose phosphate column (2.8cm. diameter x 7.0cm. height), equilibrated in 50mM K-phosphate buffer, pH 6.1. The column was washed through with this buffer until no more protein was eluted, the pH of the running buffer was then increased to pH 8.0. The pH (□) was followed throughout. The protein content was estimated by reading the absorbance at 280nm., (x). P.A.L.-activity (o) of the eluted fractions was also measured.


Cellulose phosphate column
nmole cinnamate formed/min/fraction
OD280 reading / fraction
pH
Fraction number
0
10
20
30
40
50
60
0·5
1·0
1·5
6·0
7·0
8·0
10
20
30
40
50
60
70
80
90
100
110
120

the recovery from this step. A better purification may have been possible if a more shallow pH gradient had been possible,but the use of effective buffers tends to prevent this. P.A.L.-activity was eluted in a double peak corresponding to the elution profile of the protein. This was probably due to the relatively sudden pH change which occurred during the elution, and does not indicate that P.A.L. occurs in more than one form (or isoenzymes).

C. D.E.A.E.-cellulose Gel: D.E.A.E.-cellulose is much more widely used in recent protein purifications and separations, and has been used to separate isoenzymes , including P.A.L. isoenzymes from oak leaves (Boudet et al.1971), oak roots (Ailbert et al.1972), spinach leaves (Nishizawa et al.1979) and Cuscuta chinensis vines (Nagaili et al.1977). The only other tissue from which P.A.L. isoenzymes have been separated is Aesculus hippocastamum (Charrière-Ladreix,1975, using D.E.A.E.-Sephadex A-50 gel). P.A.L. from a salt-concentrated potato tuber discs sample bound to a D.E.A.E.-cellulose column when equilibrated in 50mM Tris-HCl buffer at pH 8.0; at lower pHs P.A.L. passed straight through the column. At a lower ionic strength (20mM buffer) P.A.L.-activity was bound to the column but was not easily eluted. A 2-3-fold purification was achieved (Figure 3.6), but due to the choice of ionic strength of the starting buffer, a considerable pH change occurred during the salt gradient required for elution. Consequently there was no separation of P.A.L. from the other proteins bound to the column. The pH increased to pH 9.5, before the salt gradient was completed, and this was probably the reason for the subsequent elution of P.A.L. and the bulk of the bound protein together.

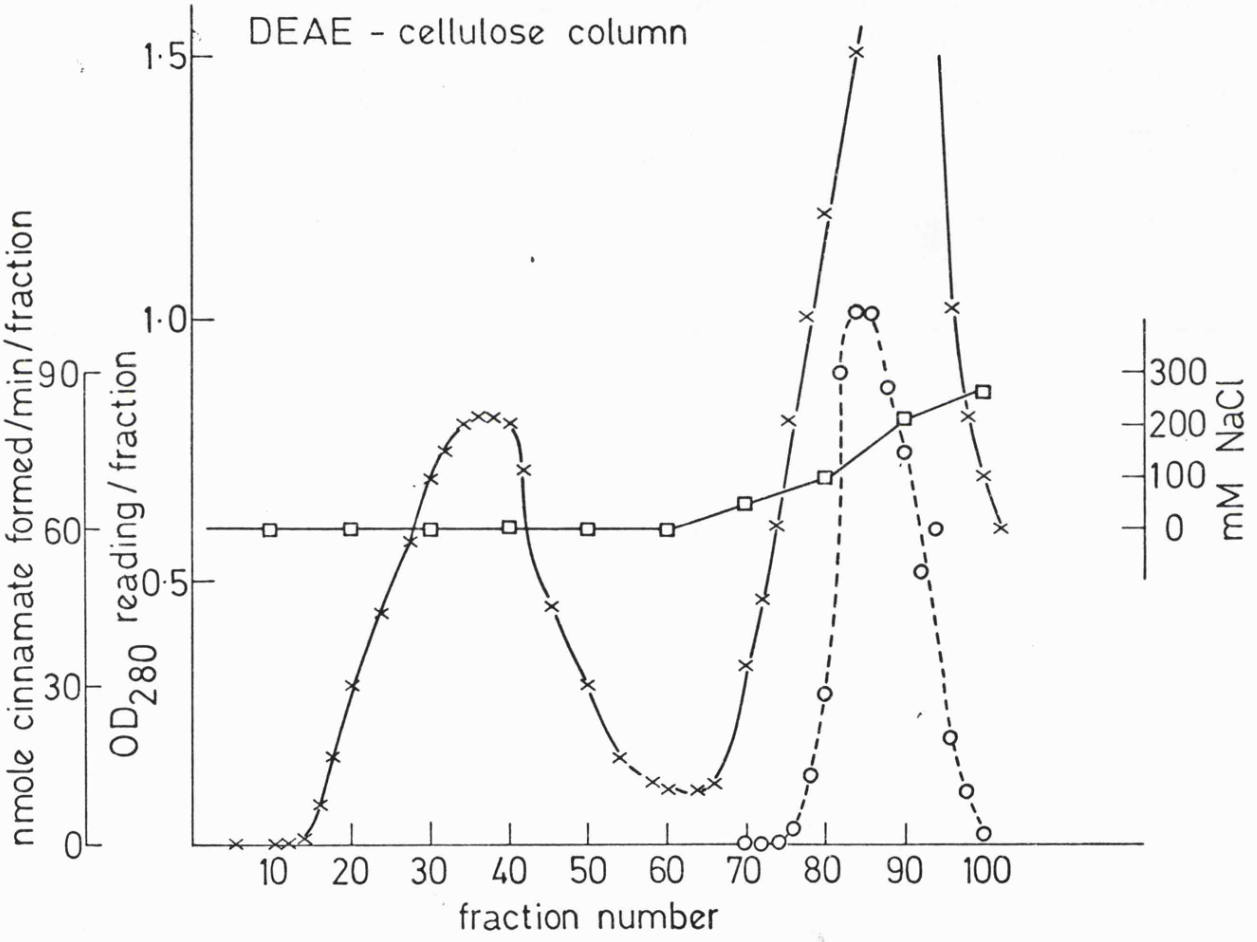
D. D.E.A.E.-Sephadex A-25 Gel: A much more reliable separation was achieved using the same type of charged group, but linked to a Sephadex gel. These gels are also much easier and quicker to prepare than cellulose gels. Two forms of Sephadex gel matrix were available, A-25 and A-50 (corresponding to Sephadex G-25 and G-50). The former is recommended for the separation of small and large MW proteins, while the latter is recommended for proteins of an intermediate MW (30,000-200,000). A salt-concentrated sample (desalted

Figure 3.6

Purification of P.A.L. from potato tuber discs.

D.E.A.E.-Cellulose column.

A salt-concentrated potato P.A.L. sample (from 200g. discs incubated in white light for 20h.)was desalted on a Sephadex G-25 column in 50mM Tris-HCl buffer,pH 8.0 (4°C), then loaded on to a D.E.A.E.-cellulose column (2.8cm. diameter x 7.0cm. height), equilibrated in the same buffer. The column was washed through with this buffer, until no more protein was eluted, a gradient of increasing salt (NaCl) concentration (□) was then set up. The protein content, estimated by reading the absorbance at 280nm.(×) and the P.A.L.-activity (●) of the eluted fractions were measured.


DEAE - cellulose column
1·5
1·0
0·5
90
60
30
0
nmole cinnamate formed/min/fraction
OD280 reading / fraction
300
200
100
0
mM NaCl
10
20
30
40
50
60
70
80
90
100
fraction number

in the starting buffer) was loaded on to a D.E.A.E.-Sephadex A-25 column equilibrated in 50mM Tris-HCl buffer, pH 8.4 (4°C), and eluted in the same way as the D.E.A.E.-cellulose column, as described in the previous section (Figure37A). The P.A.L.-activity was separated from non-binding protein and partly from non-P.A.L. protein which also bound to the column, but P.A.L.-activity eluted in a rather broad band. This may have been due to the pH change which again occurred. Instead of the salt gradiemt, a gradient of increasing Tris concentration (buffering at the same pH as the starting buffer) was used. Only a slight pH change occurred, and the P.A.L.-activity eluted in a narrow peak. A purification of 5-10-fold was possible with this column, 1mM E.D.T.A. and 1mM glutathione in the running buffers increased the recovery of P.A.L. from the column from 30-40% to between 50 and 70%. When D.E.A.E.-Sephadex A-50 was used instead of the A-25 form, under the same conditions, the P.A.L.-activity did not begin to elute until 0.25M Tris, compared to 0.15-0.2M Tris with the A-25 form, and the recovery of P.A.L. was reduced. Possibly this was due to the shrinking of the gel, to which the A-50 form is particularly susceptible over the 0-0.2 ionic strength range (see the 'Sephadex ion exchangers' pamphlet from Pharmacia Fine Chemicals AB).

3.2.6 Sucrose Density Gradient Centrifugation: Following results obtained on the partial purification of P.A.L. with this technique (Dr.C. Johnson, personal communication), it was found that potato P.A.L. could be partially purified using sucrose density gradient centrifugation. Two gradients were compared, the separation achieved was similar for both a 5-15% and a 5-20% sucrose gradient, the results from the former are shown in Figure 3.8. A 4-fold purification of P.A.L. was achieved with a recovery of P.A.L.-activity of 45-50%. The recovery may have been improved if mercaptoethanol or glutathione had been included in the gradient, as a minimum of 24h. was needed for the centrifugation. This procedure was limited by the size of samples (~1.0ml. desalted, salt-concentrated sample/tube),which was in turn limited by the capacity of the ultracentrifuge and rotors that were available. However the results were encouraging for purifications on a small scale, and possibly for the purification of P.A.L. from other tissues (such as mustard

Figure 3.7

Purification of P.A.L. from potato tuber discs.

D.E.A.E.-Sephadex A-25 column.

A salt-concentrated potato P.A.L. sample (from 200g. discs incubated in white light for 20h.) was desalted on a Sephadex G-25 column in 50mM Tris-HCl buffer, pH 8.4 (4°C), then loaded on to a D.E.A.E.-Sephadex A-25 column (4.5cm. diameter x 13.0cm. height), equilibrated in the same buffer, but also containing 1mM E.D.T.A. and 1mM glutathione . The column was washed through with this buffer until no further protein was eluted, a gradient was then set up to elute the column.

A. A gradient of increasing salt (NaCl) concentration (□).

B. A gradient of increasing Tris concentration (□).

The pH (■) was followed throughout, also the protein content(×) and the P.A.L.-activity (o).

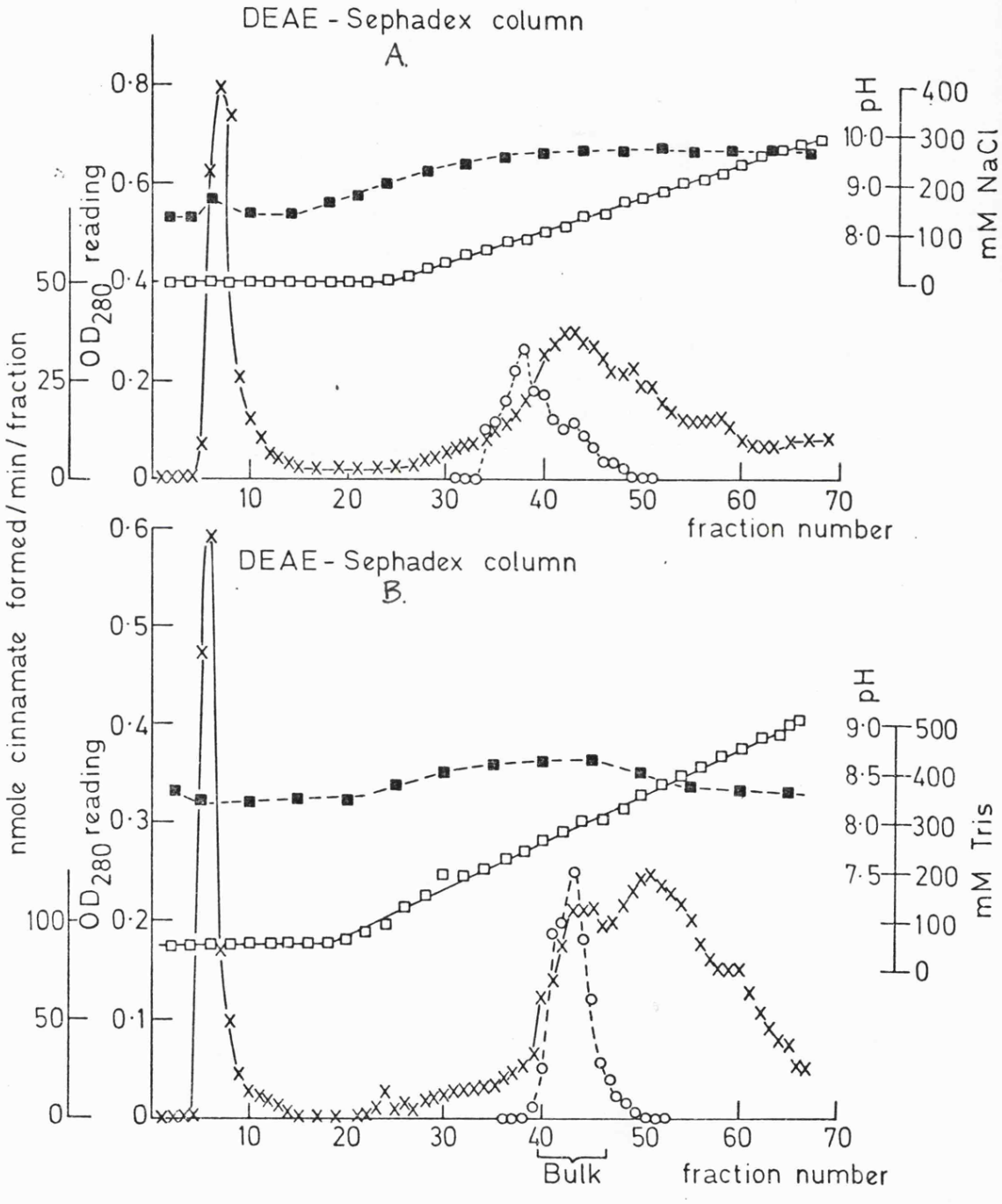
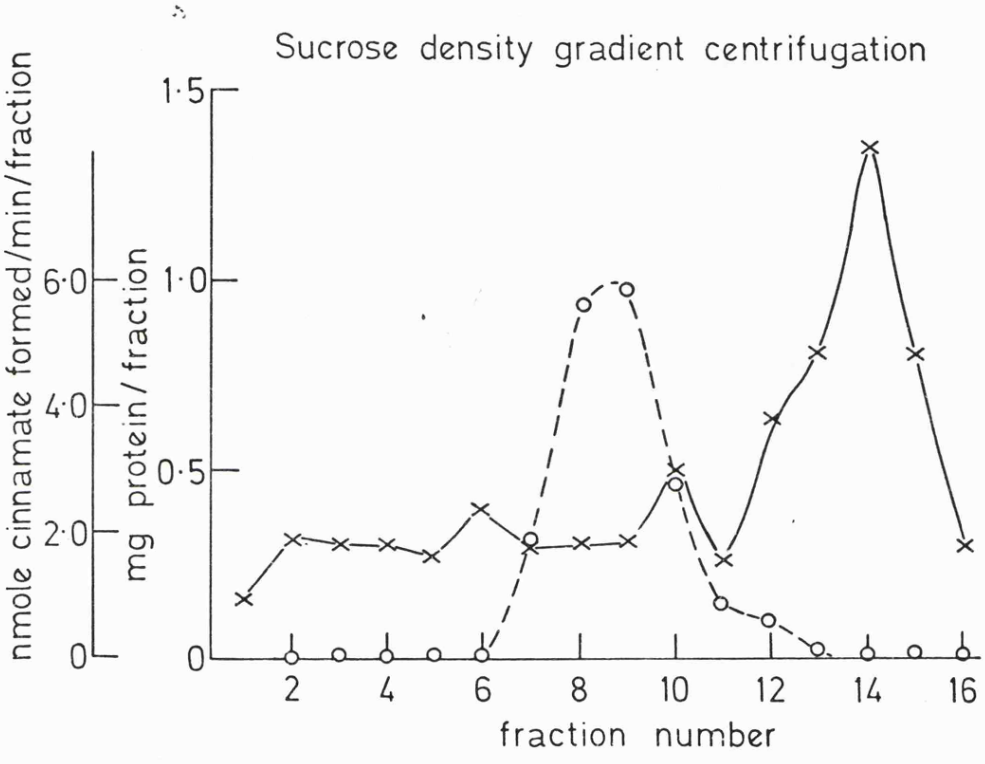

DEAE - Sephadex column
A.
0·8
0·6
0·4
0·2
0
OD280 reading
50
25
0
pH
10·0
9·0
8·0
400
300
200
100
0
mM NaCl
10
20
30
40
50
60
70
fraction number
DEAE - Sephadex column
B.
0·6
0·5
0·4
0·3
0·2
0·1
0
OD280 reading
100
50
0
nmole cinnamate formed / min / fraction
pH
9·0
8·5
8·0
7·5
500
400
300
200
100
0
mM Tris
10
20
30
40
50
60
70
Bulk
fraction number

Figure 3.8

Purification of P.A.L. from potato tuber discs.

Sucrose density gradient centrifugation.

A salt-concentrated potato P.A.L. sample (from discs incubated for 20h. in white light) was desalted on a Sephadex G-25 column in 50mM borate buffer, pH 8.8, and aliquots placed on top of a 5-15% sucrose gradient prepared in the same buffer in 17cm capacity tubes. The tubes were centrifuged at 132,000 x g. for 25h., then fractions collected by pumping the gradient out of the tubes with a syringe placed at the bottom of the tube; the denser fractions were therefore collected first. The protein content (*) and the P.A.L.- activity (o) of the fractions were measured.


Sucrose density gradient centrifugation
nmole cinnamate formed/min/fraction
mg protein/ fraction
6·0
4·0
2·0
0
1·5
1·0
0·5
0
2
4
6
8
10
12
14
16
fraction number

or gherkin), although these have not been tried yet.

3.2.7. Ultrafiltration: The ultrafiltration cell was found to be extremely useful, overcoming the problem of losses of P.A.L.-activity following column chromotography, encountered with the use of ammonium sulphate for concentrating the sample. After partially purifying the P.A.L. sample by ion-exchange chromotography, a further purification was achieved by ultrafiltration with the appropriate ultrafiltration membrane, which allowed molecules with a MW of less than 100,000 to pass through. Recoveries of P.A.L.-activity were usually between 90 and 100% when a fresh membrane was used.

3.2.8 Gel Filtration: P.A.L.-activity was eluted with the void volume , when the sample was passed through a Sephadex G-200 column, so a gel with a higher MW fractionation range was required. Sepharose 6B allows separation of proteins in the MW range 50,000-1,000,000. A large column ($\sim$700cm^3) was prepared and samples of 10ml. volume used. This was quite adequate for the potato P.A.L. purification, and was also successfully used with samples of P.A.L. from other tissues (see Section 3.3.2). P.A.L. recoveries were about 50% activity loaded on to the column, and a purification of 6-8-fold was achieved with partially purified potato P.A.L. samples (ie. following D.E.A.E.-Sephadex A-25 chromotography).

3.2.9 Polyacrylamide Gel Electrophoresis: Preparative scale, polyacrylamide gel electrophoresis (P.A.G.E.) is mow frequently used to purify enzyme samples, and obtain homogenous preparations. Previously P.A.G.E. was only carried out on a small scale, and provided a means of checking the purity of a sample; the final purification step and the check are now carried out together for small scale purifications. Homogenous samples of P.A.L. have been obtained using this technique, from parsley cell suspension cultures (Zimmermann and Hahlbrock,1974), sweet potatoes (Tanaka and Uritani,1977a) and mustard (Gupta and Acton,1979). Unfortunately no large scale apparatus, allowing a continuous flow technique to be used , was available, so several rod gels were run in parallel. One rod gel was then stained immediately for protein to check the purity of the sample; one was sliced and the sections

assayed immediately to locate the P.A.L.-activity, the remaining gels were sectioned once the position of the protein band corresponding to the P.A.L.-activity had been identified. Attempts to elute the P.A.L. from the gels by incubation of the relevant sections in buffer were not particularly successful, only low recoveries of the enzyme were obtained, possibly due to excessive dilution of the sample. The large MW of the enzyme may have prevented diffusion from the gel, in contrast to the ability of the substrate and product to diffuse in and out of the gel (as shown by the success of the P.A.L. assay). As an alternative, electrophoretic elution of P.A.L. from the gels was attempted. Gel sections containing the P.A.L.-activity were returned to the perspex rods (with great difficulty) above a 0.5cm. layer of larger pore size gel, 5% polyacrylamide gel set within the rod, 2cm. from the bottom of the rod. The bottom was sealed with a piece of ultrafiltration membrane, and electrophoresis carried out as before. The buffer from the bottom 2cm. was then collected, but again recoveries of P.A.L.-activity were poor, probably due to technical difficulties. Fully purified potato P.A.L. was therefore not available, except for the immunization in the form of the polyacrylamide gel sections containing P.A.L.-activity. The relevant sections of gel were homogenized with Freund's complete adjuvant and the mixture injected into the rabbits. P.A.G.E. was also used to analyze the samples obtained at each step during the purification procedure (see Section 3.2.11).

3.2.10 Other Purification Techniques:

A. Sephacryl Gel Chromotography: A new type of gel is currently available from Pharmacia Fine Chemicals AB, Uppsala, Sweden, which combines gel filtration with the high resolution of polyacrylamide gels: Sephacryl S-300 is suitable for the separation of proteins in the MW range 10,000-1,500,000, and is availavle in a preswollen form similar to the Sepharose gels. The gel consists of covalently cross-linked allyl dextran (using N,N'methylbis-acrylamide) which allows faster flow rates and therefore shorter separation times which should improve recoveries from gel exclusion columns and give a better resolution.

B. Affinity Chromotography: P.A.L. has beem purified by affinity chromotography

using a Sepharose-phenylalanine conjugate, from radish cotyledons (Blondel et al.1973; Iredale,1972), gherkin hypocotyls (Billett et al. 1978) and mustard cotyledons (Gupta and Acton,1979). The technique of affinity chromotography (Wilchet and Hexter,1976) can give considerable purification in a single step (eg.50-100-fold), but attempts to purify P.A.L. (as listed above) have been only partially successful, and it appears that in many of these cases,that non-specific binding occurs (Faye,1977; Huault et al.1976). It was not considered worthwhile pursuing further attempts using this technique, particularly with the types of conjugate already tried when conventional techniques were likely to be fruitful long before a successful affinity column was found.

C. Immunoaffinity Chromotography: Closely related to the type of column in the previous section, is the technique of immunoaffinity chromotography. Antibodies against a specific protein are bound to a Sepharose matrix and a crude plant extract passed through the column; only those proteins binding to the stationary antibodies are retained by the column.Elution of the desired protein is effected by increasing or lowering the pH, (depending on the effect of high or low pHs on the protein). Proteins particularly susceptible to degradation at extreme pHs may be difficult to elute. Partial purification of crude plant extracts may be necessary before this step, depending on the tissue used. Such a technique also requires large quantities of antiserum; a sheep could be used to raise a suitable volume of antiserum, rather than rabbits, as used for antibody production in these investigations (Section 3.4) The success in obtaining antibodies against P.A.L. suggests that this technique is feasible, although it requires purification of the enzyme for production of antibodies first.

3.2.11 Summary of Potato P.A.L. Purification:

A. Purification Procedure:

Step 1. Incubation of Potato Tuber Discs: Potato tuber discs were washed and sliced as described in Section 2.1.2, and placed in plastic trays, (60 discs/tray: 13.0 x 21.0cm . 1.0cm. depth) containing 20ml. distilled water,

covered with 'Cling-film' and incubated for 20-24h. at 24°C under white light. For each purification run 400-500 discs (180-220g. fresh weight of tissue)were required.

Step 2. Extraction: The discs were harvested from the trays, blotted dry and ground in an 'Ato-mix' blender at 4°C with an equal volume (1ml./1g.tissue) of the potato extraction buffer: 75mM borate buffer, pH 8.8 containing 1mM E.D.T.A., 5mM mercaptoethanol and 10mM D-isoascorbate.; three 15s. runs at 30s. intervals. The homogenate was filtered through 4 layers of muslin and then centrifuged in an M.S.E. 21 high speed centrifuge in a 6x300ml. rotor at 14,000r.p.m. (18,000 x g.) for 20 min. at 4°C. The supernatant was decanted.

Step 3. Dowex Treatment: Dowex 1x2-400 mesh, chloride form, was equilibrated in 150mM K-phosphate buffer, pH 6.5 containing 1mM E.D.T.A. and 5mM mercaptoethanol. The potato supernatant sample was added slowly to the prepared resin, with stirring (70g. moist resin in 70ml.buffer/ 100g. discs). The pH was followed throughout and held between 6.5 and 6.7 (with 1M HCl). After 15min. the resin mixture was filtered through 1 layer of 'Miracloth' inside 2 layers of muslin. The filtrate was returned to pH 8.8 (with 1M NaOH).

Step 4. Salt Fractionation: Tri-sodium citrate was added to the dowex-treated sample in two steps, firstly up to 22g./100ml. and then up to 40g./ 100ml. with centrifugation at 10,000 x g. for 15 min. to collect the precipitate after each addition (pH was held at 8.8-9.0 by adding 1M HCl). The second precipitate was redissolved in the extraction buffer (if the sample was to be stored) or in 50mM Tris-HCl buffer, pH 8.0 (4°C) containing 1mM E.D.T.A. and 1mM glutathione (for the next purification step). This sample could be kept at 4°C overnight before proceeding.

Step 5. Desalting and D.E.A.E.-Sephadex A-25 Column: Samples of up to 40ml. were desalted on a Sephadex G-25 column (2.5cm. diameter x 45.0cm. height) in 50mM Tris-HCl buffer, pH 8.0. Those fractions containing protein were loaded on to the D.E.A.E.-Sephadex A-25 column (4.5cm. diameter x 13.0cm. height) equilibrated in the same buffer, but also containing 1mM E.D.T.A.

and 0.5cm. glutathione. This column had an operating capacity of up to 40ml. desalted sample (equivalent to an extraction of 120g. incubated discs). For larger volumes two identical columns were run in parallel. Fractions were collected from the column at a flow rate of 4-5ml./min., fractions were 20ml.. The column was washed through with at least 150ml. buffer, to elute non-binding protein, before setting up the gradient, consisting of two equal volumes(700ml. each,for a single column) of 50mM and 500mM Tris buffer, both containing 1mM E.D.T.A. and 0.5mM glutathione , and adjusted to pH 8.0 (4°C). Fractions (20ml.) were then collected at a slightly faster flow rate, 6-8ml. /min.. Aliquots (0.4ml.) of each fraction were assayed for P.A.L.-activity and the protein content estimated by reading the absorbance at 280nm. Fractions containing P.A.L.-activity were pooled and concentrated by ultrafiltration.

Step 6. Ultrafiltration: The fractions above were concentrated to a final volume of 10ml., and equilibrated in 50mM borate buffer, pH 8.8. (Samples for storage were placed in the liquid nitrogen store, labelled D/U.)

Step 7. Sepharose 6B Column: Partially purified enzyme samples (10ml.) were passed through the Sepharose 6B column (3.2cm. diameter x 90.0cm. height) in 50mM borate buffer, pH 8.8, at a flow rate of 1.0-1.5ml./min., 10ml. fractions were collected. Fractions were assayed for P.A.L.-activity and protein content estimated, as above (Step 5).

Step 8. Ultrafiltration: Fractions from the previous step were pooled if they contained P.A.L.-activity and concentrated to a 10ml. volume (such samples were labelled S/U I). This sample was further concentrated in a second ultrafiltration cell, with a smaller capacity(10ml.) to a final volume of 2.0-3.0ml. Samples were placed in Eppendorf tubes, frozen and kept in the liquid nitrogen store (labelled S/U II).

Step 9. Polyacrylamide Gel Electrophoresis: P.A.L. samples, S/U II, were thawed and 85-100µl. aliquots placed on each 7% polyacrylamide rod gel (see Section 2 7.11), and electrophoresis carried out as described previously. The sections of gel containing P.A.L.-activity after electrophoresis, were frozen and kept in the deep freeze (for up to 3 weeks only) before being

used for the immunization.

B. Summary of Purification: The purification of P.A.L. achieved is summarised in Table 3.22. In terms of the increase in specific activity, a 100-fold purification was obtained, although less than 0.1% total protein(in the initial homogenate) remained in the purified sample (ie. gel sections following the final purification step, P.A.G.E.). The final amount of protein in the purified sample was estimated from the intensity of Coomassie blue stain in the P.A.L.-band, compared to that of the whole rod gel; on this basis 20% protein loaded on to each rod gel was P.A.L. The progress during the purificatiom was followed by subjecting samples from various steps to polyacrylamide gel electrophoresis, using the same conditions as used for the purification step. The results are shown in Figure 3.9. The sample volumes loaded, as shown in the accompanying table, were selected to show up as many protein bands as possible without overloading the rod gels. The protein band corresponding to P.A.L.-activity was clearly distinguishable in the rod gels for step 6 (D/U) and step 9 (S/U II). The major problem encountered in the purification involved the loses of P.A.L.-activity, that inevitably occur at each step. The more steps required, the greater the loses of P.A.L.-activity. A purification with fewer steps would therefore be highly desirable (hence the previous interest in affinity chromotography, see Section 3.2.10 B.). The most likely way of carrying this out would be using immunoaffinity chromotography (see Section 3.2 10 C.).

3.3 PROPERTIES OF P.A.L.

3.3.1 Introduction: Having purified P.A.L. from potatoes the next step in these investigations was to raise antibodies against the purified potato P.A.L., and use the antiserum to measure P.A.L. levels, not only in potatoes, but in mustard and gherkin seedlings and in tobacco cell suspension cultures. In order to be able to measure P.A.L. levels in tissues other than potato, using the antiserum to potato P.A.L., the antiserum must cross react with P.A.L. from other tissues, ie. there should be at least

Table 3.22

Summary of purification of P.A.L. from potato tuber discs.

The Table is based on an extraction of 200g.fresh weight of tissue; discs were incubated for 20h. in white light. The extraction and purification procedure was as described in Section 3.2.11. The homogenate (step 1) gave a P.A.L.-activity of 18.9 nmoles cinnamate formed/min/g. fr.wt. The purification factor achieved at the major steps is given in the second table below, in terms of protein content and the overall purification.

Purification step	P.A.L.-activity. nmoles cinnamate formed /min.	Yield of P.A.L.-activity.	Protein content. mg.protein.	Volume (ml.)
1. Homogenate	3770	=100	1820	410
2. Supernatant	3855	102	1670	395
3. Dowex-treated	3225	86	1030	560
4. Sodium citrate	2540	62	522	35
5. D.E.A.E.-Sephadex	1320	35	120	140
6. Ultrafiltration(D/U)	1250	33	59	13
7. Sepharose 6B	600	16	8	70
8. Ultrafiltration(S/U I)	506	13	4.5	10
9.Ultrafiltration(S/U II)	372	10	4.5	3
10. P.A.G.E.	187	5	0.9	gel section

Purification step	Specific activity. nmoles cinnamate formed /min./ mg. protein	Purification factor
1. Homogenate	2.1	= 1
3. Dowex-treated	3.1	x 1.5
4. Sodium citrate	4.9	x 2.3
6. D/U	21.2	x 10.1
8. S/U I	112	x 53
9. S/U II	83	x 40
10. P.A.G.E.	208	x 99

Figure 3.9

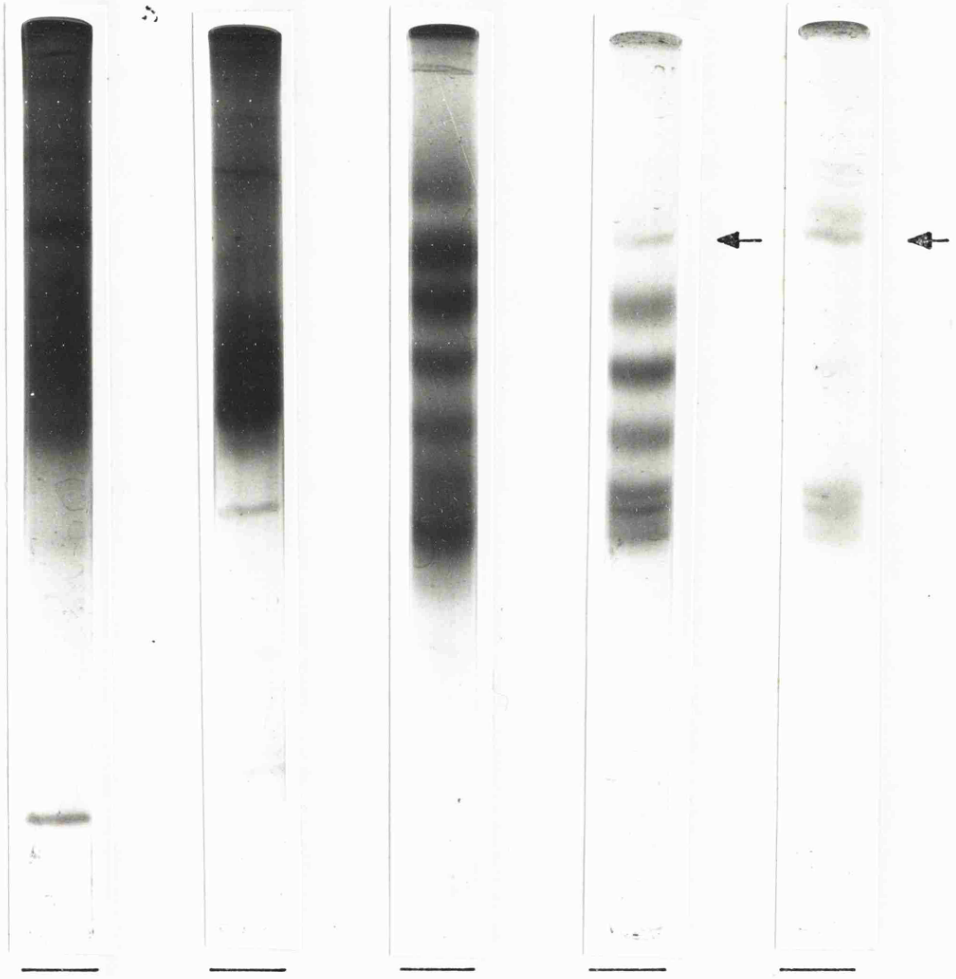
Purification of P.A.L. from potato tuber discs.

Polyacrylamide gel electrophoresis (P.A.G.E.).

Samples of potato tuber extracts and partially purified potato P.A.L. were subject to P.A.G.E. (as described in Section 2.7.11) The Table below shows the sample volumes, protein content and P.A.L.-activity loaded on to each rod gel. Arrows indicate position of P.A.L.-protein.

Sample from-	Sample volume (µl.)	Protein content /Mg. protein	P.A.L.-activity nmoles cinnamate formed/min.
Step 2 (Supernatant)	200	850	1.9
Step 3 (Dowex-treated)	200	370	1.1
Step 4 (Sodium citrate)	50	745	3.6
Step 6 (D/U)	25	110	2.4
Step 9 (S/U II)	25	35	2.9

Polyacrylamide gel electrophoresis (P.A.G.E.)



Step 2 Step 3 Step 4 Step 6 Step 9

Purification steps

one, if not more, common immunological determinant on the P.A.L. protein molecule, regardless of the source of the enzyme. As it is not possible to predict which regions of a macromolecule are involved in these determinants, the only tests which can be used to check for cross-reactivity are immunological tests, but as a preliminary study several of the molecular properties of P.A.L. from different sources were compared. These included molecular weights (MWs), pH optima and Km values for the P.A.L.-activity. One would expect that if a large number of differences were found in the molecular properties of P.A.L. from various tissues, then the degree of cross-reactivity of the antiserum, raised against P.A.L. from potatoes, would be low. Conversely if the differences were few, a high degree of cross-reactivity would be expected.

3.3.2. Molecular Weights: Using the Sepharose 6B column (see Section 2.7.9) it was possible to compare the molecular weights of P.A.L. from the several plant sources of interest in these investigations. Molecular weights were determined from the elution profiles of extracts containing P.A.L.-activity, using the MW calibration curve (Section 2.7.9), and are given in Table 3.23. Also given in the table are MW values obtained elsewhere, as given in the references. There is close agreement between the values for potato and Rhodotorula P.A.L.. No high MW forms of P.A.L. from potato were detected (Havir and Hanson, 1968a). Figure 3.10 shows the elution profile for potato P.A.L. from a relatively crude supernatant extract (Fig. 10A) and from a partially purified sample (Fig.10B); there is no difference in the MW values observed. P.A.L. from mustard cotyledon extracts (Figure 3.11) has a MW similar to that of Rhodotorula P.A.L., this value is slightly higher than recently reported by Gupta and Acton (1979). Again no high MW forms were detectable (Schopfer, 1971). The value for gherkin P.A.L. was slightly less than previously reported. This was checked by running a mixture of potato and gherkin P.A.L. (Figure 3.12B)as the results suggested that the MWs were significantly different from each other. Gherkin P.A.L. alone, tended to elute in a broad peak (Figure 3.12A), and was not separated

Table 3.23

Molecular weights of P.A.L. from several plant sources.

Crude supernatant extract samples (10ml.) from potato tuber discs (incubated for 20h. in white light); gherkin hypocotyls (from seedlings 3 days-old which had received a 3h. white light treatment); mustard cotyledons (from seedlings 36h-old which had received a further 24h. in far-red light); and tobacco cell suspension cultures (taken from cultures on stock medium, 24h. after transfer to fresh medium) and a commercial preparation of Rhodotorula P.A.L. (Sigma Chemical Ltd.) were passed through the calibrated Sepharose 6B column in 50mM borate buffer, pH 8.8.(see Section 2.7.9). The MWs, given in column 1, were determined from the position of the peak of P.A.L.-activity eluted from the column, (samples run separately), with an error of ±10,000. Also shown are the MW values obtained for P.A.L. from the same plant sources, see references.

Plant source	MW obtained from the Sepharose 6B column	MW obtained elsewhere--	References
Potato	320,000	330,000	Havir and Hanson,1970
Rhodotorula	280,000	275,000	Hodgins,1971
Gherkin	270,000	316,000	Iredale and Smith,1973
Mustard	275,000	240,000	Gupta and Acton,1979
Tobacco	310,000	-	-

Figure 3.10

Elution profile of potato P.A.L. on the Sepharose 6B column.

A crude supernatant extract from potato tuber discs incubated for 20h. in white light (A.) and a partially purified sample of potato P.A.L.,D/U (B.) were passed through the calibrated Sepharose 6B column in 50mM borate buffer, pH 8.8 (see Section 2.7.9). The protein content,estimated from the absorbance at 280nm. (×) and the P.A.L.- activity (○) of the eluted fractions were measured.

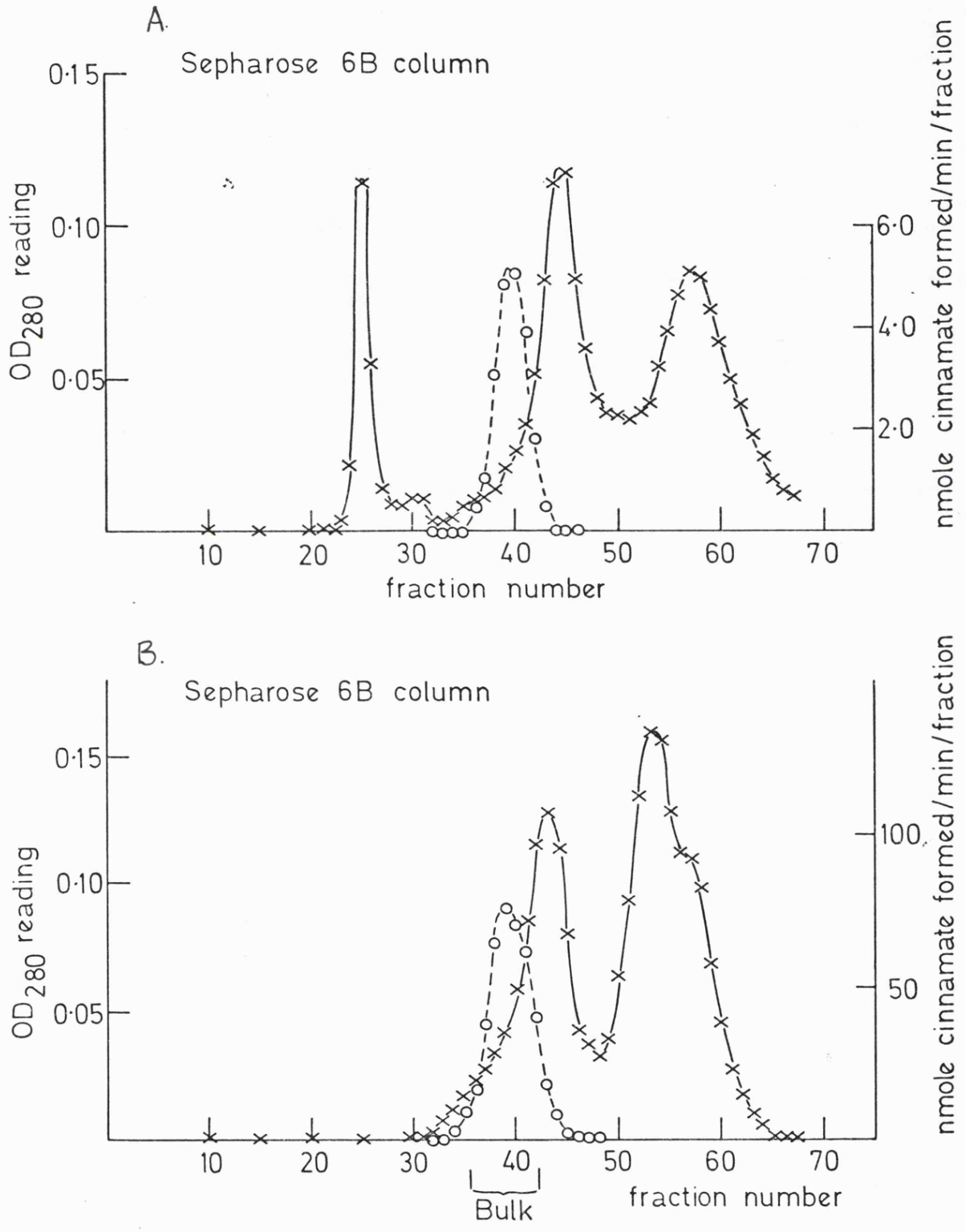

A.
Sepharose 6B column
0·15
0·10
0·05
OD_{280} reading
6·0
4·0
2·0
nmole cinnamate formed/min/fraction
10
20
30
40
50
60
70
fraction number
B.
Sepharose 6B column
0·15
0·10
0·05
OD_{280} reading
100
50
nmole cinnamate formed/min/fraction
10
20
30
40
50
60
70
Bulk
fraction number

Figure 3.11

Elution profile of mustard P.A.L. on the Sepharose 6B column.

A crude supernatant extract from mustard cotyledons (taken from seedlings given a light treatment of 36h. darkness and 24h. far-red light) was passed through the calibrated Sepharose 6B column in 50mM borate buffer, pH 8.8 (see Section 2.7.9). The protein content, estimated from the absorbance at 280nm. (x) and the P.A.L.-activity (o) of the eluted fractions were measured.

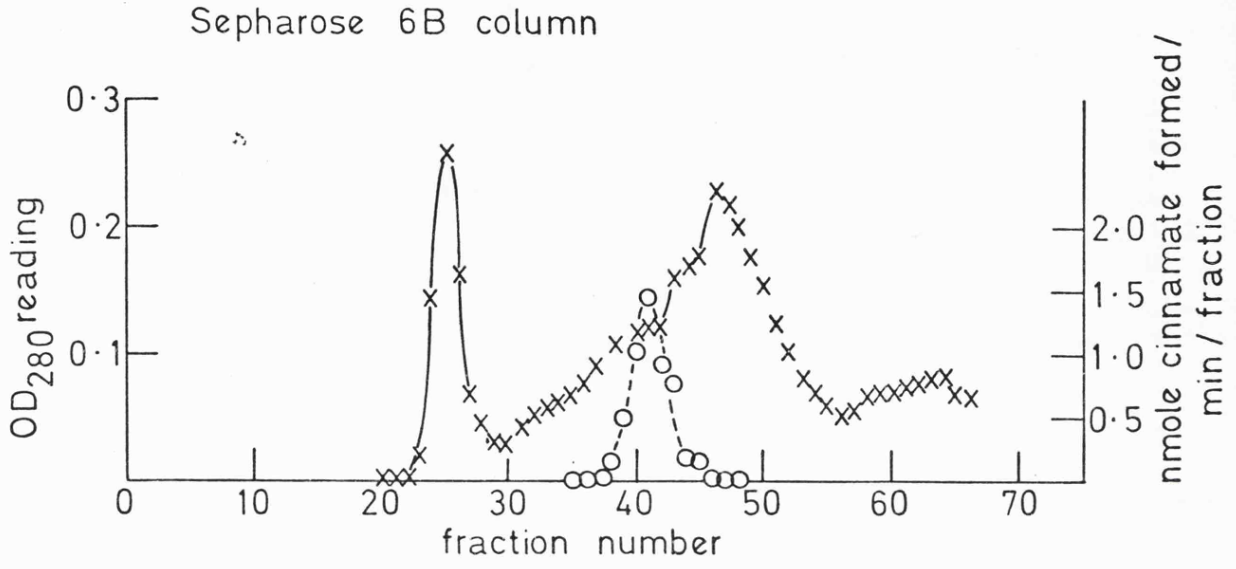
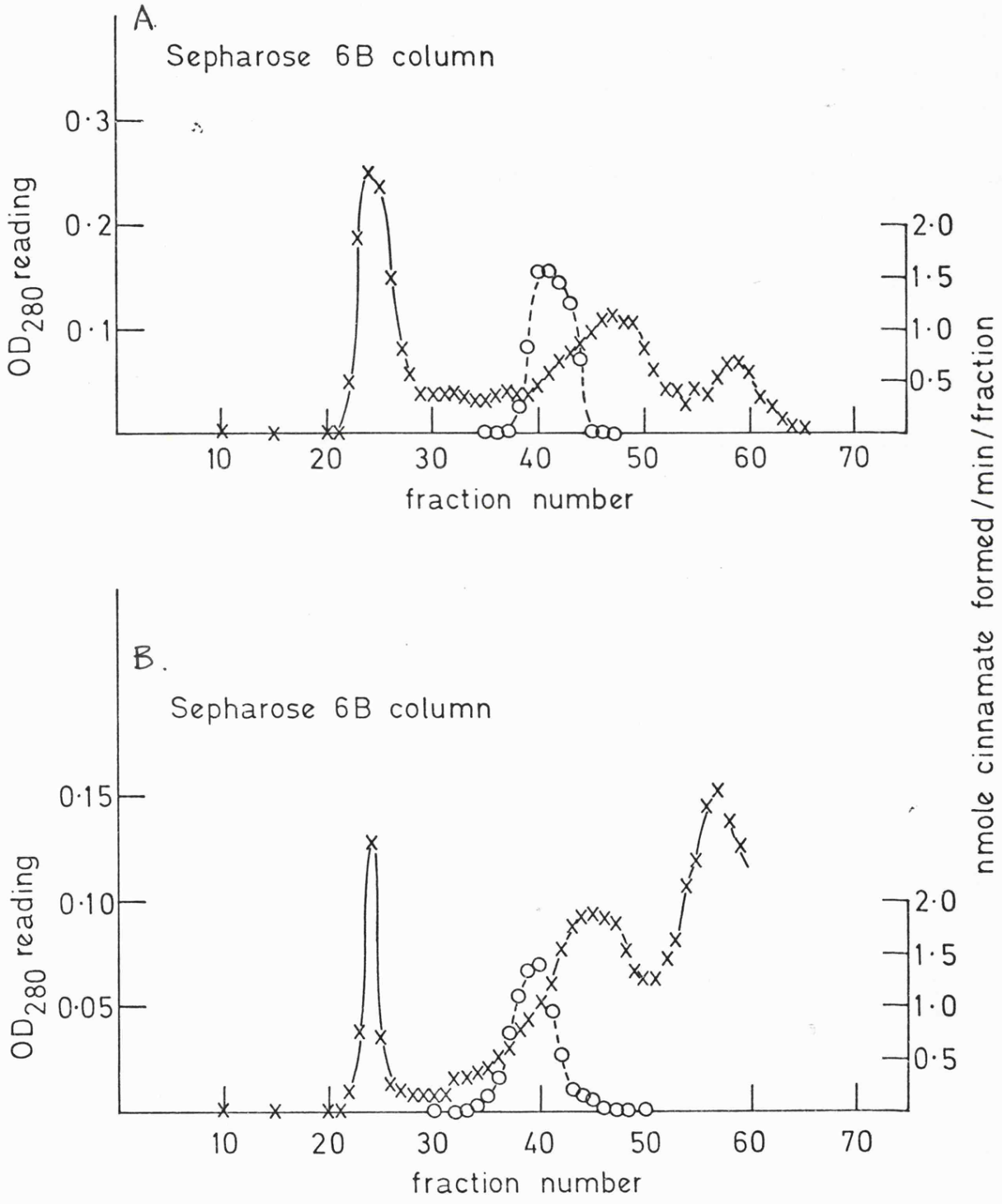

Sepharose 6B column
0·3
0·2
0·1
OD_{280} reading
0
10
20
30
40
50
60
70
fraction number
2·0
1·5
1·0
0·5
nmole cinnamate formed / min / fraction

Figure 3.12

Elution profile of gherkin P.A.L. on the Sepharose 6B column.

A crude supernatant extract from gherkin hypocotyls (taken from 3 day-old seedlings given a 3h. white light) was passed through the calibrated Sepharose 6B column in 50mM borate buffer, pH 8.8 (see Section 2.7.9).(A.) A mixture of partially purified potato P.A.L., D/U and a crude supernatant extract from gherkin hypocotyls (each containing an equal amount of P.A.L.-activity) was also passed through the column (B.). Thr protein content, estimated from the absorbance at 280nm. (x) and the P.A.L.-activity (o) of the eluted fractions were measured.


A.
Sepharose 6B column
0·3
0·2
0·1
OD_{280} reading
2·0
1·5
1·0
0·5
10
20
30
40
50
60
70
fraction number
B.
Sepharose 6B column
0·15
0·10
0·05
OD_{280} reading
2·0
1·5
1·0
0·5
10
20
30
40
50
60
70
fraction number
nmole cinnamate formed/min/fraction

from potato P.A.L. when the mixture was run. The MWs are probably quite close to each other, contaminating proteins in the gherkin extracts may have contributed to the rather broad band of P.A.L.-activity in the gherkin extract. The MWs of P.A.L. from the different sources examined, from both yeast and higher plants, are similar, although not identical.

3.3.3 pH Optima: The pH optima of P.A.L.-activity in extracts from dark- and light-incubated potato tuber discs (Figure 3.13) and also from dark-grown and far-red treated mustard cotyledons (Figure 3.14) were determined. There was a slight difference in the position of the peak of P.A.L.-activity for the two plants; that for mustard being pH 9.0-9.5, and that for potato being pH 8.5-9.0. This may reflect differences in the molecular structure of the P.A.L. from these two sources. The most interesting result here was the difference in the overall shapes of the curves for dark and far-red treated mustard cotyledons, P.A.L.-activity in extracts from etiolated tissue was only significantly active at pHs above 8.0. The pH optimum curve for P.A.L.-activity in extracts from far-red treated tissue was much broader, similar in shape to the pH optima curves for potato P.A.L. This may reflect a difference in the sensitivity of P.A.L. from dark-grown and far-red treated tissue, whether this has any physiological significance or is due to factors arising from the disruption of the tissue during extraction is not yet known.

3.3.4 Km Values: P.A.L. has been recognized as a likely regulatory enzyme in many plants due to the non-Michaelis-Menten kinetics of this enzyme (Camm and Towers, 1977; Huault et al. 1976). An attempt was therefore made to see whether mustard P.A.L. behaved in a similar manner to potato P.A.L. in showing negative co-operativity (Havir and Hanson, 1968b; Lamb and Rubery, 1976c), ie. at higher substrate concentrations the affinity for substrate is reduced, and a higher Km value is obtained; the Km value represents the substrate concentration giving half the maximum rate of enzyme reaction. (The role of negative co-operativity in higher plants is discussed by Lamb and Rubery, 1976d, using P.A.L. as an example.) Initially potato P.A.L. was

Figure 3.13

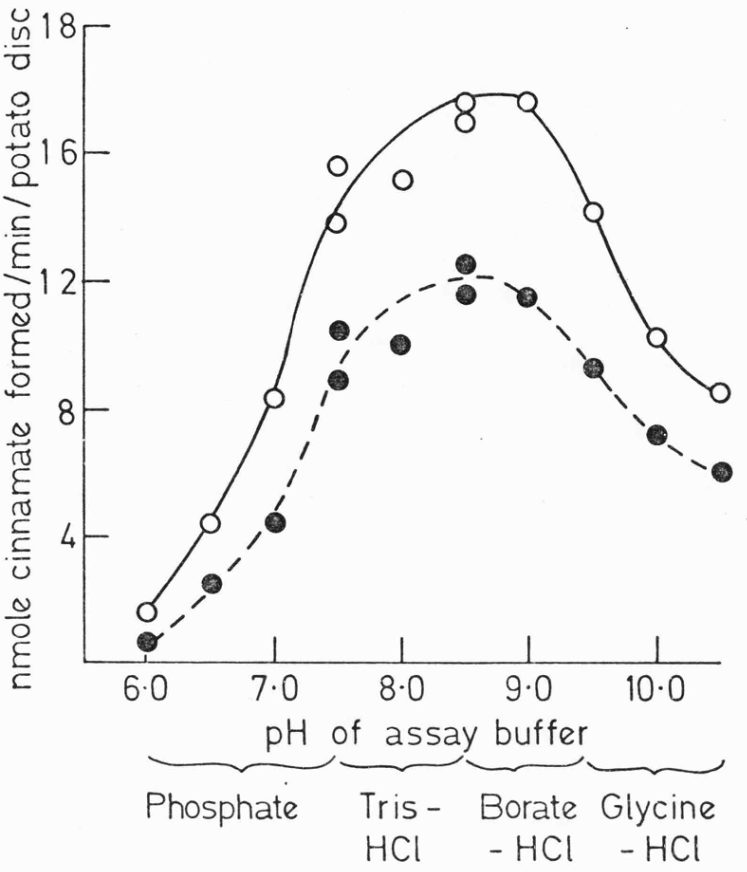
pH Optima curves for potato P.A.L.

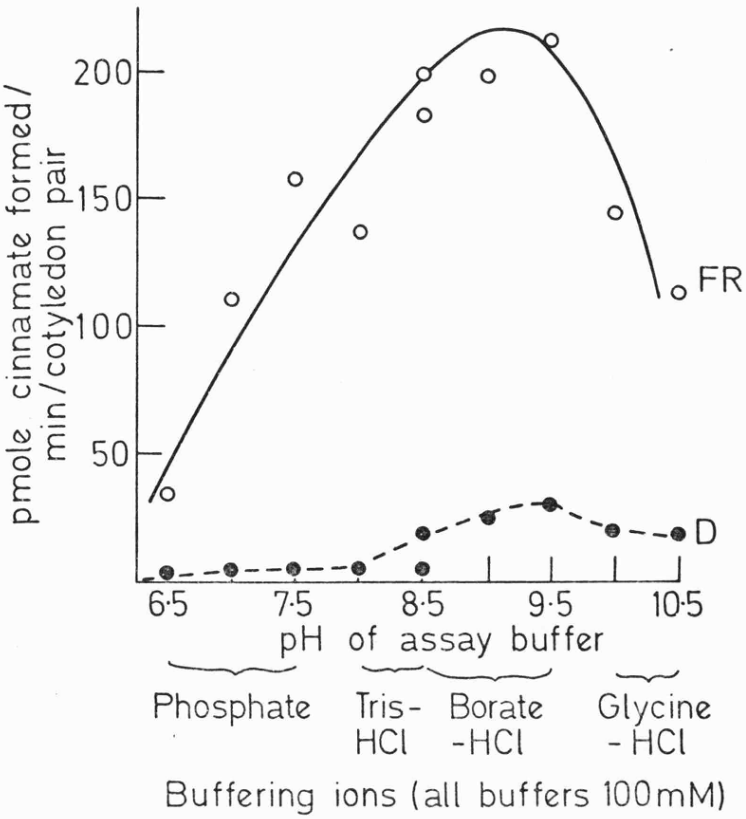
Crude supernatant extracts from potato tuber discs incubated in darkness (closed symbols) or in white light (open symbols) for 20h., were desalted in 50mM borate buffer, pH 8.8. The desalted samples were assayed at a range of pHs as indicated in the figure; the assays were carried out using 0.1ml sample in a 1.0ml. assay volume with 100mM buffers, to ensure that the correct pH was obtained.

Figure 3.14

pH Optima curves for mustard P.A.L.

Crude supernatant extracts from mustard cotyledons taken from seedlings 36h.-old which then received a further 24h. darkness (•) or in far-red light (o), were desalted in 50mM borate buffer, pH 8.8, and D-isoascorbate was added to 10mM. The desalted samples were assayed at a range of pHs as indicated in the figure; the assays were carried out using 0.3ml. sample in a 1.5ml. assay volume with 100mM buffers, to ensure that the correct pH was obtained.


nmole cinnamate formed/min/potato disc
18
16
12
8
4
6·0
7·0
8·0
9·0
10·0
pH of assay buffer
Phosphate
Tris-HCl
Borate-HCl
Glycine-HCl
Buffering ions (all buffers 100 mM)


pmole cinnamate formed/min/cotyledon pair
200
150
100
50
FR
D
6·5
7·5
8·5
9·5
10·5
pH of assay buffer
Phosphate
Tris-HCl
Borate-HCl
Glycine-HCl
Buffering ions (all buffers 100 mM)

assayed at a range of substrate concentrations between 0 and 6mM L-phenylalanine, using a salt-concentrated sample (desalted in borate buffer, pH 8.8). The data obtained were plotted using several different types of plot which give a linear plot with such data.(Plowman,1972), and from which Km values and maximum rates of reaction, Vmax. can be obtained. The results are given in Table 3.24A. The different plots are biased towards either high or low substrate concentrations, the most balanced plot is the Hanes plot; but similar values were obtained with all three plots (based on the same data). The results were similar to those reported previously (0.26mM and 0.038mM, Havir and Hanson,1968b), and clearly show negative co-operativity. When crude supernatant extracts of potato tuber discs,which had been desalted to remove any inhibitory or stimulatory low MW compounds, including any substrate or product of the enzyme which may have been in the crude extract, were used instead of the concentrated sample, lower Km values were obtained, and when the assays were carried out at a lower pH (7.5 instead of 8.8), only a single Km value was obtained.This latter result was in agreement with the results of Lamb and Rubery,1976c, the results are given in Table 3.24B. Similar assays were carried out with mustard seedling extracts over a substrate range of 0-1.5mM L-phenylalanine. The maximum rate of reaction was obtained with 1.0-1.2mM L-phenylalanine which was slightly lower than the substrate concentration required for maximum P.A.L.-activity for potato P.A.L. (where 2.0-4.0mM gave Vmax., a higher concentration was required for P.A.L.-activity assayed at pH7.5.) The Km values for the mustard extracts were determined from Hanes plots, and are given in Table 3.24C. For each extract a single Km value was obtained, in several sets of assays the plots were curved, not linear, nor was it possible to obtain two Km values alone. These results are from a limited number of assays, but indicate that further investigation is necessary, to check the significance of the different Km values obtained for extracts from different parts of the mustard seedling, suggesting that allosteric regulation of enzyme activity may occur. A lack of negative co-operativity

Table 3.24

Enzyme kinetics for P.A.L. from potato and mustard.

Linear plots used to determine Km values:

Lineweaver-Burke plot 1/[S] against 1/(Vi)

Eadie-Hofstee plot (Vi) against (Vi)/[S]

Hanes plot [S]/(Vi) against [S]

([S] = substrate concentration, (Vi) = initial rates of reaction)

3.24A Salt-concentrated potato P.A.L. sample, from potato tuber discs incubated for 20h. in white light; desalted prior to assays.

Type of plot	Km, [S] high*	Km, [S] low*
Lineweaver-Burke	0.31mM	0.028mM
Eadie-Hofstee	0.29mM	0.033mM
Hanes	0.30mM	0.024mM

* [S] high $\geqslant$ 0.2mM; [S] low $<$ 0.2mM L-phenylalanine

3.24B Crude supernatant extract from potato tuber discs incubated for 20h. in white light, desalted and assayed in either 50mM borate buffer, pH 8.8 or 100mM Tris-HCl buffer, pH 7.5 (4°C).

Type of plot	pH 8.8: Km, [S] high	Km, [S] low	pH 7.5: Km
Lineweaver-Burke	0.23mM	0.015mM	0.52mM
Eadie-Hofstee	0.26mM	0.018mM	0.42mM
Hanes	0.21mM	0.014mM	0.52mM

3.24C Crude supernatant extracts from sections of mustard seedlings, 36h.-old given a further 24h. in darkness or in far-red light, desalted and assayed in 50mM borate buffer, pH 8.8 + 10mM D-isoascorbate.

Tissue section	Light treatment	Km value (from Hanes plots)
Cotyledon pairs	dark	0.15mM
	far-red	0.09mM
Hypocotyls	dark	0.16mM
	far-red	0.09mM
Radicles	dark	0.08mM

for mustard P.A.L. was recently reported by Gupta and Acton (1979), using a purified sample of P.A.L. from far-red treated cotyledons. As it is not possible to duplicate the in vivo situation, the best that can be attempted is carrying out the assays at more appropriate temperatures and pHs (ie. nearer to physiological conditions), at a range of likely substrate and product concentrations.

3.3.5 Comparison of P.A.L. from dark- or light-treated tissue: The technique of ion-exchange chromatotgraphy was used to see whether P.A.L. from dark- and white-light-treated potato tuber discs was the same in both cases. Samples of salt-concentrated extracts were desalted and loaded on to a D.E.A.E.-Sephadex A-25 column. P.A.L.-activity was eluted with a gradient of increasing Tris concentration. (Figure 3.15). No significant difference in the elution profiles were obtained.

3.3.6 Summary: P.A.L. from several plant sources were compared, with particular emphasis on potato and mustard. No isoenzymes of P.A.L. were separated from these tissues. P.A.L.from potato tuber discs was not affected by the light treatment, but mustard P.A.L. from dark- and far-red-treated tissue gave different pH optima profiles, and from the different sections of the seedling gave different Km values. The failure to obtain only one Km value suggests that more than one form of P.A.L. may be present in mustard, but the differences may be more subtle than the techniques so far used can properly distinguish. The lack of negative co-operativity may be due to the temperature and conditions used for the assay. It is difficult to look for possible molecular differences when there are many variables due to the extraction and purification which must also be taken into account, but this may be necessary to resolve the question of regulation of enzyme levels.

3.4 IMMUNOLOGICAL STUDIES

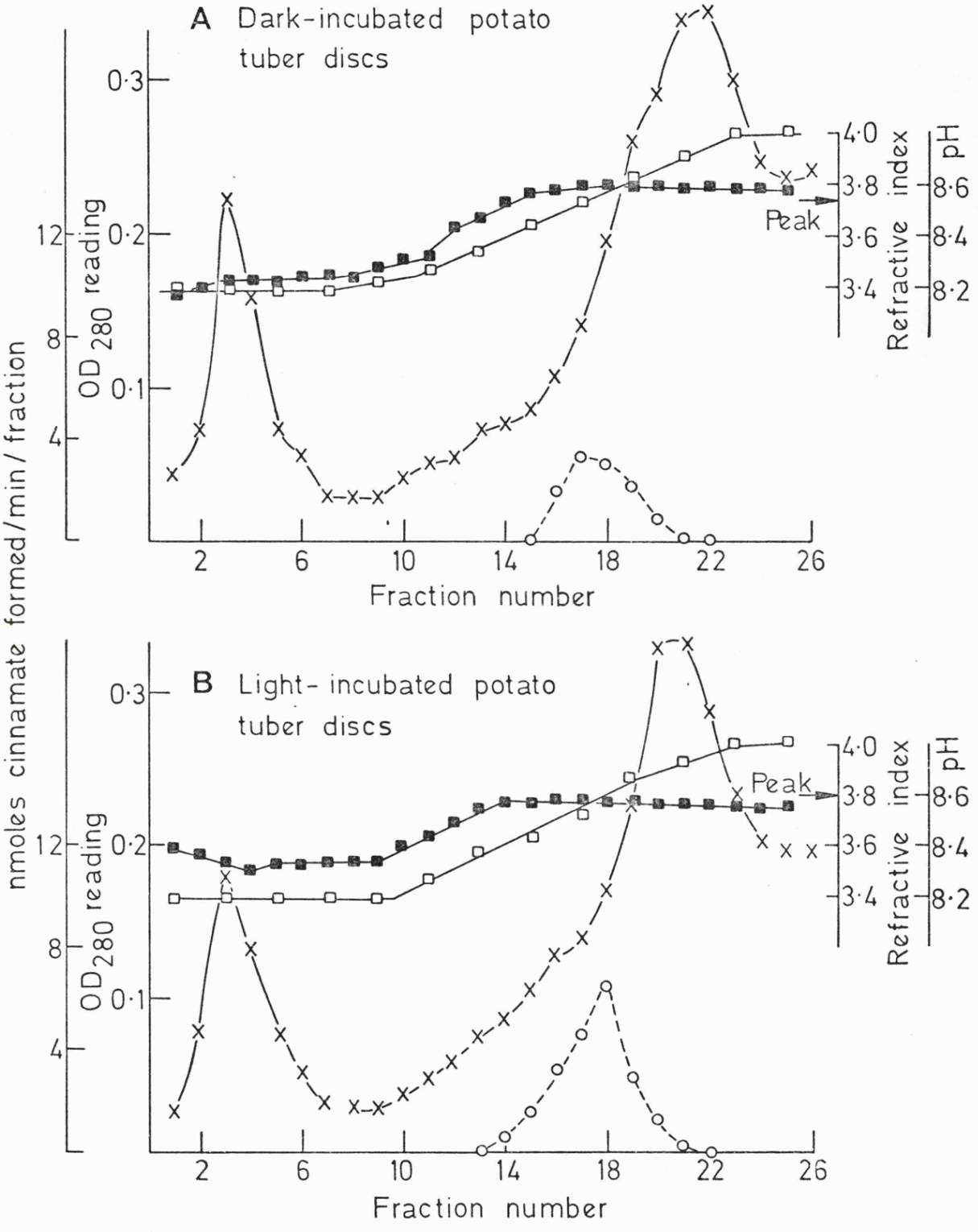
3.4.1 Introduction: Antibodies against potato P.A.L. were raised in rabbits as described in Section 2.9. Initially serum from trial bleeds was tested against samples of P.A.L. using double diffusion plates. Precipitin

Figure 3.15

Elution profile of potato P.A.L. on a D.E.A.E.-Sephadex A-25 column.

Crude supernatant extracts from 25g. potato tuber discs, incubated for 20h. in darkness (A.) or in white light (B.) were concentrated with tri-sodium citrate , desalted on a Sephadex G-25 column in 50mM Tris-HCl buffer, pH 8.4 (4°C), and then loaded on to separate D.E.A.E.-Sephadex A-25 columns (1.6cm. diameter x 7.5cm. height), equilibrated in the same buffer, but containing 1mM E.D.T.A. and 1mM glutathione. The columns were washed through with buffer, then eluted with a gradient of increasing Tris concentration (□); the refractive index was used as a measure of Tris concentration. The pH (■) was followed throughout, the protein content, estimated by reading the absorbance at 280nm. (×) and the P.A.L.-activity (○) of the eluted fraction were measured.

DEAE - Sephadex A-25 columns



lines were obtained against potato P.A.L., with serum from the second or third trial bleeds (taken two weeks after completion of the injection schedule) and in subsequent bleeds, but not with serum from the control, normal rabbit serum (N.R.S.), see Figure 3.16. More than one precipitin line was visible using crude antiserum, and the high protein content meant that overall staining of the plates for protein hampered ,rather than aided the visualization of the precipitin lines. Therefore the antiserum was partially purified by ammonium sulphate fractionation and D.E.A.E.-cellulose chromatography. The initial salt fractionation removed most of the haemoglobin from the serum. The ion-exchange chromatography separated the remaining proteins, mainly immunoglobulins. The fractions eluted from the D.E.A.E.-cellulose column (Figure 3.17) were tested against anti-rabbit γ-globulin serum and anti-rabbit whole serum using double diffusion plates (Figure 3.18); those fractions containing only γ-globulins (IgG fraction) were pooled and concentrated by ultrafiltration. The antiserum was then tested against potato P.A.L., as before on double diffusion plates; two precipitin lines were obtained again, but usually only when antibody or antiserum were in excess (see Section 3.4.2). Immunotitration experiments were carried out to check the specificity of the antiserum.After several months storage in the deep freeze, the antiserum was less effective in ability to immunoprecipitate the P.A.L.-activity, suggesting that the antiserum was undergoing degradation during storage (although antiserum is generally stable for longer periods than this). Fluctuations in storage temperature may have been partly resonsible, or possibly the antiserum was less stable than expected. The results of the immunotitration experiments to determine P.A.L. levels in extracts of plant tissues are presented, but complete quantitative results were prevented by the progressive decline in antiserum effectiveness.

3.4.2 Double Diffusion Tests: Precipitin lines were obtained between the antiserum and partially purified and concentrated potato P.A.L. samples. The next step was to use relatively crude extract samples, ie. supernatant (equivalent to step 2 in the purification procedure, Section 3.2.11). Figure

Figure 3.16

Double Diffusion Plates with potato P.A.L.

The central well of each set of wells, contains 50µl. serum from trial bleeds from rabbit 1, bleed 3 (R1 iii), rabbit 2, bleeds 2 and 3 (R2 ii, R2 iii) and serum from a normal rabbit (N.R.S.). The surrounding wells contain 50µl. potato P.A.L. sample, D/U, at a range of dilutions; the top well contains undiluted P,A.L. sample with progressive 2-fold dilutions , in a clockwise direction around the central well.

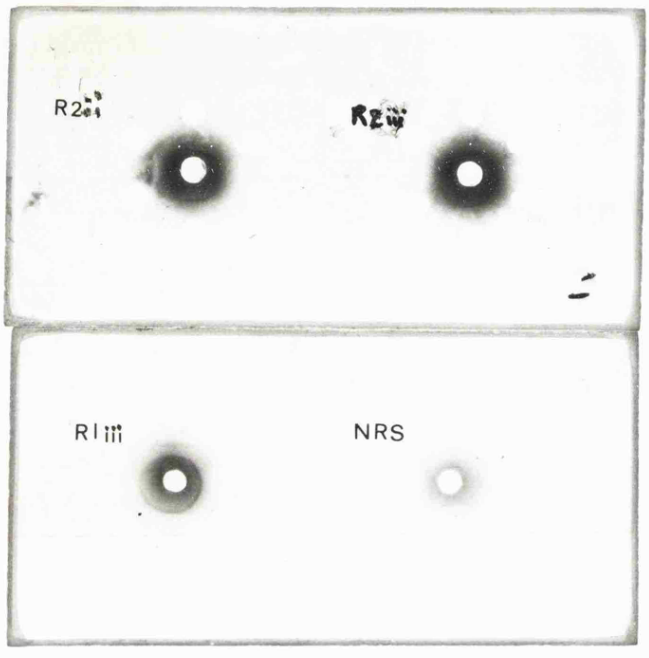

R2 i
R2 ii
R1 iii
NRS

Figure 3.17

Purification of crude anti-potato P.A.L. serum.

D.E.A.E.-cellulose column.

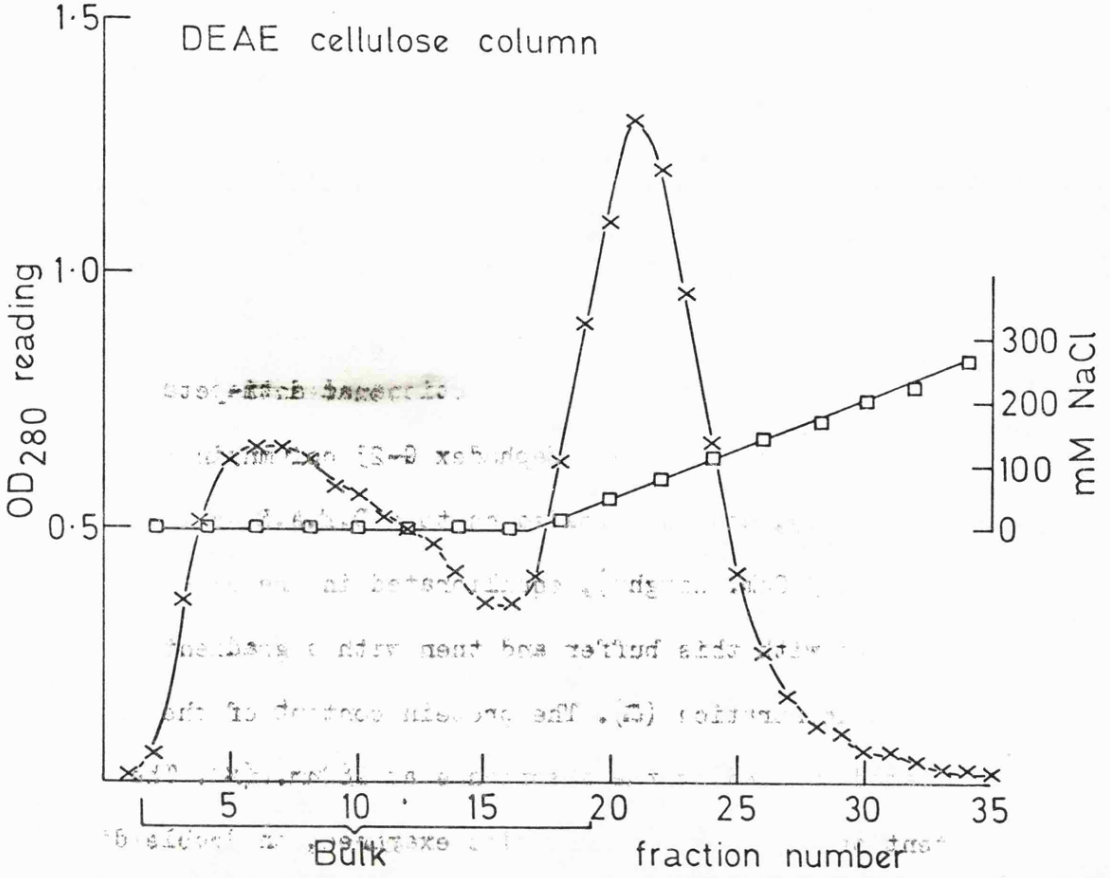
A sample of ammonium sulphate fractionated anti-potato P.A.L. serum (8.0ml.) was desalted on a Sephadex G-25 column in 50mM Tris-HCl buffer, pH 7.6 (18°C), and then loaded on to a D.E.A.E.-cellulose column (4.8cm. diameter x 3.0cm. height), equilibrated in the same buffer. The column was eluted with this buffer and then with a gradient of increasing salt (NaCl) concentration (□). The protein content of the eluted fractions was estimated by reading the absorbance at 280nm. (×). The immunoglobulin content of the fractions was also examined, on double diffusion plates see below Figure 3.18.

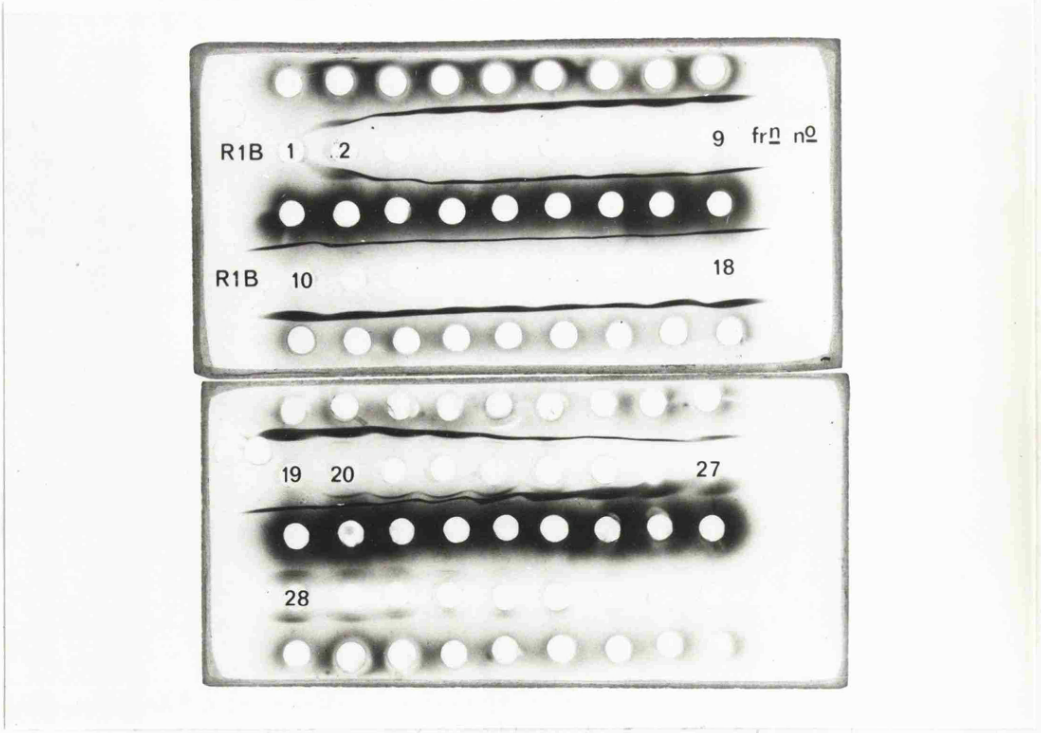
Figure 3.18

Double Diffusion Plates with fractions from the D.E.A.E.-cellulose column.

Two 5x10cm . plates were prepared, each divided into 5 rows of wells. The second and fourth rows contain 20µl. aliquots of the fractions eluted from the D.E.A.E.-cellulose column above, Figure 3.17 (fr^{n} n^{o}), R1B refers to the batch of anti-potato P.A.L. serum, taken from rabbit 1, and partially purified by ammonium sulphate fractionation. The other rows contain 20µl. aliquots of anti-rabbit whole serum, from goats; rows one and five contain goat serum diluted 2 times, the central row, row three contains goat serum diluted 10 times.

Fractions 2-19 gave a single precipitin line, and therefore contain only IgG (or γ) immunoglobulins. An identical set of plates was prepared for tests with anti-rabbit γ-globulin serum, from goats; a single precipitin line was obtained against fractions 2-30.


DEAE cellulose column
1·5
1·0
0·5
OD280 reading
300
200
100
0
mM NaCl
5
10
15
20
25
30
35
Bulk
fraction number


R1B 1
2
9
frn nº
R1B 10
18
19
20
27
28

3.19 shows the results of double diffusion tests using the purified antiserum and supernatant extracts from potato tuber discs incubated in darkness or white light for 12, 18 and 24h.; two precipitin lines were visible after staining the plates for protein (with Coomassie blue or the B.D.H. equivalent). This result was obtained with purified samples of potato P.A.L. and crude antiserum, and is common to both, so it would appear that the antiserum is responsible. As it is not possible to identify the precipitin line corresponding to P.A.L. (as there is no suitable staining technique for the enzyme), immunoelectrophoresis was used in an attempt to separate the two precipitin lines obtained on simple double diffusion plates. The results of such an experiment are shown in Figure 3.20. Partially purified and concentrated samples of potato were used because the precipitin lines obtained were more distinct, and smaller volumes were more suited to immunoelectrophoresis. Unfortunately only a single precipitin line was obtained; this was, however, coincident with the position of P.A.L.-activity (located by assaying sections of the agarose gel from an identical plate run in parallel for the electrophoresis). In the two samples of P.A.L. used, the migration rates of P.A.L. were different, this does not reflect any difference in the properties of P.A.L. itself because the sample giving the slower migration rate was a salt-concentrated preparation (which was not desalted prior to the experiment), in which aggregation of the enzyme molecules had probably occurred, hence the slower migration rate. In both cases the P.A.L.-activity was coincident with the precipitin line, supporting the hope that the antiserum was specific for P.A.L. The second precipitin line obtained in the simple double diffusion tests may have been due to aggregation of P.A.L. molecules, or due to degradation products of P.A.L., such as the enzyme subunits (these processes may have occurred following injection of the purified P.A.L. sample into the rabbits). Alternatively minor contaminants in the purified P.A.L. sample injected may have resulted in the formation of 'contaminating' antibodies. The double diffusion tests themselves were not suitable for quantitative experiments, but did show that P.A.L. from

Figure 3.19

Double Diffusion Plates with extracts from potato tuber discs.

The central wells contain 40µl. purified anti-potato P.A.L. serum, R1C2, serum from rabbit 1, purified by D.E.A.E. cellulose chromatography, second run. The surrounding wells contain crude supernatant extracts from potato tuber discs incubated for 12, 18 or 24h. in darkness (D) or in white light (L), 40µl aliquots in the set of wells on the left, and 80µl. aliquots in the set of wells on the right.

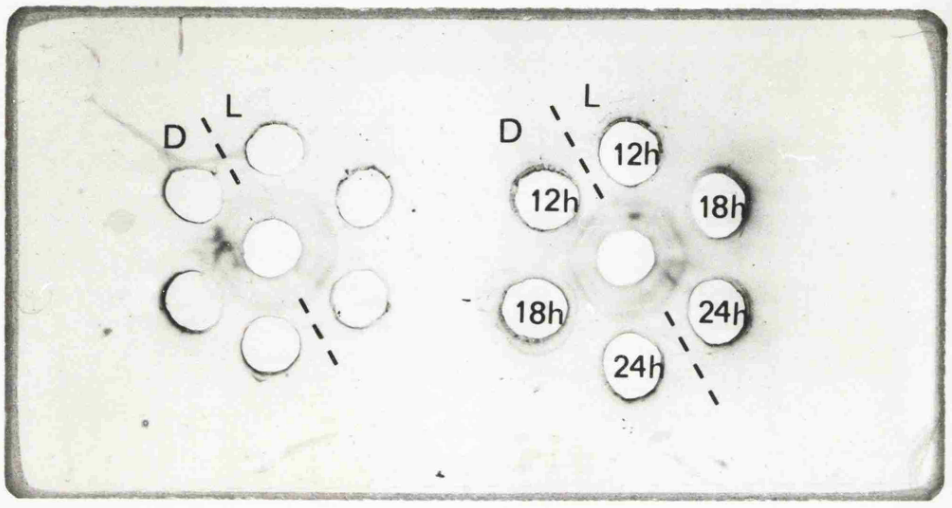
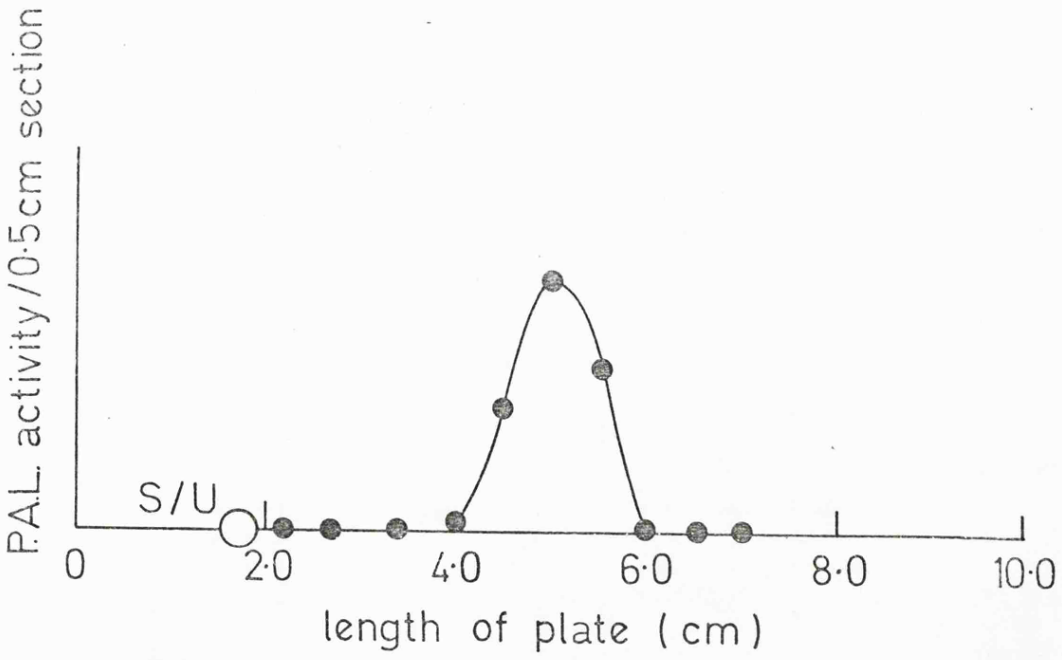
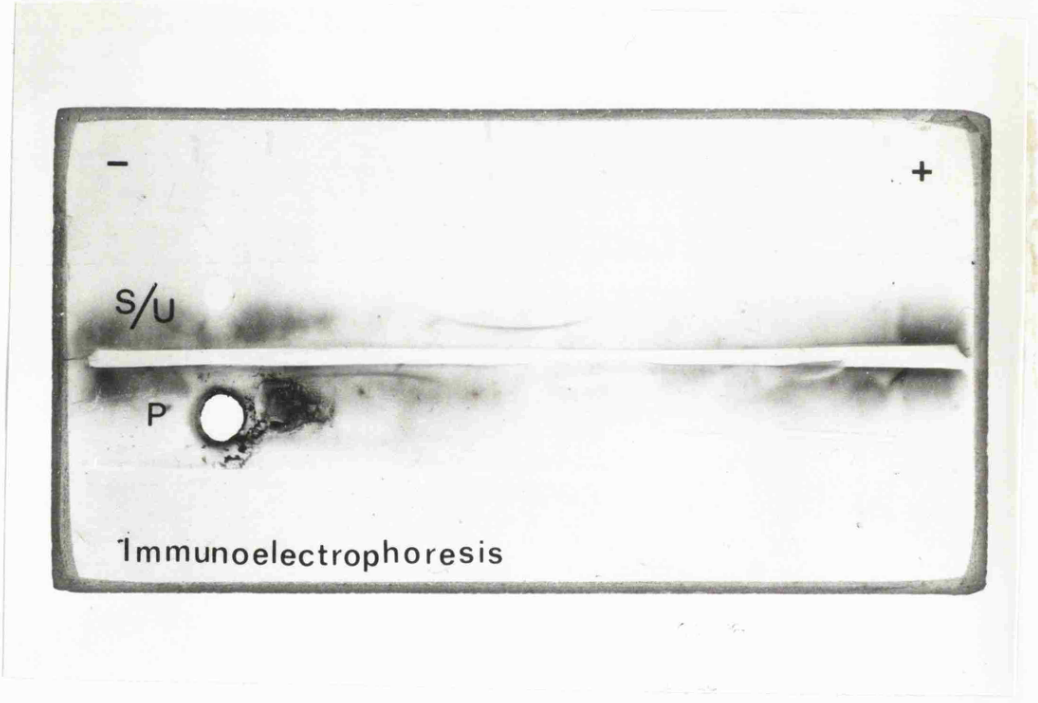

D
L
D
L
12h
12h
18h
18h
24h
24h

Figure 3.20

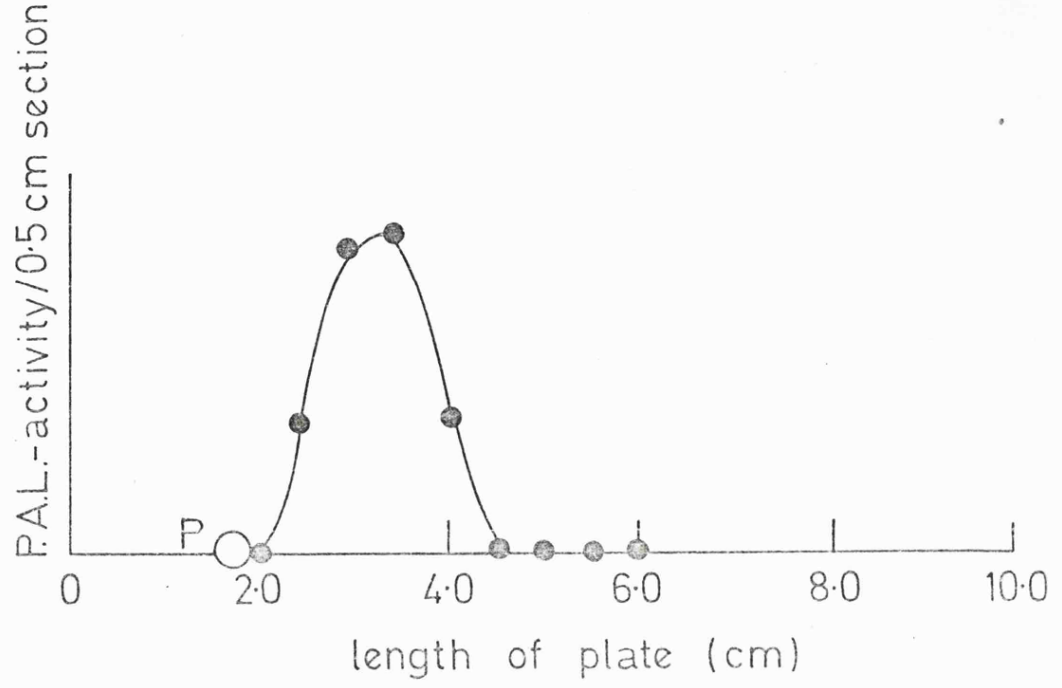
Immunoelectrophoresis Plates with potato P.A.L.

The immunoelectrophoresis plate is shown in the centre, the direction of electrophoresis was from left to right. Potato P.A.L. samples were placed in the wells, the upper well contained a 20µl. aliquot of purified P.A.L. S/U I, the lower well contained a 50µl. aliquot of concentrated potato P.A.L. extract (sodium citrate fractionated). Electrophoresis was carried out as described in Section 2.12.3. After electrophoresis the agarose gel was removed from the troughs from between the wells and on the outside edges of the plate, these were filled with anti-potato P.A.L. serum, R1C (serum from rabbit 1, purified by D.E.A.E.-cellulose chromatography, run 1). The central trough contained 250µl. antiserum, the outer troughs contained 250µl. antiserum at 1:20 dilution.
The two graphs show the P.A.L.-activity on an identical plate, following electrophoresis: the agarose gel was cut into 0.5cm. wide sections, across the plate, from left to right.,the gel was also cut in a line across the plate between the wells, and sections assayed for P.A.L.-activity as described in Section 2.5.3.





Immunoelectrophoresis



potato tuber discs, was unchanged immunologically, during the incubation period of the discs (Figure 3.19). The precipitin lines were confluent, forming a complete circle around the central antiserum well. The stronger of the two precipitin lines was assumed to be the one corresponding to P.A.L. following the results of the immunoelectrophoresis experiment, where no second, minor precipitin line was obtained. The intensity of the precipitin line provides an indication of the amount of antigen present. (By using serial dilutions of sample-antigen against a constant volume of antiserum, it is possible to find the equivalence point and obtain quantitative results, but the sensitivity is limited, and purely dependent on the formation of precipitin lines.) In Figure 3.21 the intensity of the precipitin lines increases as the sample volume increases. P.A.L. from different potato varieties was also immunologically indistinguishable (Figure 3.22); the precipitin lines between the two varieties were confluent. The precipitin line against an extract from the red-potatoes was slightly stronger than that against an extract from white-fleshed potatoes, as expected as the red variety contained twice the amount of P.A.L.-activity.

3.4.3 Immunotitration Tests: The specificity of the antiserum can be checked more precisely by immunoprecipitation, ie. by the ability of the antiserum to precipitate P.A.L. from the P.A.L.-containing extract. Using a single extract from potato tuber discs incubated for 24h. in white light (24h.,L.) at three dilutions, it was shown that the volume of antiserum required to precipitate P.A.L. was proportional to the amount of P.A.L.-activity precipitated (Figure 3.23). Following incubations of 20h., of the crude supernatant extract (or dilution thereof) from incubated tuber discs and antiserum at a range of volumes, the amount of P.A.L.-activity in the supernatant was found to decline with increasing volumes of antiserum (Figure 3.23A). The greater the amount of P.A.L.-activity present, the greater the volume of antiserum required to precipitate all of the P.A.L.-activity. At lower antiserum volumes P.A.L.-activity was not precipitated as well as may be expected, this was probably due to the formation of soluble enzyme-antibody

Figure 3.21

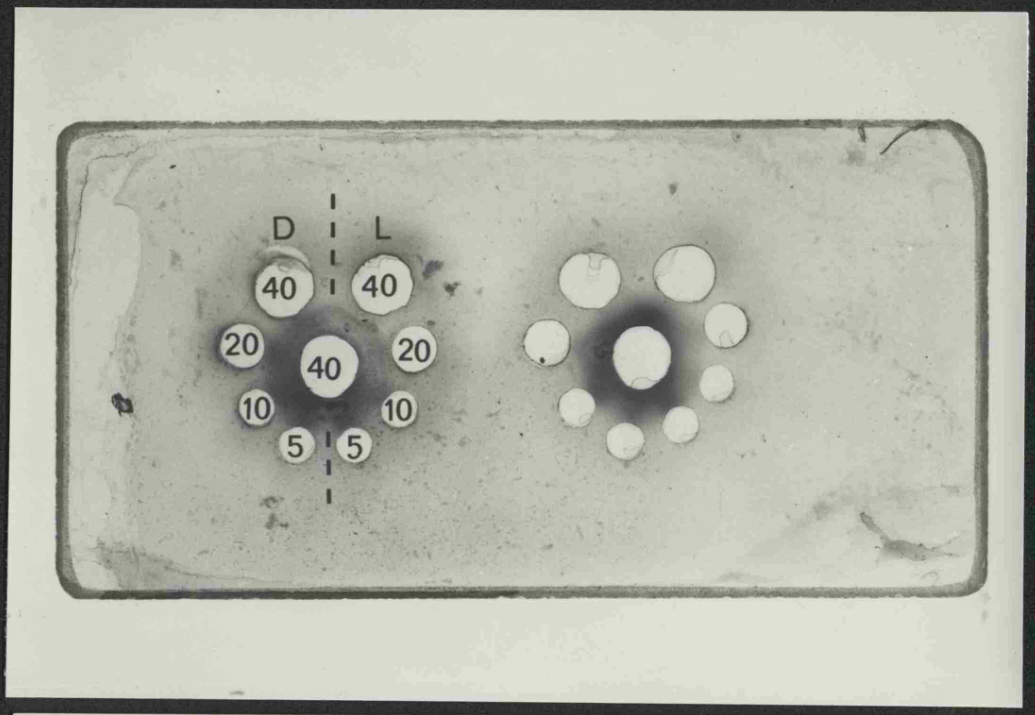
Double Diffusion Plates with extracts of potato tuber discs.

The central wells contain 40µl. aliquots of serum: anti-potato P.A.L. serum (R1C2) on the left, and normal rabbit serum (N.R.S.) on the right. The surrounding wells contain aliquots (5,10,20, or 40µl.) of crude supernatant extracts from potato tuber discs incubated for 20h. in darkness (D) or in white light (L).

Figure 3.22

Double Diffusion Plates with extracts of potato tuber discs.

The central wells both contain 50µl. aliquots of anti-potato P.A.L. serum (R1C2). The surrounding wells contain various aliquots (volume in µl. is given on the plates) of extracts from two varieties of potatoes, a red-skinned variety with yellow flesh, Desiree,(r) and a white-fleshed variety, King Edward's, (w). Extracts were made after incubating the discs in white light for 20h..


D
L
40
40
20
40
20
10
10
5
5

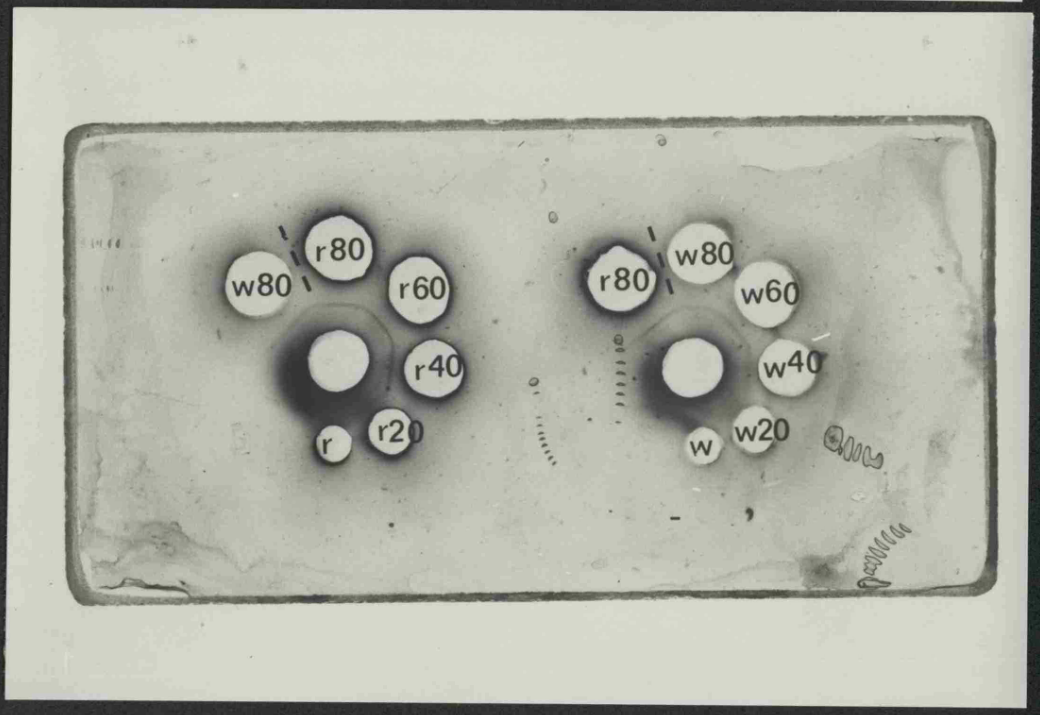
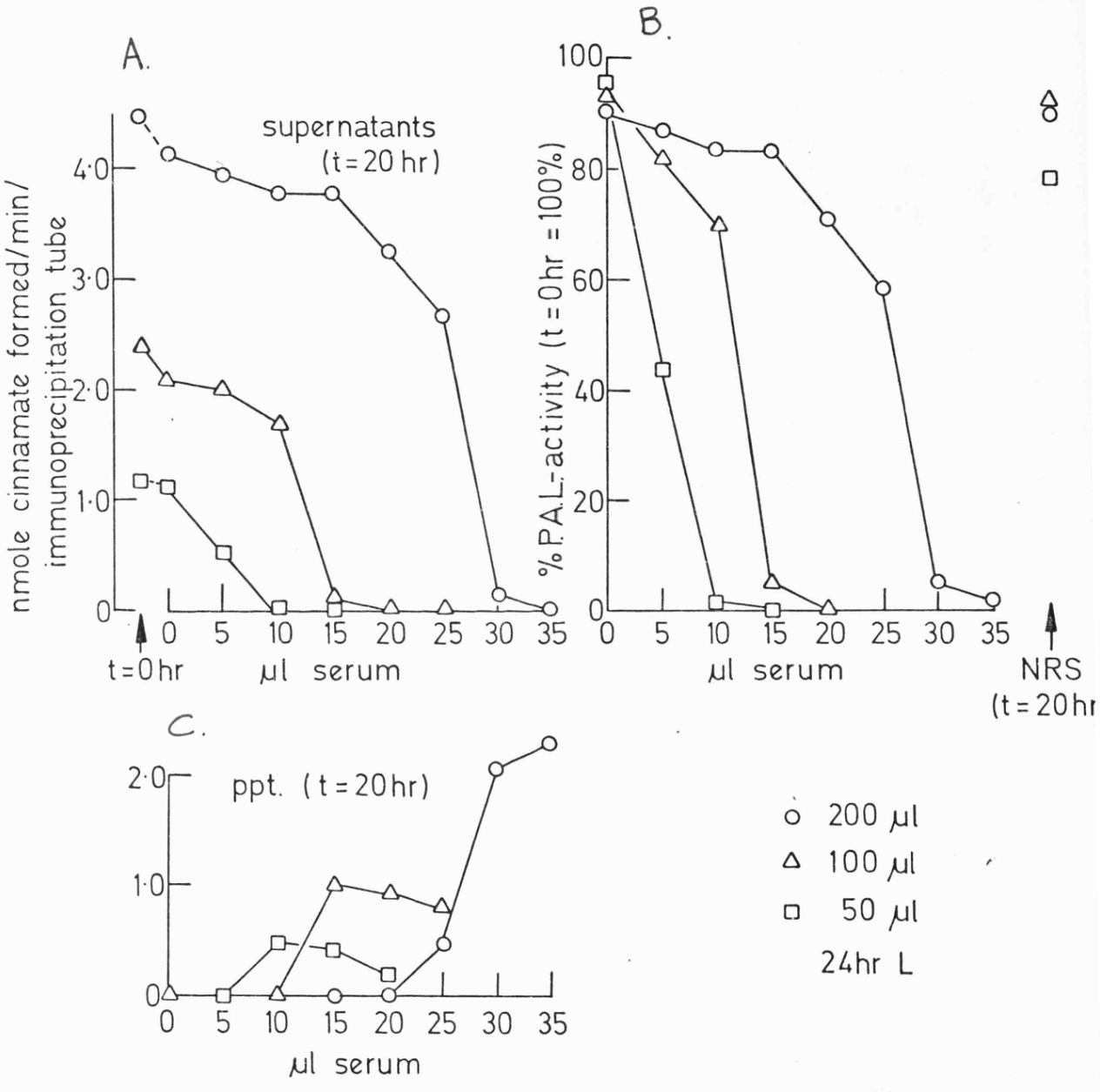

r80
w80
r60
r40
r
r20
r80
w80
w60
w40
w
w20

Figure 3.23

Immunotitration Experiment with extracts of potato tuber discs.

A mixture of an extract from potato tuber discs, incubated for 24h. in white light, at a range of dilutions (50,100 or 200µl. aliquots of extract made up to a final volume of 200µl. with extraction buffer), and anti-potato P.A.L. serum (R1C) or normal rabbit serum (N.R.S.), was incubated for 20h. at 4°C. The mixture was then centrifuged and the supernatant assayed for P.A.L.-activity (A.). The P.A.L.-activity was also plotted as a % of the activity in the sample volume used (B.). The immunoprecipitates were then washed and resuspended in buffer at pH 9.5, and reassayed for P.A.L.-activity (C.). For full details of the procedure see Section 2.12.4.


A.
supernatants
(t = 20 hr)
nmole cinnamate formed/min/
immunoprecipitation tube
4·0
3·0
2·0
1·0
0
0 5 10 15 20 25 30 35
t = 0 hr
µl serum
B.
100
80
60
40
20
0
% P.A.L.-activity (t = 0 hr = 100%)
0 5 10 15 20 25 30 35
µl serum
NRS
(t = 20 hr
C.
2·0
1·0
0
ppt. (t = 20 hr)
0 5 10 15 20 25 30 35
µl serum
200 µl
100 µl
50 µl
24hr L

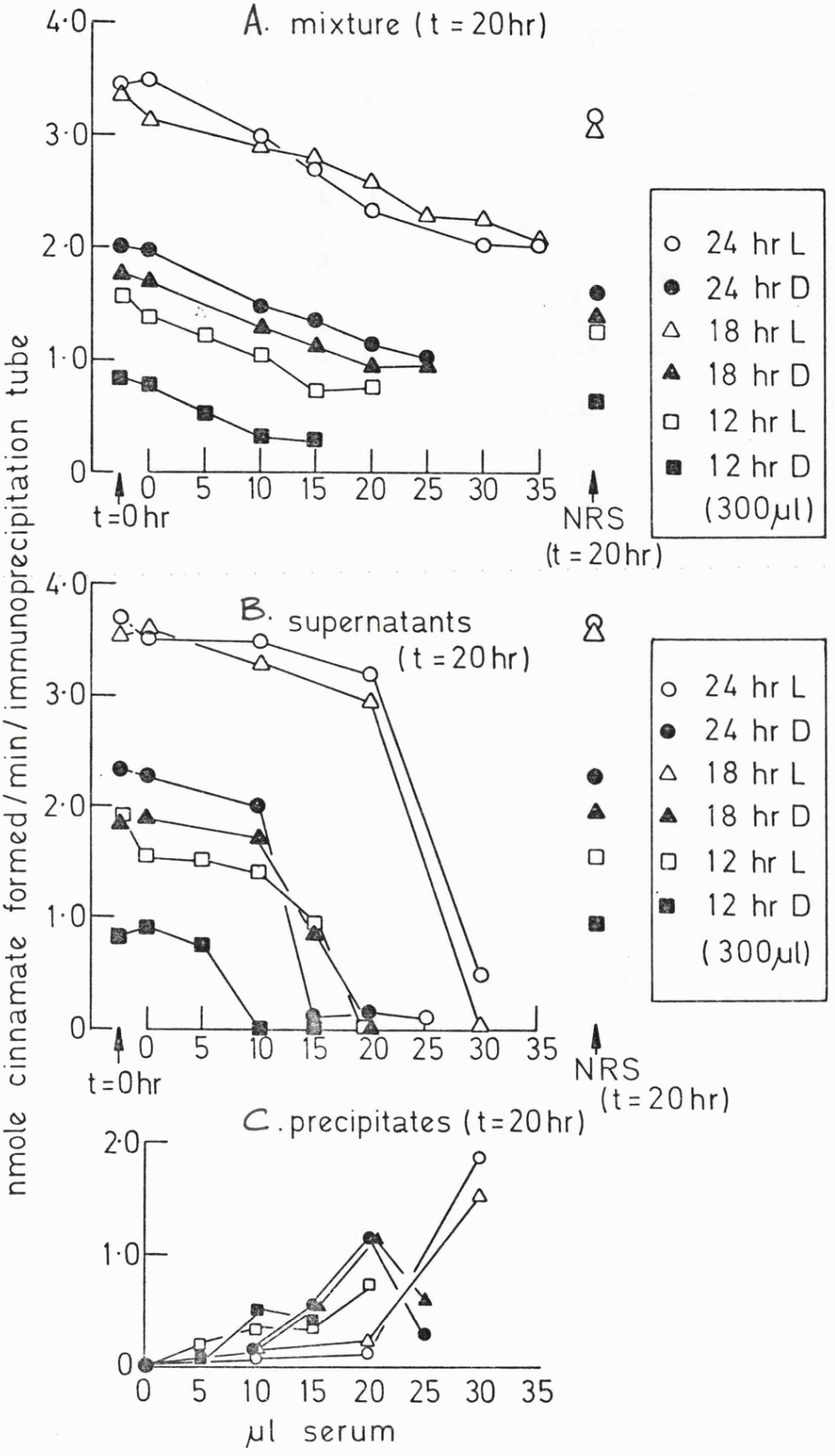
complexes, rather than insoluble complexes which are formed when the amount of antiserum and antigen are present in roughly equivalent proportions, corresponding to the 'equivalence zone' of the reaction between antibody and antigen. This was later verified using a second antibody, anti-rabbit serum from goats, to precipitate the rabbit anti-potato P.A.L. serum, which would also precipitate any soluble antigen(enzyme)-antibody complexes. The shapes of the immunoprecipitation curves could be altered depending on the amount of second antibody used. As the immunoprecipitation curves were obtained using a constant sample volume and increasing volumes of antiserum, no suitable volume of second antibody could be found which did not interfere with the shapes of the immunoprecipitation curves, so second antibody was not used routinely. (Similar problems of soluble and insoluble complexes due to varying proportions of antigen (rabbit serum) and antibody (second antibody) may have contributed to these effects.) That the loss of P.A.L.-activity from the supernatants corresponded to precipitation of P.A.L. was shown by washing the immunoprecipitates and re-suspending them in a higher pH buffer (to help solubilize the immunocomplexes formed) and assaying for P.A.L.-activity. The results are shown in Figure 3.23C., the amount of P.A.L.-activity recovered in the immunoprecipitates was $\sim$50% that precipitated, ie. lost from the supernatant, and follows the pattern of the immunoprecipitation curves in Figure 3.23A.. Complete recovery of the P.A.L. from the immunoprecipitates was not possible because the immunoprecipitates were not completely solubilized in the buffer used, a higher pH buffer may have been more successful in breaking down the immunocomplexes and freeing the P.A.L., but at higher pHs P.A.L.-activity itself was reduced. It can also be seen from the results that at higher antiserum volumes the recovery of P.A.L. is lower than expected, this is due to inhibition of the enzyme activity by the antiserum (see Figure 3.24A.). In the presence of normal rabbit serum, N.R.S. negligible inhibition or precipitation of P.A.L.-activity occurred, showing that the effect of anti-potato P.A.L. serum was probably specific, further supported by the

correlation between the amount of antiserum required to precipitate the P.A.L. from an extract of potato tuber discs. The immunoprecipitation curves are plotted in a slightly different manner in Figure 3.23B., the P.A.L.-activity in each sample volume at the beginning of the experiment before mixing (t=0h.) is taken as 100%. the decrease in P.A.L.-activity is then given as a % of this value. If all the samples contained the same amount of P.A.L., then the curves would be identical, instead the amount of P.A.L.-protein appears to be roughly proportional to the amount of P.A.L.-activity present in each sample volume. In order to check that the amount of protein present did not affect the results obtained by immunoprecipitation, a similar series of experiments were carried out using a range of potato P.A.L. supernatant extracts from discs aged for 12, 18 or 24h. in darkness or in white light, in which the P.A.L.-activity, but not the protein content, changed. The results are shown in Figure 3.24.. Following incubation of the mixture of the extract and antiserum for 20h. at 4°C., the P.A.L.-activity in the mixture (prior to centrifuging) was found to decline with increasing volumes of antiserum, the amount of inhibition being proportional to the amount of antiserum present; the inhibition plots are roughly parallel to each other, (A.) and normal rabbit serum, N.R.S. was without effect. As mentioned earlier the anti-potato P.A.L. serum inhibits the P.A.L.-activity, whether the immunocomplexes are soluble or insoluble. In order to precipitate the immunocomplexes in the mixture, the samples were centrifuged. The immunoprecipitation curves are shown in Figure 3.24 (B.), and are very similar to those shown in Figure 3.23A. The curves are roughly parallel to each other, showing little tendency to verge towards each other, only overlapping where equal amounts of P.A.L.-activity were present in the extracts (such as in extracts from discs incubated for either 18 or 24h. in darkness and 12h. in white light). The P.A.L.-activity in the immunoprecipitates (Figure 3.24C.) follows the same pattern as before; corresponding to the amount of P.A.L.-activity in the extracts, with inhibition of P.A.L.-activity greatest in those mixtures containing lowest P.A.L.-activity

Figure 3.24

Immunotitration Experiments with extracts of potato tuber discs.

A mixture of an extract from potato tuber discs, incubated in darkness (closed symbols) or in white light (open symbols), for 12, 18 or 24h. (as indicated in the key), and antiserum: either anti-potato P.A.L. serum (R1C) or normal rabbit serum (N.R.S.), was incubated for 20h. at 4°C. The mixture was then assayed for P.A.L.-activity (A.) or was then centrifuged and the supernatant assayed for P.A.L.-activity (B.). The precipitates were then washed and resuspended in buffer and assayed for P.A.L.-activity (C.). For details of the procedure see Section 2.12.4.


A. mixture (t = 20hr)
B. supernatants (t = 20hr)
C. precipitates (t = 20hr)
nmole cinnamate formed / min / immunoprecipitation tube
4·0
3·0
2·0
1·0
0
0 5 10 15 20 25 30 35
t = 0 hr
NRS (t = 20hr)
24 hr L
24 hr D
18 hr L
18 hr D
12 hr L
12 hr D
(300µl)
µl serum

or highest antiserum volume. From these results it was shown that the ability of the antiserum to precipitate P.A.L. from extracts from potato tuber discs was a property of the anti-potato P.A.L. serum (and could not be obtained with N.R.S.) and therefore the antiserum was specific. When immunoprecipitation curves were obtained using a constant volume of antiserum and increasing volumes of extract, the amount of P.A.L.-activity in the supernatant after centrifuging the mixtures, increased with increasing volume of extract. By carefully selecting appropriate antiserum volumes the P.A.L.-activity in the lowest extract volumes was completely removed from the supernatant, as the amount of P.A.L.-activity present increased,(as the extract volume increased), the P.A.L.-activity in the supernatant increased. The protein content of the immunoprecipitates was measured and although there was a gradual increase in protein content with increasing extract volume used there was no direct correlation between the amount of P.A.L.-activity precipitated and the amount of total protein in the immunoprecipitates. This shows that proteins other than P.A.L. and the specific antibodies were present in the immunoprecipitates, however there was no reason to suggest that this interferred with the results obtained by immunotitration experiments (as described for experiments shown in Figures 3.23 and 3.24). The use of relatively crude supernatant extracts may have been responsible, especially as the antiserum even the partially purified antiserum used here (R1C or R1C2) probably contained non-specific antibodies or immunoglobulin molecules which combined with determinants not found on the P.A.L. protein.

3.4.4 Immunological Determination of P.A.L. Levels.

A. In incubated potato tuber discs: Immunoprecipitation curves, referred to as immunotitration curves because a range of antiserum volumes were used, were prepared for a range of potato tuber disc extracts, taken from discs incubated for between 0 and 42 h., either in darkness or in white light. The results are presented in Figures 3.25 and 3.26; the data were plotted in terms of total P.A.L. activity (absolute activity) in Figure3.25 and as a

Figure 3.25

Immunotitration Experiments to measure P.A.L. levels in extracts from potato tuber discs.

Mixtures of extracts from potato tuber discs and anti-potato P.A.L. serum (R1 C2), were incubated for 20h. at 4°C. The mixtures were then centrifuged and the supernatants assayed for P.A.L.-activity. The potato extracts were made from discs incubated for between 0 and 42h. in darkness (closed symbols, D.) or in white light (open symbols, L.), and the volumes used are indicated on each graph.

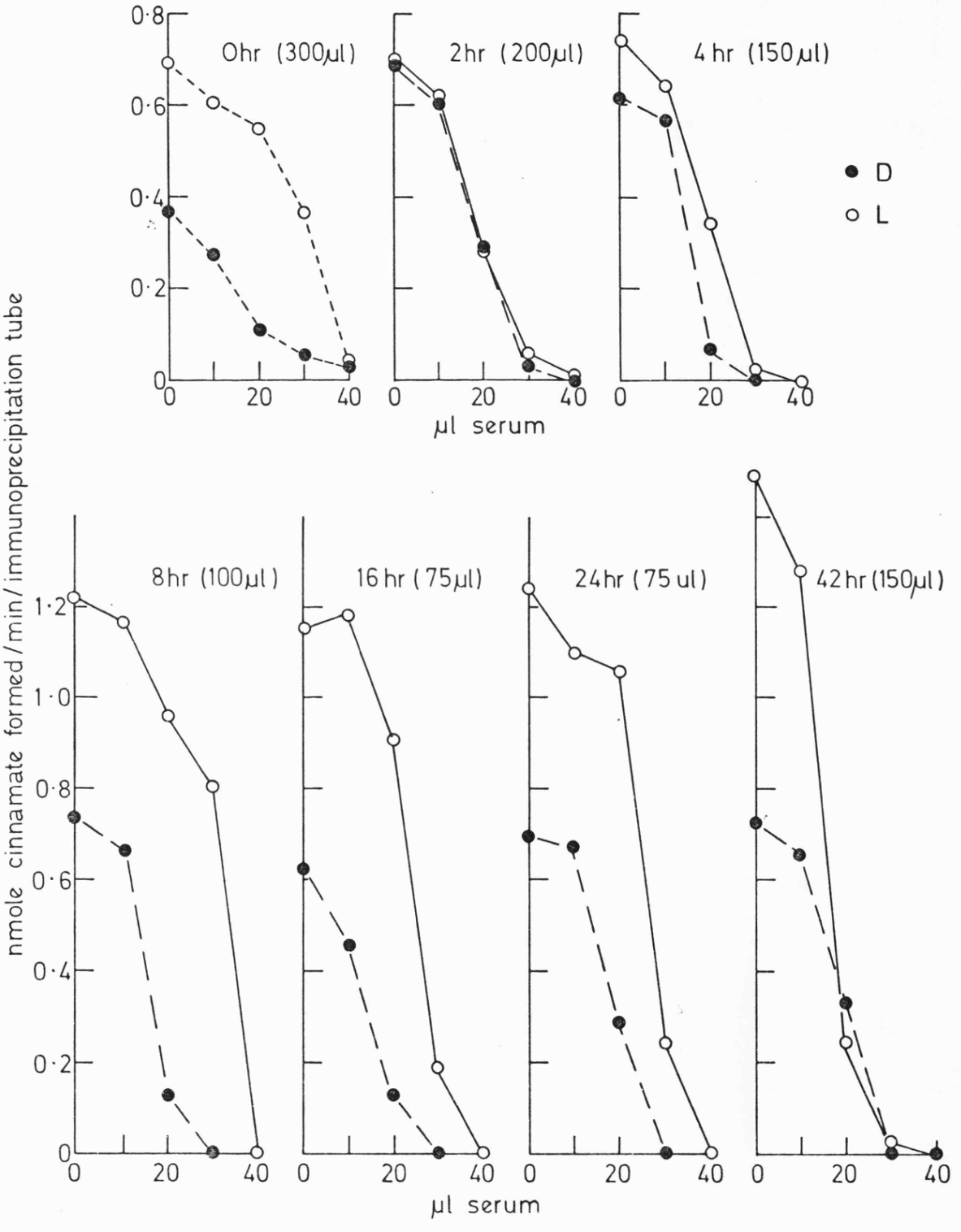
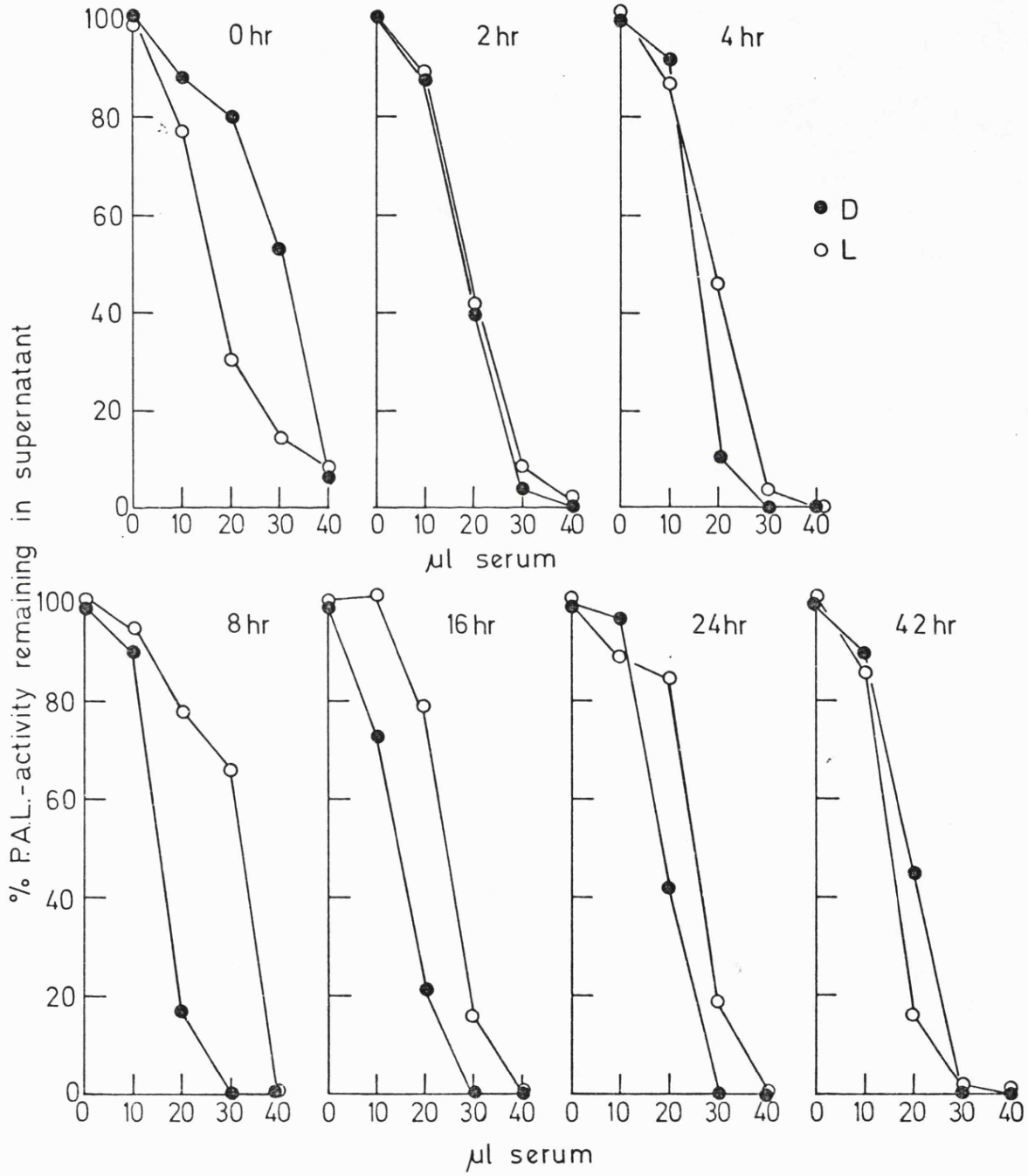

0hr (300µl)
2hr (200µl)
4 hr (150µl)
8hr (100µl)
16 hr (75µl)
24hr (75 ul)
42 hr (150µl)
D
L
nmole cinnamate formed/min/immunoprecipitation tube
µl serum
µl serum
0·8
0·6
0·4
0·2
0
1·2
1·0
0·8
0·6
0·4
0·2
0
0
20
40

Figure 3.26

Immunotitration Experiments to measure P.A.L. levels in extracts from potato tuber discs.

The data plotted in this set of graphs is taken from the previous figure (3.25); mixtures of extracts from potato tuber discs and anti-potato P.A.L serum were incubated for 20h. at $4^{\circ}C$, then the mixtures were centrifuged and the supernatants assayed for P.A.L.-activity. The potato extracts were made from discs incubated for between 0 and 42h. in darkness (closed symbols,D.) or in white light (open symbols,L.) as indicated on each graph. The P.A.L.-activity was plotted as a % of the activity in the sample volumes used, ie. at t=0h. the beginning of the experiment.


0 hr
2 hr
4 hr
8 hr
16 hr
24 hr
42 hr
D
L
% P.A.L.-activity remaining in supernatant
µl serum
µl serum
0
20
40
60
80
100
0 10 20 30 40

% of the activity in each extract in Figure 3.26. The sample volumes (given in Figure 3.25) were chosen so that the same amount of P.A.L.-activity was present in all the samples from the extracts of dark-incubated discs, for easier visualization of the results. The volume of antiserum required to precipitate the P.A.L. increases as the amount of P.A.L.-activity present in the extracts increases; the curves for the dark extracts are all very similar to each other, and the curves for the other extracts are roughly parallel to them. There are two exceptions to this, the extracts from freshly sliced discs (t=0h.) and those from discs incubated in white light for 42h. The amount of P.A.L.-activity in extracts from discs incubated for 2h. is the same whether incubated in darkness or in continuous white light, and so is the P.A.L. protein, detected immunologically. Then as the P.A.L.-activity increases,(note that a smaller volume of extract is used,) then the amount of P.A.L.-protein also increases (there is an increase in the volume of antiserum required to precipitate the P.A.L.-activity completely from the extract sample). The activity in extracts incubated in white light is noticeably higher than extracts from dark tissue, after 4h. The difference between the light-incubated extracts and the dark-incubated extracts in terms of the amount of antiserum required to precipitate all of the P.A.L.-activity, tends to fall in the extracts from older disc extracts (longer incubations), and is seen as a tendency for the immunotitration curves of the dark and light extracts to converge, until in the extracts from 42h-long incubations, there is no apparent difference in the amount of antiserum required to precipitate the P.A.L.-activity in dark or white light extracts, although there is a 2-fold difference in the level of P.A.L.-activity. The rather peculiar result with the t=0h. extracts was obtained using two separate extracts from freshly sliced discs, one of which had been prepared the day before (stored overnight at 4°C). The lower immunotitration curve was obtained using the stored sample, the P.A.L.-activity of this sample was lower than that of the extract freshly prepared for the experiments, and the amount of antiserum required was

also less. The upper immunotitration curve was obtained with a freshly prepared extract, as were all the other immunotitration curves. The overall shape of the t=0h. extracts differed from those containing the same amount of activity, in being less steep, indicating that more antiserum was required to precipitate the P.A.L. in these extracts. These results suggest that the changes in P.A.L.-activity are not due solely to an increase in the amount of P.A.L.-protein. The absolute amounts of P.A.L. protein in the extracts were determined from the immunotitration curves as plotted in Figure 3.26. The amount of antiserum required to precipitate 50% P.A.L.-activity was determined and from this the amount of P.A.L. protein was calculated, based on the results of immunotitration curves obtained with partially purified P.A.L. samples, S/U II (from step 9 of the purification procedure, see Section 3.2.11). These samples did not contain fully purified P.A.L., but the amount of P.A.L. protein was estimated following the final purification step (see Section 3.2.11B). From this the amount of antiserum required to precipitate 1µg. potato P.A.L. protein was found to be 10µl. anti-potato P.A.L. serum. However as mentioned earlier, the antiserum became less effective with storage, and as the experiments desrcibed above were carried out at a latter date than the immunotitration with partially purified P.A.L. it was necessary to adjust this figure. The calculation for the absolute amounts of P.A.L. protein in the extracts is based on a figure of 37µl. anti-potato P.A.L. serum/µg. potato P.A.L. protein. The results are given in Table 3.25, and plotted out in Figure 3.27. The interpretation of the results is based on the specific activity estimates. The specific activity remains fairly constant for extracts taken from potato tuber discs incubated for 2h. in darkness or white light, and up to 24h. in white light and 42h. in darkness. A constant specific activity indicates that as the P.A.L.-activity increases (or decreases), so does the amount of P.A.L. protein. This is most easily explained by de novo synthesis of the enzyme during the period of increasing P.A.L.-activity, and by an increase in the rate of removal (degradation) of the enzyme during the period of

Table 3.25

Immunotitration Experiments to measure P.A.L. levels in extracts from potato tuber discs.

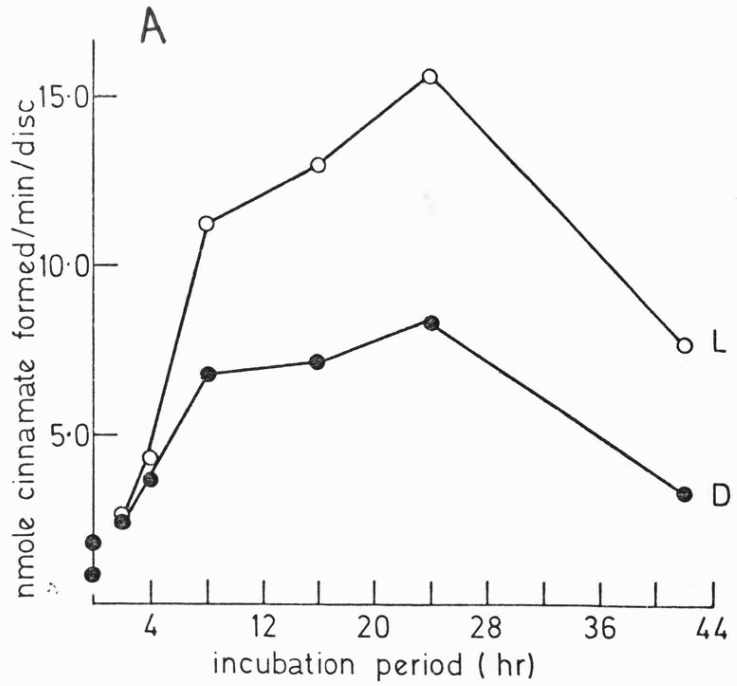
The data in this table are taken from the immunotitration experiment as described in Figures 3.25 and 3.26. From these graphs the amount of antiserum required to precipitate 50% activity was determined for each of the potato tuber extracts, and from this the quantity of P.A.L.protein present was calculated, using a value of 37 µl. serum/µg. P.A.L. protein with purified potato P.A.L. The specific activity of the P.A.L. in the extracts was determined.

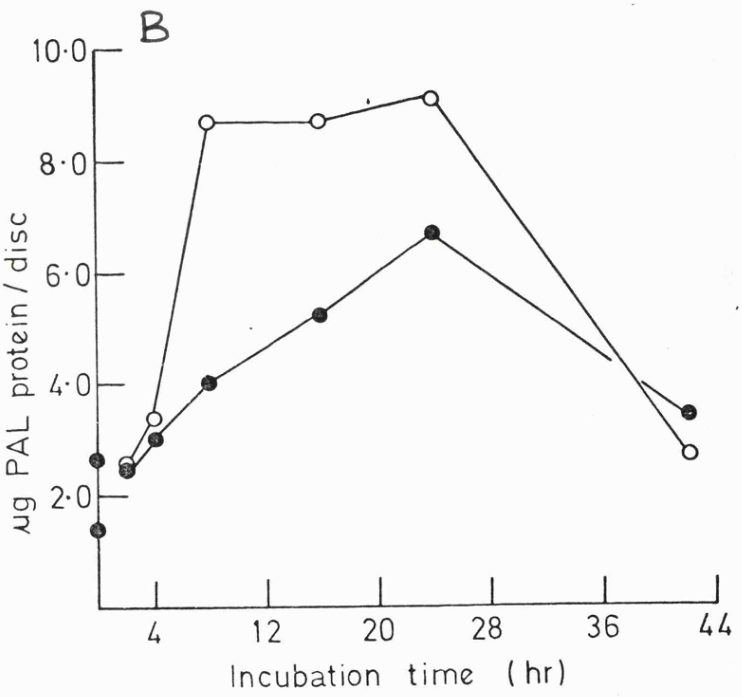
Incubation period (h.).	Light treatment.	Volume of antiserum required (µl.).	P.A.L.-protein (µg.).	Specific activity: P.A.L.-activity /P.A.L.-protein.
0	-	16.0	0.43	0.42
0	-	30.5	0.82	0.37
2	dark	17.5	0.47	0.73
2	light	18.5	0.50	0.70
4	dark	16.5	0.45	0.69
4	light	19.0	0.51	0.73
8	dark	15.0	0.40	0.91
8	light	32.0	0.87	0.67
16	dark	14.5	0.39	0.79
16	light	24.5	0.66	0.87
24	dark	18.5	0.50	0.70
24	light	25.0	0.68	0.91
42	dark	19.0	0.51	0.71
42	light	15.0	0.40	1.87

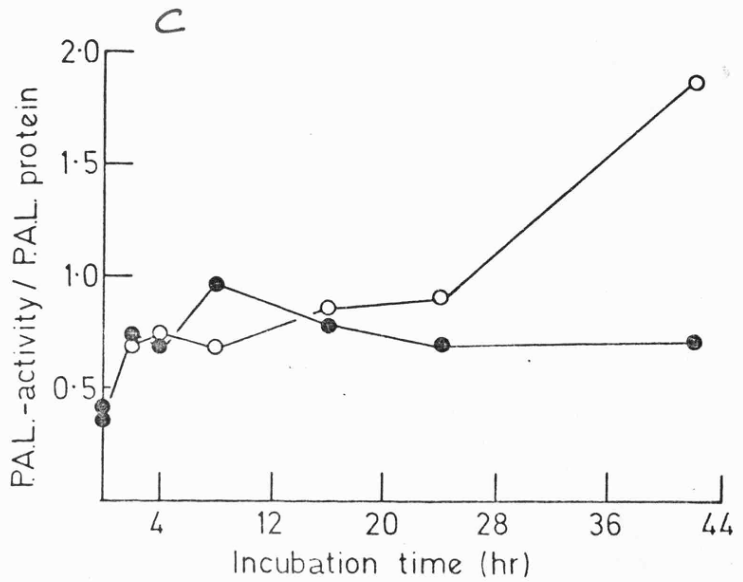
Figure 3.27

Immunotitration Experiments to measure P.A.L. levels in extracts from potato tuber discs.

The data in this figure are taken from the immunotitration experiment as described in Figures 3.25 and 3.26, and shows the P.A.L.-activity (A.), the P.A.L. protein (B.) and the specific activity of P.A.L. (C.) in the extracts. Closed symbols for dark-extracts and open symbols for light-extracts.


A
15·0
10·0
5·0
nmole cinnamate formed/min/disc
L
D
4
12
20
28
36
44
incubation period (hr)


B
10·0
8·0
6·0
4·0
2·0
µg PAL protein / disc
4
12
20
28
36
44
Incubation time (hr)


C
2·0
1·5
1·0
0·5
P.A.L.-activity / P.A.L. protein
4
12
20
28
36
44
Incubation time (hr)

decreasing P.A.L.-activity. The specific activity does not remain constant throughout the period of incubation in the presence of white light; there is an increase in specific activity between 24 and 42h. for those discs incubated in white light, accompanied by a large decrease in the amount of P.A.L. protein. In fact there is no difference in the amount of P.A.L. protein in discs incubated in darkness or in white light for 42h. although there is twice as much P.A.L.-activity in the light-incubated discs. This suggests that the activity of those enzyme molecules present in the light, is twice that of the enzyme molecules present in the dark. As the specific activity does not change between 2 and 42h. in the dark it is unlikely that there is a pool of inactive enzyme molecules present, and there is also a reasonably good correlation between the P.A.L.-activity (Figure 3.27A) and the P.A.L. protein (Figure 3.27B) over this period. There is also an increase in specific activity between 0 and 2h. after slicing (with incubation in darkness or in white light). In this case there is no decrease in P.A.L. protein during this period, so the increase in P.A.L.-activity must arise from those enzyme molecules already present. Therefore although the main increase in P.A.L.-activity in darkness and white light, can be explained by <u>de novo</u> synthesis of the enzyme, the initial increase (which occurs before activity from newly synthesised enzyme proteins would be available) and the relative increase in P.A.L.-activity in 42h-old light-incubated discs, appears to be due to some other mechanism, involving changes in the properties of the P.A.L. molecules--enabling twice as much enzyme activity to be expressed. Further experiments are obviously necessary, labelling experiments in conjunction with immunoprecipitation would allow rates of enzyme synthesis to be measured (or rates of enzyme turnover). Further studies on the properties of P.A.L. from freshly sliced tissue and older tissue (ie.42h-old or more) would also be valuable in explaining the apparent increases in enzyme activity in the absence of synthesis or activation. The possibility that there is a pool of inactive enzyme molecules has not been ruled out: a change in the proportions of inactive and active enzyme molecules could also explain the increases in

specific activity, but is unlikely ,as the specific activity is roughly the same, during the main increase in P.A.L.-activity in both dark- and light-incubated discs. The increase in P.A.L.-activity over that obtained in the dark appears to be due to a higher rate of de novo synthesis, occurring in the presence of white light.

B. In mustard seedlings: Double diffusion tests were carried out between mustard seedling extracts and anti-potato P.A.L. serum, to check whether the antiserum would cross-react with mustard P.A.L. Figure 3.28 shows the results: a precipitin line was obtained against both the dark (D) and far-red (FR) cotyledon extracts. A second, very faint precipitin line was also visible, as in the case with potato extracts, but is only seen at high antiserum volumes. Double-diffusion plates with mustard samples were not always successful, (this may be related to the decreasing effectiveness of the antiserum with storage) but when precipitin lines were obtained the intensity of the precipitin lines was the same for extracts from both dark- and far-red-treated tissue. There was no immunological difference between the P.A.L. from dark- or far-red-treated tissue (Figure 3.29); the precipitin lines were confluent. No precipitin lines were obtained against normal rabbit serum (N.R.S.). By carrying out extractions at pH 8.8 it was possible to perform immunoprecipitation experiments with mustard seedling extracts. Quantitative results were obtained, but only relative values for the amounts of mustard P.A.L. protein were determined as no standard (ie. purified mustard P.A.L.) was available. The results are presented in Figure 3.30: several dilutions of the cotyledon extract, from far-red-treated seedlings were used to check the specificity of the antiserum. At the three dilutions used, the amount of antiserum required to remove the P.A.L.-activity from the immunotitration supernatant was the same, ie. the decrease in P.A.L.-activity was proportional to the volume of antiserum used. Unfortunately it was not possible to recover any P.A.L.-activity in the immunoprecipitates, probably because the activities were low. In the case of the extracts of cotyledons from far-red treated seedlings, 4041.

Figure 3.28

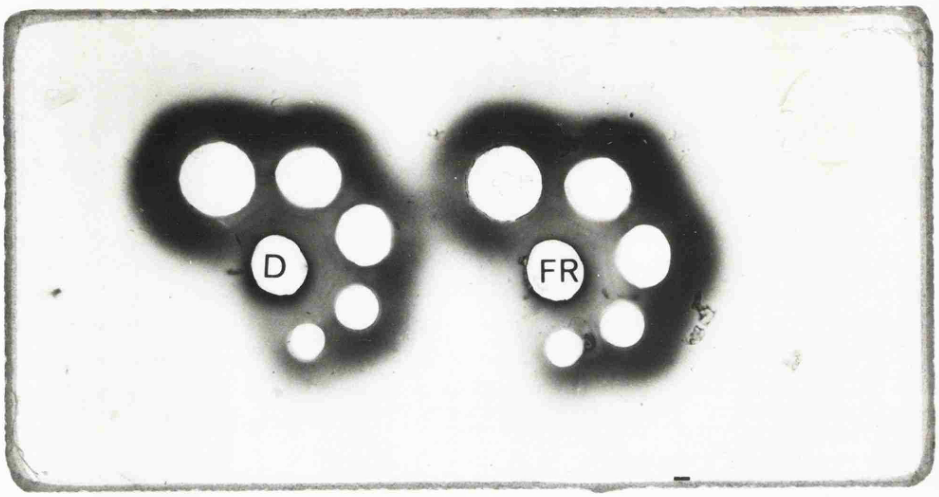
Double Diffusion Plates with extracts of mustard cotyledons.

The central wells contain 50µl. aliquots of crude supernatant extracts, prepared by extracting cotyledons from seedlings grown for 60h. in the dark (D) or 36h in dark + 24h. in far-red light (FR), in 75mM borate buffer, pH 8.8 and desalted in the same buffer. The outer wells contain aliquots (100,75,50,25 and 10µl.)of anti-potato P.A.L. serum (R1B; serum from rabbit 1, partially purified by ammonium sulphate fractionation), the highest volume being in the largest well, with decreasing volumes in a clockwise direction.

Figure 3.29

Double Diffusion Plates with extracts of mustard cotyledons.

The central wells contain aliquots of anti-potato P.A.L. serum, 80µl. on the left and 40µl. on the right. The surrounding wells contain extracts from seedlings grown in darkness (D) or given a far-red treatment (FR),(see above for details,)aliquots in these wells were all 80µl. but diluted with extraction buffer, undiluted at the top, a 2-fold dilution below, and a 4-fold dilution below this.


D
FR

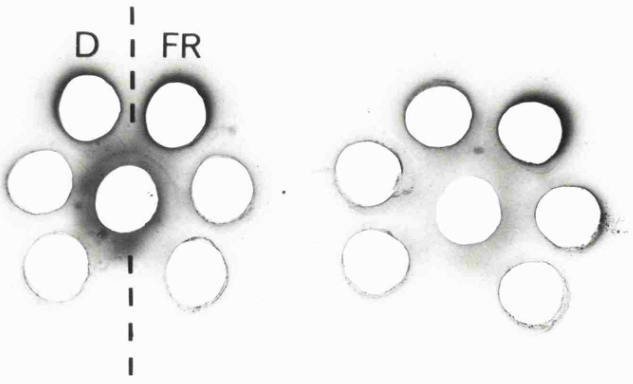
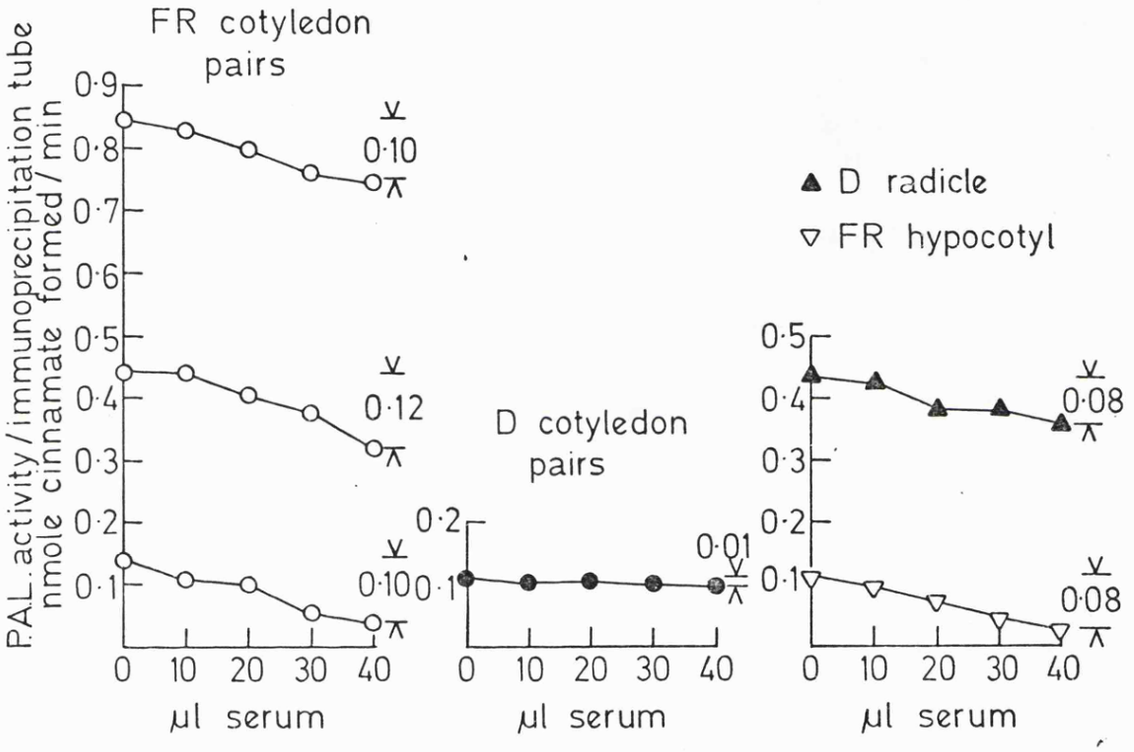

D
FR

Figure 3.30

Immunotitration Experiments to measure P.A.L. levels in extracts from mustard seedlings.

A range of extracts were prepared from mustard seedlings,(divided into cotyledon pairs, hypocotyl sections and radicles,)taken from seedlings grown either in continuous darkness for 60h. (D) or in darkness for 36h. followed by 24h. in far-red light (FR). The extracts were prepared as described in Section 3.1.9, and desalted in 50mM borate buffer, pH 8.8 (D-isoascorbate was added immediately after desalting to 10mM). Mixtures of the extracts and a range of volumes of anti-potato P.A.L. serum were incubated for 20h. at 4°C, then centrifuged and the P.A.L.-activity remaining in the supernatant assayed.


FR cotyledon pairs
P.A.L. activity / immunoprecipitation tube
nmole cinnamate formed / min
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·10
0·12
0·10
0 10 20 30 40
µl serum
D cotyledon pairs
0·2
0·1
0·01
0 10 20 30 40
µl serum
D radicle
FR hypocotyl
0·5
0·4
0·3
0·2
0·1
0·08
0·08
0 10 20 30 40
µl serum

anti-potato P.A.L. serum precipitated 0.10 nmole cinnamate formed/min., P.A.L.-activity. This compares with a value of 40µl. antiserum precipitating ~1.0 nmole cinnamate formed/min potato P.A.L.-activity (see Section 3.4.4.A). Immunotitration curves were also prepared for cotyledon extracts from dark-grown mustard seedlings, and for hypocotyl and radicle extracts (Figure 3.30). The dark cotyledon extract contained approximately the same amount of P.A.L-activity as the third dilution of the far-red cotyledon extract (with a similar total protein content to the undiluted far-red cotyledon extract), but the antiserum was much less effective in precipitating the P.A.L.-activity, by a factor of 1/10. With the hypocotyl and radicle extracts, the antiserum precipitated amounts of P.A.L.-activity comparable to those precipitated from the far-red cotyledon extracts. Table 3.26 shows the amount of P.A.L.-activity in each of the sample extracts used in the immunotitration experiments, and the amounts of anti-potato P.A.L. serum required to precipitate the P.A.L.-activity (amount of antiserum required to precipitate all the P.A.L.-activity in each sample was obtained by extrapolation of the immunotitration curves). There was little loss of P.A.L.-activity during the experiment, as the extracts were reasonably stable during this period. An estimate of specific activity of the P.A.L. was made by dividing the P.A.L.-activity by the amount of antiserum required to precipitate that amount of activity, for each extract; the amount of antiserum required being directly proportional to the amount of P.A.L. protein present. The specific activity for extracts of cotyledons from the dark-grown seedlings is $1/10^{th}$ that obtained with extracts of cotyledons from far-red-treated seedlings, there is also a similar difference in the measurable P.A.L.-activity. The most obvious conclusion to draw from this, is that there is a pool of inactive P.A.L. present in cotyledons of mustard seedlings, which is activated by treatment with far-red light. The values obtained with the dark radicle extract and the far-red hypocotyl extract indicate that there is little inactive P.A.L. in these tissues, ie. that most of the P.A.L. present is in an active form. This is in agreement with

Table 3.26

Immunotitration Experiments to measure P.A.L. levels in extracts from mustard seedlings.

The data in this table are taken from the immunotitration experiments described in Figure 3.30. From these graphs the amount of antiserum (anti-potato P.A.L. serum) required to precipitate the P.A.L.-activity from these extracts was determined, by extrapolation, and from this the amount of P.A.L. protein present has been estimated-the relative amounts of P.A.L. protein are given in terms of the amount of antiserum required. The specific activities have been estimated and are given as the ratio of P.A.L.-activity (present in the absence of antiserum at the end of the experiment, t=20h.) : volume of antiserum required. The P.A.L.-activity present in the sample extracts at the beginning of the experiment is also given.

Extract from- (sample volume used in µl.).	Light treatment.	P.A.L.-activity pmoles cinnamate formed/min. t=0h.	t=20h.	Volume of antiserum required(µl.)	Specific activity.
Cotyledon pair (400)	dark	128	110	440	0.25
Cotyledon pair (400)	far-red	920	845	340	2.5
Cotyledon pair (200)	—"—	460	445	150	3.0
Cotyledon pair (56)	—"—	130	140	55	2.5
Radicle (200)	dark	470	435	220	2.0
Hypocotyl (200)	far-red	120	110	55	2.0

the smaller changes in P.A.L.-activities in these sections of the mustard seedling, compared to the cotyledons, following a far-red light treatment. Further experiments are necessary to measure the amounts of P.A.L. protein present in all sections of the mustard seedling, during these early stages of development. A comparison of the effects of growing seedlings in the dark, far-red light and 'normal sunlight' would be extremely interesting, for photomorphogenetic studies. The evidence from both double diffusion tests and preliminary immunotitration experiments suggests that in cotyledons the amount of P.A.L. protein present in dark-grown seedlings is sufficient to account for the increase in P.A.L.-activity during far-red light treatment. The main limitation of the immunotitration method for measuring P.A.L. levels, is the need to measure the actual enzyme activity, a technique based solely on measuring the degree of immunochemical reaction, such as rocket immunoelectrophoresis or radioimmunoassay would provide more convincing results.

C. In gherkin seedlings: Preliminary experiments were also carried out with gherkin hypocotyl extracts. Double diffusion tests between these extracts and anti-potato P.A.L. serum, showed that gherkin P.A.L. also cross-reacted (Figure 3.31). Extracts from 3 day-old dark-grown gherkin hypocotyls, gave a precipitin line which was confluent with those from extracts of hypocotyls from slightly older seedlings, either left in continuous darkness for 5h. or placed in white light for 3 or 5h.. Only a single precipitin line was obtained, this may be because these extracts contain less protein (than those from potato or mustard cotyledons), and therefore less chance of the extract containing 'contaminating' proteins, which may have been responsible for the two precipitin lines obtained with other extracts (potato and mustard cotyledon). Although the P.A.L.-activity increases 3-4-fold after a 3h.-white light treatment, there was no increase in the intensity of the precipitin line obtained. The results of immunotitration experiments are given in Figure 3.32. The actual P.A.L.-activity is given in Figure 3.32D. Figures 3.32A and B. show the immuno-

Figure 3.31

Double Diffusion Plates with extracts of gherkin hypocotyls.

The central wells contain 40µl. aliquots of serum: anti-potato P.A.L. serum, R1C2 (serum from rabbit 1, purified by D.E.A.E.-cellulose, run 2) on the left, and R1A (serum from rabbit 1, unpurified) in the middle and normal rabbit serum, N.R.S. on the right. Thesurrounding wells contain 100µl. aliquots of gherkin hypocotyl extracts, from 3 day-old seedlings (D,0h.) which had then received a 3h. (L,3h.) or 5h. (L,5h.) white light treatment or remained in darkness for a further 5h. (D,5h.). The extractions were carried out in 100mM Na-phosphate buffer, pH 7.2 containing 0.5mM glutathione.

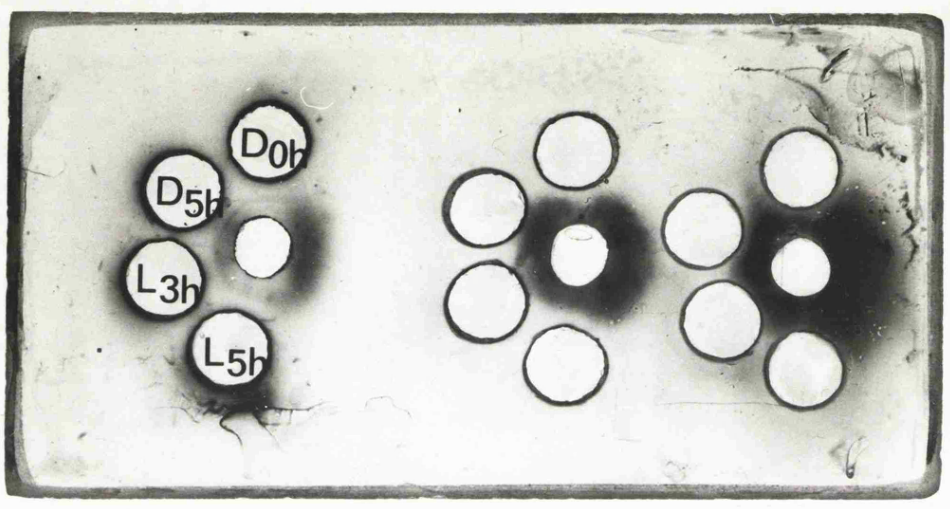
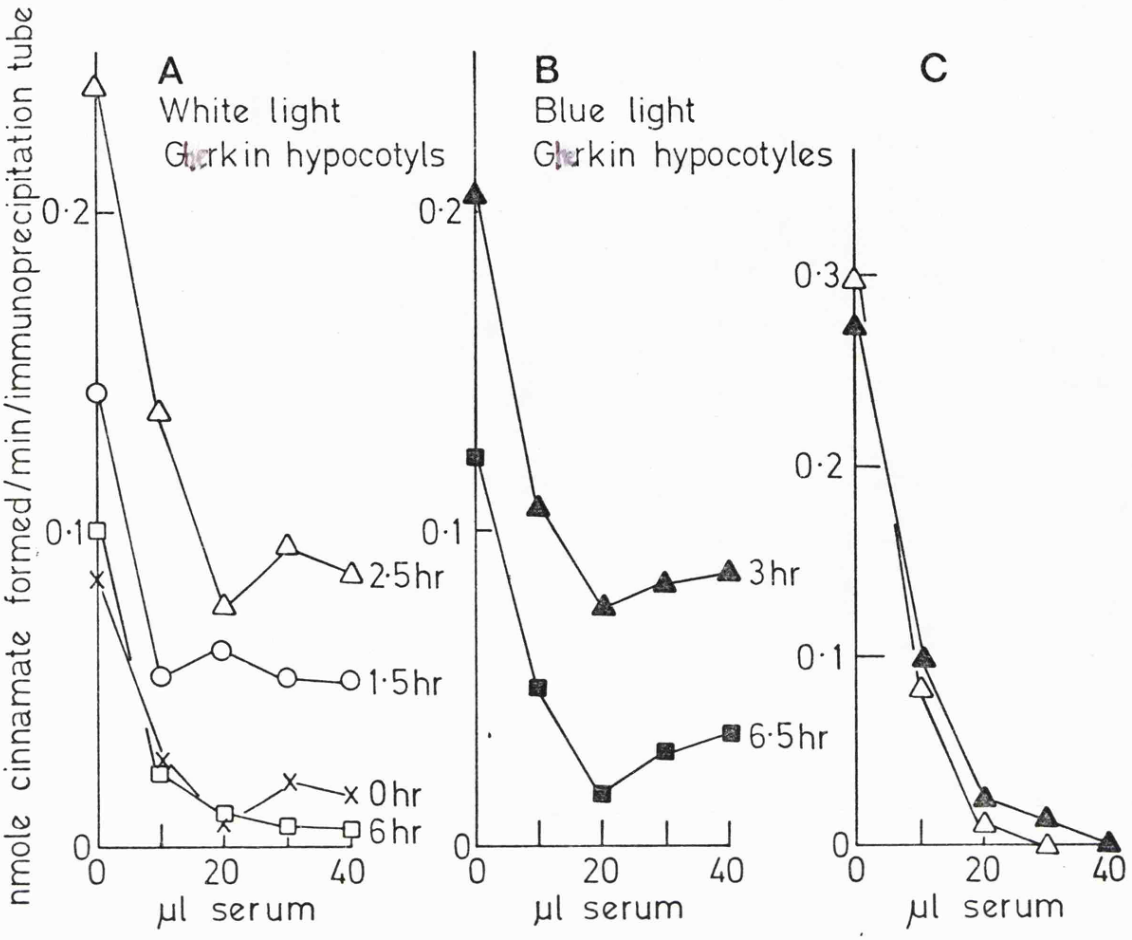

D0h
D5h
L3h
L5h

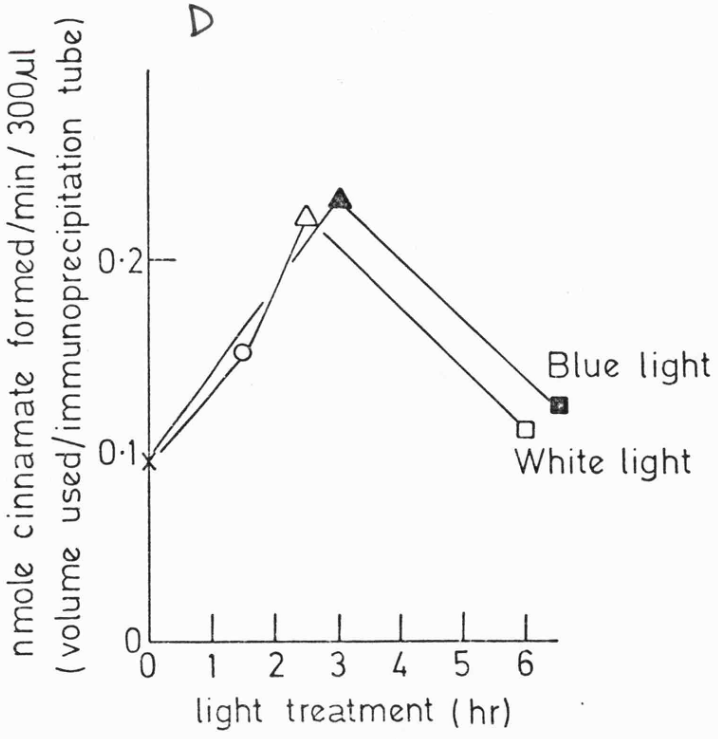
Figure 3.32

Immunotitration Experiment to measure P.A.L. levels in extracts from gherkin hypocotyls.

A range of extracts were prepared from gherkin hypocotyls, taken from 3 day-old seedlings, which had been given a white light treatment (0,1.5, 2.5 or 6h.) or a blue light treatment (3 or 6.5h.). The extracts were prepared as described in Section 3.1.9, and desalted in 50mM borate buffer, pH 8.8 (D-isoascorbate was added immediately after desalting to 10mM). Mixtures of the extracts and a range of volumes of anti-potato P.A.L. serum were incubated for 14h. then 100µl. anti-rabbit whole serum, from goats was added (Figures A. and B.) or 100µl. buffer added (Figure C.) and the mixtures incubated for a further 6h. (all incubations at 4°C) The mixtures were then centrifuged and the supernatants assayed for P.A.L.-activity.

Also shown are the amounts of P.A.L.-activity in the extracts (after desalting) as used in the immunotitration experiments (Figure D.).


A
White light
Gherkin hypocotyls
B
Blue light
Gherkin hypocotyles
C
nmole cinnamate formed/min/immunoprecipitation tube
0·2
0·1
0
0·3
2·5hr
1·5hr
0hr
6hr
3hr
6·5hr
0
20
40
µl serum


D
nmole cinnamate formed/min/ 300µl
(volume used/immunoprecipitation tube)
0·2
0·1
0
Blue light
White light
0
1
2
3
4
5
6
light treatment (hr)

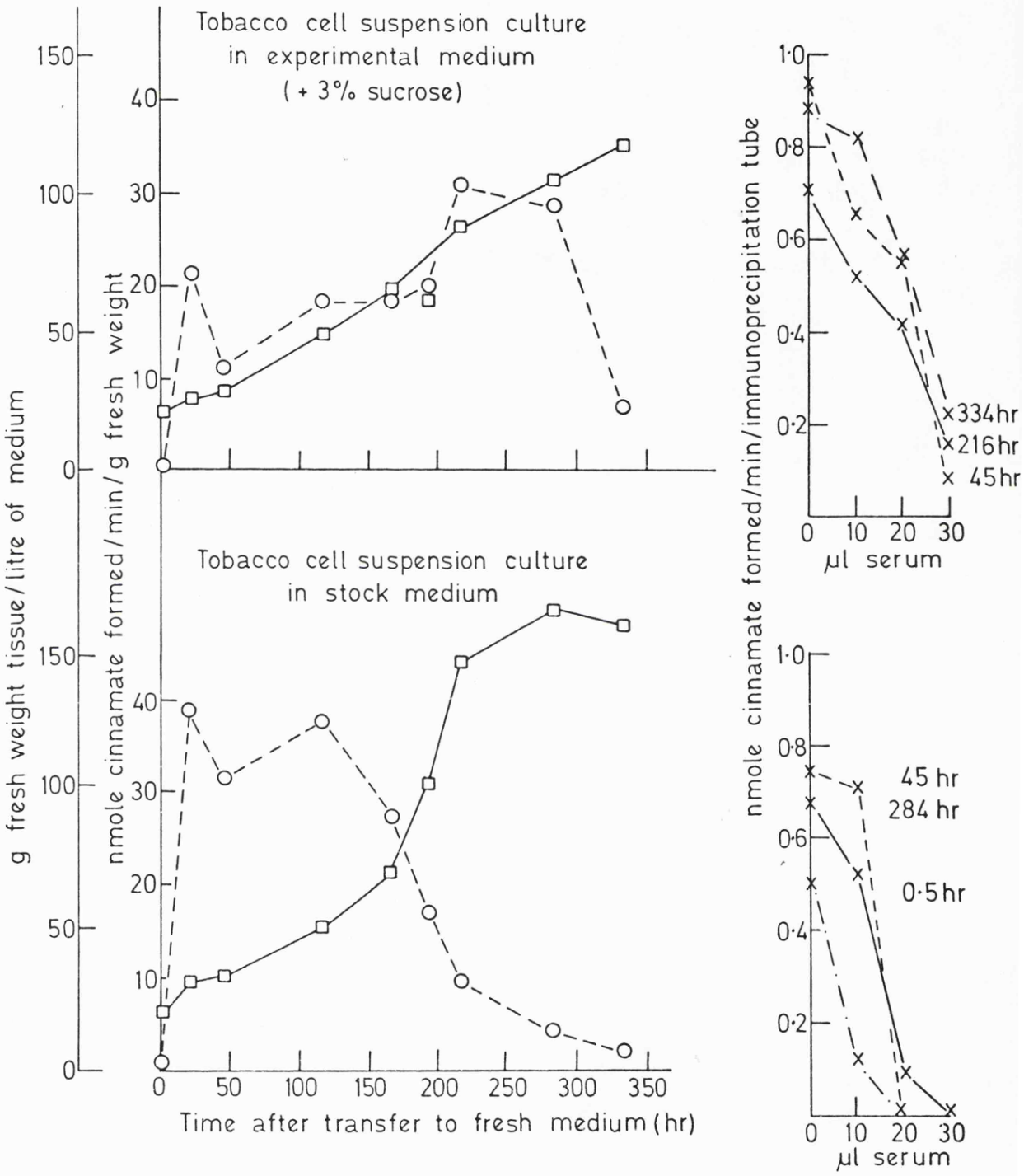
titration curves with hypocotyl extracts from seedlings given a white, or a blue light treatment, but a second antibody (anti-rabbit whole serum, from goats) was added after the normal incubation period for the immunoprecipitation in order to ensure complete immunoprecipitation took place, the immunotitration curve in Figure 3.32C was obtained in the absence of second antibody. In the presence of second antibody, at the higher volumes of anti-potato P.A.L. serum, P.A.L.-activity was not completely precipitated, this result was unexpected and appears to indicate that second antibody released enzyme activity. The reason for this is unclear, but is possibly an indication of the poor binding between gherkin P.A.L. and anti-potato P.A.L. serum. Ignoring this effect, the results suggest that there is a reasonable correlation between the amount of P.A.L.-activity in the extracts and the amount of antiserum required to precipitate that activity; the immuntitration curves are roughly parallel to each other. This may be taken as evidence for the increase in P.A.L.-activity in gherkin hypocotyls being due to an increase in P.A.L. protein, ie. that the increase in P.A.L.-activity arises via de novo synthesis of new enzyme molecules, and the decrease in P.A.L.-activity, being due to a decrease in P.A.L. protein; but again futher experiments are required before drawing any conclusions. It would be interesting to compare the amounts of P.A.L. protein present in the hypocotyls and in the cotyledons, as there was no decrease in P.A.L.-activity in the cotyledons over the same period (see Section 3.1.9B.).

D. In tobacco cell suspension cultures: Following the transfer of tobacco cells to fresh medium, there was a dramatic increase in P.A.L.-activity. Tissue culture techniques provide relatively homogeneous tissue with which to work, and the growth rate and enzyme levels may be manipulated by varying the growth substance concentrations. Figure 3.33 shows the time courses for changes in P.A.L.-activity in two different media. In the experimental medium there were two distinct peaks in P.A.L.-activity, the first within 1 day of transfer, and the second 8-10 days after transfer to the medium. In the stock medium, there was only one peak of P.A.L.-activity, which

Figure 3.33

Immunotitration Experiments to measure P.A.L. levels in extracts from tobacco cell suspension cultures.

A range of extracts were prepared from samples taken from tobacco cell suspension cultures. The increase in fresh weight (□) and the P.A.L.-activity (o) in two culture mediums is shown, the main difference in the media was a 10-fold higher concentration of auxin and cytokinin in the stock medium. For details of the extraction and culture technique, see Sections 2.1.6. and 2.4.1). Mixtures of the extracts (taken from the cultures at the times indicated) and a range of volumes of anti-potato P.A.L. serum were incubated for 20h. at 4°C, then centrifuged and the P.A.L.-activity (×) in the supernatants measured. The immunotitration curves for extracts from the two cultures are placed alongside the culture from which they were taken.


Tobacco cell suspension culture
in experimental medium
(+ 3% sucrose)
Tobacco cell suspension culture
in stock medium
g fresh weight tissue / litre of medium
nmole cinnamate formed / min / g fresh weight
Time after transfer to fresh medium (hr)
nmole cinnamate formed/min/immunoprecipitation tube
×334hr
×216hr
× 45hr
µl serum
45 hr
284 hr
0·5hr
µl serum

lasted from day 1 to day 5, before declining. The pattern of P.A.L.-activity was altered by the auxin and cytokinin levels, which were 10-fold higher in the stock medium. Double diffusion tests between a salt-concentrated preparation of tobacco P.A.L. and the anti-potato P.A.L. serum gave a single precipitin line, showing that tobacco P.A.L. cross-reacted. Immunotitration experiments were then carried out, and the immunotitration curves are given alongside the time-course plots in Figure 3.33. The P.A.L. samples were diluted with the extraction buffer, to give roughly equal amounts of P.A.L.-activity in the sample volumes used for immunotitration. The amount of anti-potato P.A.L. serum required to precipitate the P.A.L.-activity appears to be similar in each case. The data obtained from these plots was tabulated (Table 3.27), in a manner similar to the results of the mustard immunotitration experiments: the ratio of P.A.L.-activity : volume of antiserum required, being used as an indication of the specific activity. In the stock medium, the specific activity remains fairly constant throughout. This suggests that the changes in P.A.L.-activity are due to changes in P.A.L. protein levels. In the experimental medium a similar specific activity is obtained for P.A.L. after the initial peak of P.A.L.-activity, suggesting again that the amount of P.A.L. protein changes, ie. that de novo synthesis and then degradation can account for the changes in P.A.L.-activity. The second peak of P.A.L.-activity is accompanied by a lower specific activity; this indicates that there is less P.A.L.-activity than expected for the amount of P.A.L. protein present. From this it is possible to infer that there is a pool of inactive enzyme molecules present at this stage, and this could be due to a difference in the stability of the P.A.L. in these extracts. It should be pointed out that the extracts had been stored before the immunotitration experiments were carried out. These results require confirmation using freshly prepared samples, but serve as a basis for further experiments, which may provide evidence for the role of growth substances in regulating the rate of inactivation and subsequent degradation, if indeed such a mechanism is found

Table 3.27

Immunotitration Experiments to measure P.A.L. levels in extracts from tobacco cell suspension cultures.

The data in this table are taken from the immunotitration experiments described in Figure 3.33. From these graphs the amount of antiserum (anti-potato P.A.L. serum) required to precipitate the P.A.L.-activity from the extracts was determined. From this the amounts of P.A.L. protein present were estimated-the relative amounts of P.A.L. protein are given in terms of the volume of antiserum required. The specific activit es have been estimated, and are given as the ratio of P.A.L.-activity (present in the absence of antiserum at the end of the experiment) : volume of antiserum required.

Extract from cells in culture medium for-	Sample volume used (µl.)	P.A.L.-activity nmoles cinnamate formed/min.	Volume of antiserum required(ml.)	Specific activity
Experimental medium:				
45h.	115	0.94	0.034	27.6
216h.	45	0.71	0.040	17.8
334h.	300	0.87	0.042	20.7
Stock medium:				
0.5h.	200	0.49	0.015	32.7
45h.	21	0.74	0.027	27.4
284h.	183	0.67	0.020	33.5

to exist. This mechanism could be used to explain the present results: in the stock medium there is no delay between inactivation of enzyme and degradation, and so the specific activity remains unchanged; in the experimental medium there is a delay. If the inactivation process is reversible this pool of inactive enzyme molecules could be responsible for the second increase in P.A.L.-activity. As yet this explanation remains purely speculative. The initial increase in P.A.L.-activity is however, probably due to an increase in the number of enzyme molecules, due to de novo synthesis of the enzyme.

E. Rhodotorula P.A.L.: Double diffusion tests were carried out between samples of Rhodotorula P.A.L. (available commercially, Sigma Chemical Ltd.) and the anti-potato P.A.L. serum, R1B (serum from rabbit 1, partially purified by ammonium sulphate fractionation), but no precipitin lines were obtained. The measurable P.A.L.-activity in the samples was comparable to that of purified potato P.A.L. samples (Section 3.4.4 A.). Immunotitration experiments were also tried, but there was no inhibition or precipitation of P.A.L.-activity from Rhodotorula P.A.L. samples, identical results were obtained with normal rabbit serum (N.R. S.). These results show that there was no cross-reaction of the anti-potato P.A.L. serum with Rhodotorula P.A.L..

3.4.5 Summary: The results presented in this section, show that anti-potato P.A.L. serum was raised and successfully used to measure P.A.L. levels in extracts from potato tuber discs. The changes in P.A.L.-activity were largely due to changes in the amount of P.A.L. protein present, although an initial increase in P.A.L.-activity and a higher level of P.A.L.-activity in older discs incubated in white light, could not be explained in these terms. The properties of each P.A.L. molecule appeared to change during these periods. The degree of cross-reaction of the anti-potato P.A.L. serum with P.A.L. from other plants was dependent on the source of P.A.L..An equal amount of tobacco P.A.L.-activity was precipitated by the antiserum (compared to potato P.A.L.-activity). In cell suspension cultures of this tissue, the

changes in P.A.L.-activity were also due to changes in the amount of P.A.L.-protein, but the results with cultures at lower auxin and cytokinin concentrations, suggested that a reversible inactivation/activation mechanism developed after several days in the culture medium. The cross-reaction of the anti-potato P.A.L. serum with mustard and gherkin extracts was poor, especially after storing the antiserum for several months, this meant that larger quantities of antiserum were required to measure the P.A.L. levels in these tissues. Insufficient quantities of antiserum had been raised to meet this requirement, so only a few experiments were possible,(without raising more antiserum.)In mustard cotyledons the results suggested that unlike other tissues examined, the changes in P.A.L.-activity were not accompanied by changes in the amount of immunologically detectable P.A.L. protein; inactive P.A.L. protein may have been present in these tissues when grown in the dark.

However there is obviously great potential for the use of immunological techniques in measuring enzyme levels in plants. There are several methods available for using specific antibodies to measure enzyme levels which do not rely on the measurement of the enzyme activity, as does the immunotitration method used here, which would provide valuable results. A combination of radio-isotopic labelling and immunological methods would provide even more rewarding results.

SECTION 4 RESULTS: A.A.O.

4.1 PROPERTIES OF A.A.O.

4.1.1 Introduction: A.A.O. was available commercially, and therefore was readily available in sufficient quantities for attempts to raise antibodies, and to develop a suitable method for labelling the enzyme, before proceeding to develop a radioimmunoassay technique for this enzyme. The source of commercially available, purified A.A.O. was given as Cucurbita sp., most probably the yellow crook-neck squash, Cucurbita pepo condensa, or the green summer squash, Cucurbita pepo medullosa, from which this enzyme has previously been purified (Dunn and Dawson,1951; Tokuyama et al.1965; Marchesini and Kroneck,1979). A.A.O. has also been purified from cucumber, Cucumis sativa (Nakamura et al. 1968). The plant of particular interest with respect to measuring A.A.O. levels was however, mustard, Sinapis alba. It was therefore desirable to know whether antiserum raised against Cucurbita A.A.O. would cross-react with A.A.O. from other plant species. Before proceeding with the immunological experiments then, a few properties of the Cucurbita and mustard A.A.O. were compared.

4.1.2 Molecular Weights: Cucurbita A.A.O. has molecular weight of approx.140,000 (Strothkamp and Dawson,1974). A similar result was obtained using Cucurbita A.A.O. and the calibrated Sephadex G-150 column (Figure 4.1), the peak of P.A.L.-activity eluted, corresponds to a MW of 150,000 (using the MW calibration curve in Section 2.8.2). The molecular weight of mustard A.A.O. was slightly less, but hardly distinguishable from that of Cucurbita A.A.O. on this size column. Both A.A.O. from dark-grown and far-red-treated mustard cotyledons eluted from the column in a similar elution volume, corresponding to a MW of 140,000 (Figures 4.2A and B).

4.1.3 pH Optima: The pH optima of Cucurbita A.A.O. and of mustard A.A.O. from a far-red-treated cotyledon extract, were determined. The results are shown in Figures 4.3A and B., the pH optimum of the Cucurbita A.A.O. was slightly higher ,at pH 6.0, than that of mustard A.A.O., which was between pH 5.0 and 5.5. (A comparison of the pH optima for Cucurbita and mustard

Figure 4.1

Elution profile of Cucurbita A.A.O. on the Sephadex G-150 column.

A sample of Cucurbita A.A.O. was dissolved in 50mM Na-phosphate buffer, pH 7.0 (2.0mg./ml.) and was then passed through a Sephadex G-150 column (1.25cm. diameter x 21.0cm. height) in the same buffer (see Section 2.8.2 for calibration). The protein content, estimated from the absorbance at 280nm.(×) and the A.A.O.-activity (○) of the eluted fractions were measured.

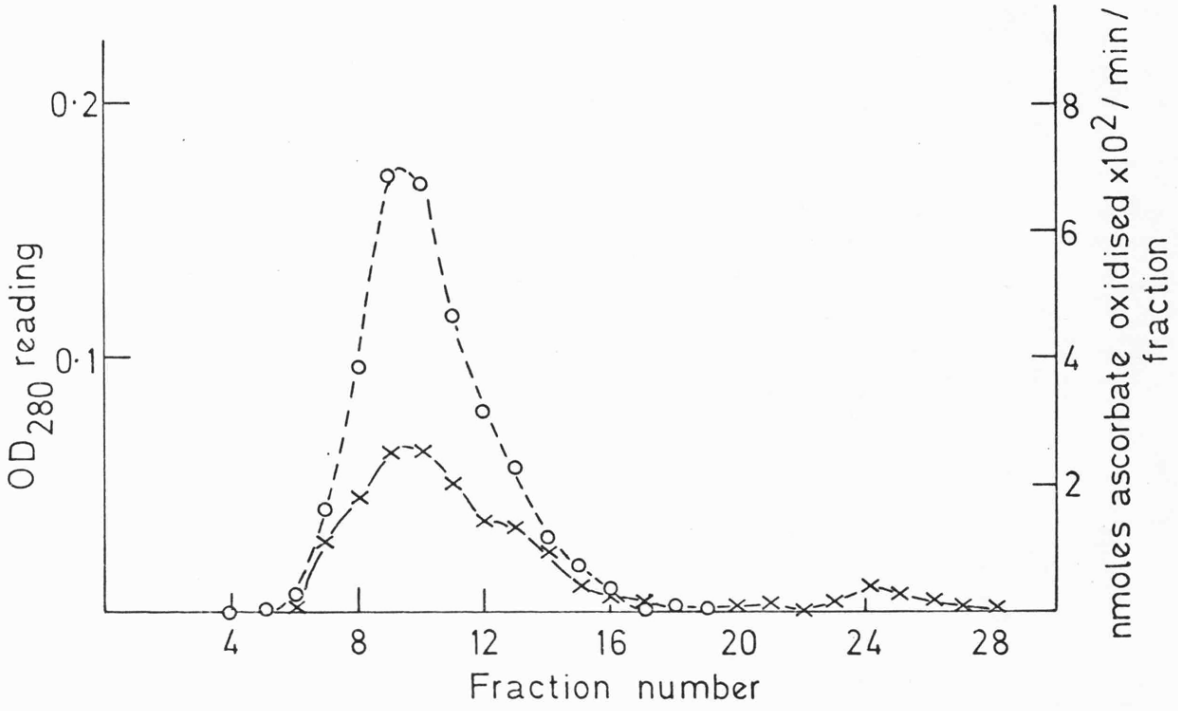

0·2
0·1
OD_{280} reading
4
8
12
16
20
24
28
Fraction number
8
6
4
2
nmoles ascorbate oxidised x10^2/ min/ fraction

Figure 4.2

Elution profile of mustard A.A.O. on the Sephadex G-150 column.

Crude supernatant extracts of cotyledons were prepared from mustard seedlings which had been grown in continuous dark (60h. dark) or in far-red light (36h. dark + 24h. far-red), in 100mM citrate -phosphate buffer, pH 5.0. Samples of the extracts, dark-grown (A.) or far-red-treated (B.) were passed through a Sephadex G-150 column (1.25cm. diameter x 21.0cm. height) in 50mM Na-phosphate buffer, pH 7.0 (see Section 2.8.2 for calibration). The protein content, estimated from the absorbance at 280nm. (×) and the A.A.O.-activity (o) of the eluted fractions were measured.

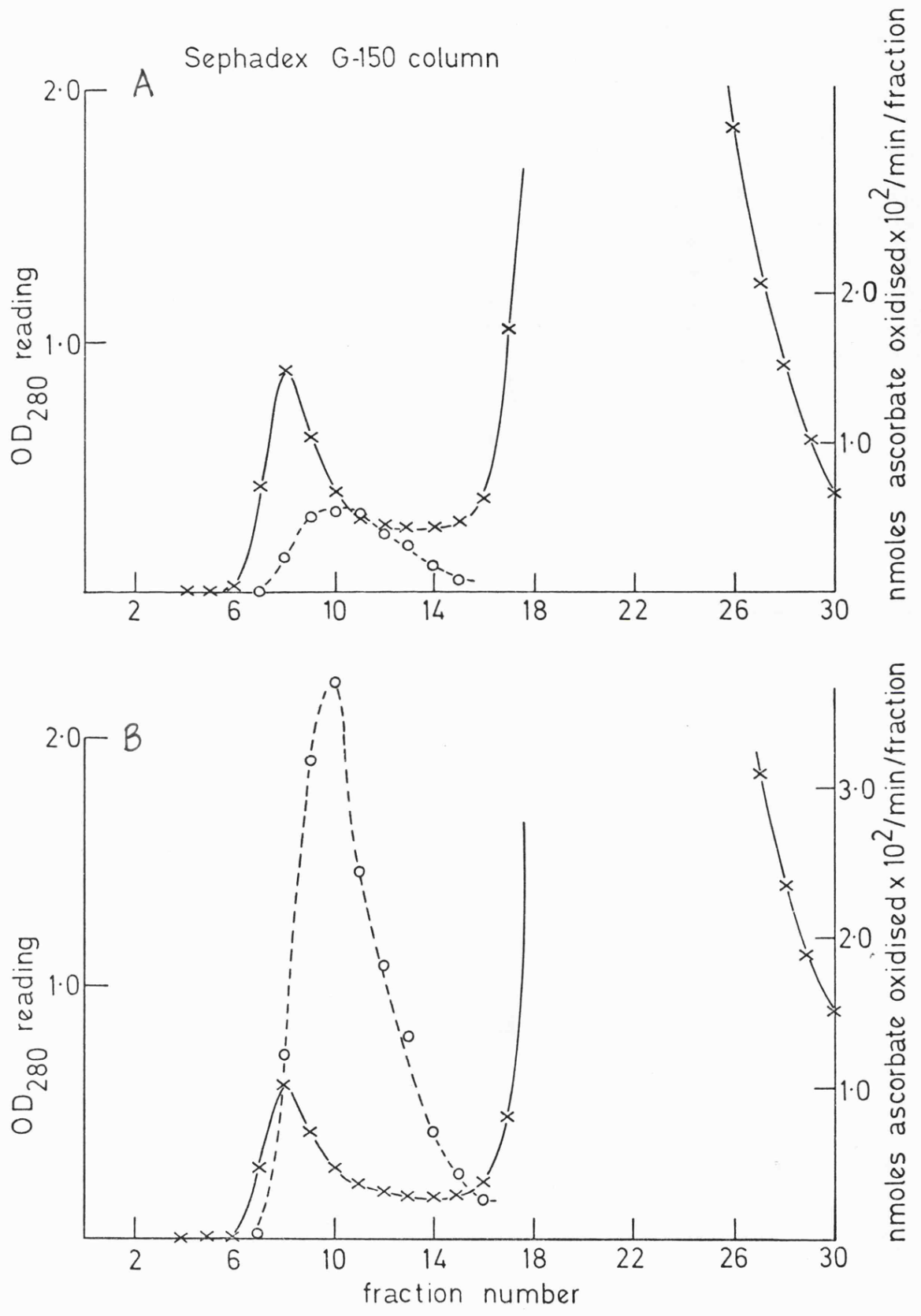
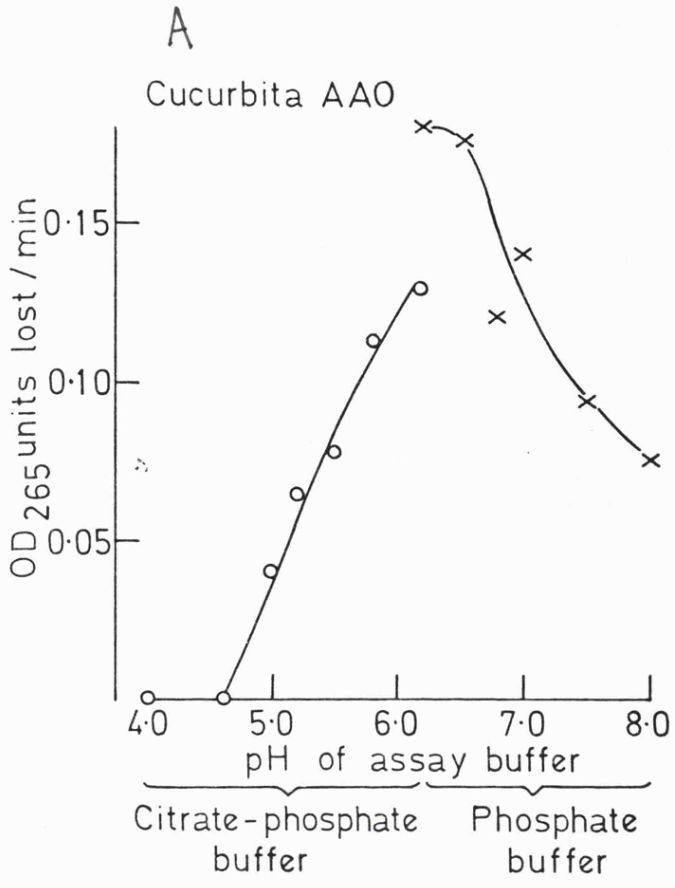
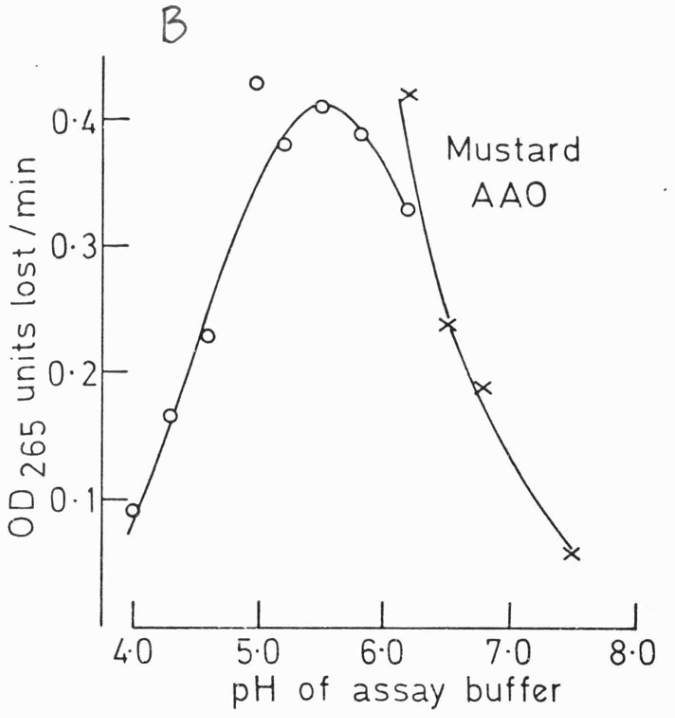

Sephadex G-150 column
A
B
2·0
1·0
OD_{280} reading
nmoles ascorbate oxidised x 10^2/min/fraction
2·0
1·0
3·0
2·0
1·0
2
6
10
14
18
22
26
30
fraction number

Figure 4.3

pH Optima curves for Cucurbita and mustard A.A.O..

A sample of Cucurbita A.A.O. was dissolved in 50mM Na-phosphate buffer, pH 7.0 (1mg./ml.). A crude supernatant extract of cotyledons from far-red-treated mustard seedlings (36h.dark + 24h.far-red) was prepared in 50mM citrate-phosphate buffer, pH 5.0 and concentrated by ammonium sulphate fractionation(see Section 2.8.1). The extract was desalted on a Sephadex G-25 column in 50mM Na-phosphate buffer. The enzyme assay was carried out as described in Section 2.5.5, using a 25µl. aliquot of Cucurbita A.A.O. (A.) or 20µl. aliquot of mustard A.A.O. (B.), in an assay volume of 2.5ml. using 100mM buffers, as indicated in the figure to obtain the desired pH.


A
Cucurbita AAO
OD 265 units lost / min
0·15
0·10
0·05
4·0
5·0
6·0
7·0
8·0
pH of assay buffer
Citrate-phosphate buffer
Phosphate buffer


B
OD 265 units lost / min
0·4
0·3
0·2
0·1
Mustard AAO
4·0
5·0
6·0
7·0
8·0
pH of assay buffer

by Moller and Van Poucke,1970, showed a slightly greater difference, with a similar value for mustard, but a higher value for Cucurbita A.A.O., of pH 7.0).

4.1.4 Summary: This brief investigation of the properties of A.A.O. from Cucurbita and mustard, indicates that the two enzymes are not identical. The results obtained for mustard may be partly due to the use of relatively crude supernatant extracts for the investigations, compared to the use of a purified sample of Cucurbita A.A.O.. Immunological tests were required next, to see whether anti-Cucurbita A.A.O. serum would cross-react with mustard A.A.O.

4.2 IMMUNOLOGICAL STUDIES

4.2.1 Introduction: Cucurbita A.A.O. was used to raise antibodies, but first the purity of the commercial preparation was checked. No detectable protein contaminants were present on polyacrylamide-S.D.S.-slab gels, following electrophoresis and staining of the gel for protein with Coomassie blue. A single protein band was visible, migrating on the gel with a MW of 65,-68,000. Minor contaminants were detectable in Cucurbita A.A.O. samples which had been stored in solution (J.Newbury, personal communication). These were probably degradation products of the enzyme; the stability of the Cucurbita A.A.O. was considerably reduced when the enzyme was kept in solution. Under non-denaturing conditions of electrophoresis, Cucurbita A.A.O. again migrated as a single protein band, and the position of A.A.O. activity on an identical gel was concomitant with this protein band. The purity of the enzyme preparation was therefore established.

4.2.2 Preparation of Antiserum: Antibodies were raised in rabbits, as described in Section 2.10. The immunization schedule allowed serum to be collected over a period of a couple of years, provided a booster injection of the antigen was given a month before taking the serum. A choice of bleeds were available, and in order to optimize the assay, the one giving the highest specificity and binding ability was desired. Purification of the antiserum was not considered necessary, as only very low dilutions

of the antiserum were required in the assay, and normal rabbit serum (N.R.S.) was used as a control. Antiserum was stored in a deep freeze in 100µl. aliquots, and was only thawed and diluted immediately before carrying out the assays.

4.2.3 Simple Diffusion in Tubes: For preliminary qualitative tests on trial bleeds of serum, simple diffusion in tubes was used. This method normally provides results within several hours. Table 4.1 shows the results of tests using serum from the first set of trial bleeds following immunization. All three rabbits used, gave a definite antibody reaction against a sample of Cucurbita A.A.O., with serum from the second or third bleed. If the tubes were incubated for more than a few hours, the results were obscured, control tubes also began to show precipitation or cloudiness at the interface between antiserum and enzyme sample, but no distinct precipitin arcs were formed with the control (N.R.S.). The technique was useful here as a quick test, but otherwise it's use is limited, especially where either the serum or antigen solutions themselves are cloudy (as would be the case if crude supernatant extracts of mustard cotyledons were used,)and requires rather large volumes of serum (0.5-1.0ml.) for each tube.

4.2.4 Double Diffusion Tests: The double diffusion method has been used to provide quantitative results (Ouchterlony, 1949; Mancini,1965). Two methods can be used; in one,serial dilutions of the antigen solution are placed in wells, equidistant from a central well, containing the antiserum (or vice versa), and the lowest dilution giving a precipitin line, provides a measure of the antigen concentration (or antibody titre). In the other method the agar gel contains antiserum, the radius of the precipitin ring surrounding the antigen well, is proportional to the concentration of antigen. To avoid using large volumes of antiserum, the first method described was used. A single precipitin line was obtained between the antiserum and Cucurbita A.A.O.. The A.A.O. solution used (100µg./100µl.) also gave a precipitin line at 5-fold dilution, but not at a 10-fold dilution (see

Table 4.1

Simple Diffusion in Tubes: with Cucurbita A.A.O..

Test-tubes pre-coated with a layer of agar, were prepared with a layer of agar-serum, using serum from a range of bleeds from the immunized rabbits and from a non-immunized rabbit(N.R.S.). On top of the set agar-serum was placed a sample of Cucurbita A.A.O. (100µg./4.0ml. buffer. The tubes were sealed and left for 3-4h. at room temperature, the tubes were then examined, and any reaction at the interface between the two layers was reported.(For full details of the procedure see Section 2.12.1)

Rabbit	Bleed number	Reaction vs. saline	Reaction vs. antigen	Antibodies present.
1	1	clear	faint arc	?
	2	cloudy	distinct arc	+
	3	—"—	———"———	+
	4	—"—	———"———	+
2	1	clear	cloudy	-
	2	cloudy	faint arc	?
	3	—"—	distinct arc	+
3	1	cloudy	cloudy	-
	2	—"—	faint arc	?
	3	—"—	distinct arc	+
N.R.S.		clear	cloudy	-

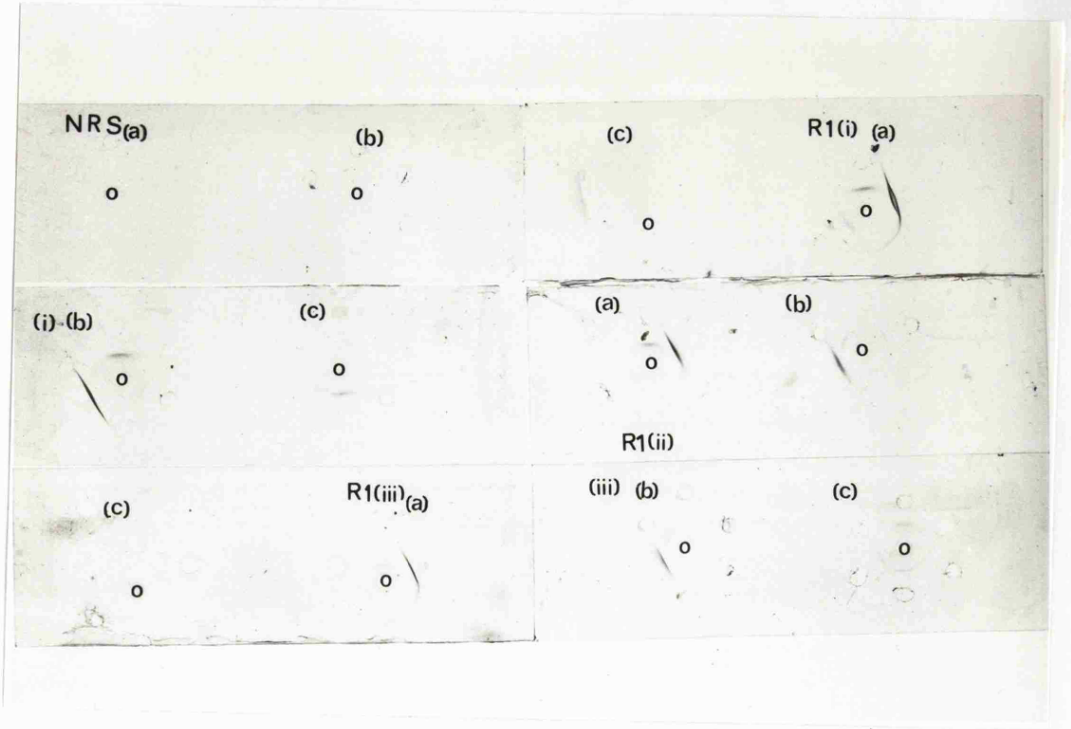
Figure 4.4). Also shown are double diffusion tests with extracts from mustard seedlings. A single precipitin line was obtained using these extracts, the preciptin lines tended to overlap between neighbouring wells. Confluence of the precipitin lines, as between undiluted and 5-fold diluted Cucurbita A.A.O. (Figure 4.4, R1, bleed(i)(a)) and between neighbouring mustard extracts (Figure 4.4, eg. R1, bleed(iii)(c)), indicates complete immunological identity. 'Spurring' of the precipitin lines, as suggested on several of the plates(Figure 4.4, eg. R1(i)(b) and R1(ii)(a) and (b)) is an indication of only partial immunological identity, in these cases, between mustard and Cucurbita A.A.O.. Spurious results can be obtained if the wells are filled twice, as in the case of the undiluted Cucurbita A.A.O. sample in Figure 4.4, R1(i)(a), where a double or split precipitin line was obtained: the ends of the precipitin line join indicating identity of the two lines in the middle. The Cucurbita A.A.O.and mustard A.A.O. are therefore recognized as distinct proteins immunologically, with some degree of cross-reaction (ie. with only some antigenic determinants on the two enzymes being the same).

4.2.5 Specificity of the Antiserum: Immunoelectrophoresis was attempted to check the specificity of the antibody reaction, as detected by the double-diffusion method. Conditions for electrophoresis under which the mustard A.A.O. would migrate from the origin, and still retain enzyme activity were not obtained. Therefore following electrophoresis (at pH7.5) the A.A.O.-activity in the mustard sample run was still at the origin, and a single precipitin line was obtained in this position. A single preciptin line was also obtained when Cucurbita A.A.O. was used instead. In order to obtain more evidence of specificity, immunoprecipitation of samples of A.A.O. was tried. The anti-Cucurbita A.A.O. serum was shown to precipitate A.A.O.-activity from a solution of Cucurbita A.A.O.(Table 4.2). A.A.O.-activity was not only precipitated by the antiserum, but also showed inhibition of the A.A.O.-activity in the presence of antiserum, immediately after mixing of the two solutions. There was negligible inhibition or

Figure 4.4

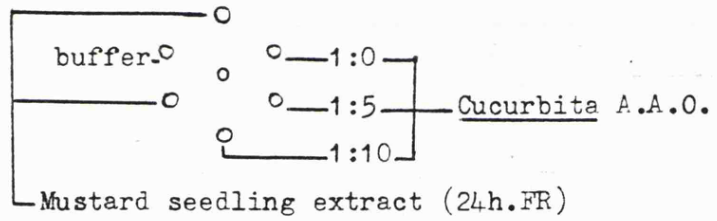
Double Diffusion Plates with Cucurbita and mustard A.A.O.

The central wells (o) contain 5µl. aliquots of anti-Cucurbita A.A.O. serum from rabbit 1 (R1) from a series of bleeds taken 3 months after the initial immunization injection, and 2, 4 or 6 weeks after a booster injection (bleeds (i),(ii),(iii)); or of normal rabbit serum (N.R.S.), taken from a non-immunized rabbit. The surrounding wells contain 4µl. aliquots of either Cucurbita A.A.O. (100µg./100µl.) undiluted(1:0) or diluted 5- or 10-fold(1:5 or 1:10), or extracts of sections of mustard seedlings, the seedlings were germinated in darkness for 42h. then transferred to far-red light for 0 -24h. and extracted. The extractions and dilutions were carried out using 50mM Na-phosphate buffer, pH 7.0.
A key to show the positions of the samples in the wells is given below the plates, a series of 3 sets of wells (a-c) were used for each sample of serum.

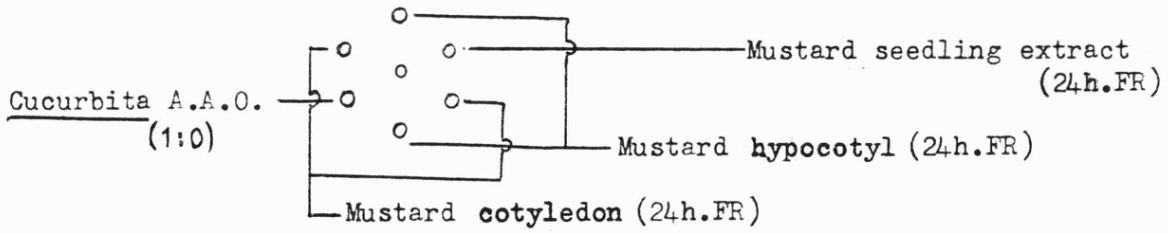


Key to show positions of Cucurbita A.A.O. and mustard extracts:

(a)



(b)



(c) Mustard extracts:

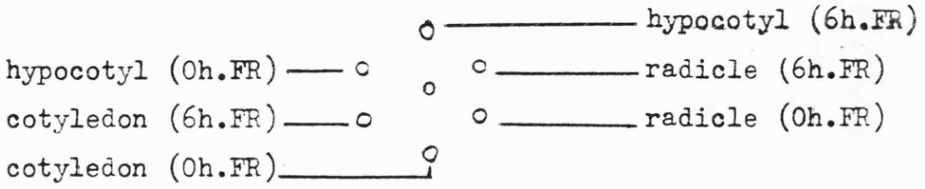


Table 4.2

Immunotitration Experiments: with Cucurbita A.A.O.

Mixtures of Cucurbita A.A.O. and serum from rabbit 3 (bleed taken on date indicated, 3 or 6 months after the initial immunization, and 4 weeks after a booster injection) or from a non-immunized rabbit (N.R.S.) or buffer, 50mM Na-phosphate buffer, containing 0.15M NaCl (P,B.S.=phosphate-buffer-saline), were incubated at 4°C for 2h. or 20h.. The mixture was then assayed, or centrifuged and the supernatant assayed. The mixtures contained 10µl. aliquots of Cucurbita A.A.O. (1mg./ml.P.B.S.; giving an A.A.O.-activity of 0.41 nmoles ascorbate oxidised/min./10µl.) and 10µl. serum or buffer, in a final volume of 500µl. made up with P.B.S.

Serum	A.A.O.-activity: nmoles ascorbate oxidised/min./500µl.				
	t=0h.	t=2h.	t=2h.+ spin.	t=20h.	t=20h.+ spin.
N.R.S.	0.43	0.38	0.37	0.38	0.43
P.B.S.	0.40	0.42	0.38	0.43	0.39
R3 20/10/78	0.18	0.12	0.007	0.037	0.007
R3 18/01/79	0.275	0.12	0.006	0.018	0.010

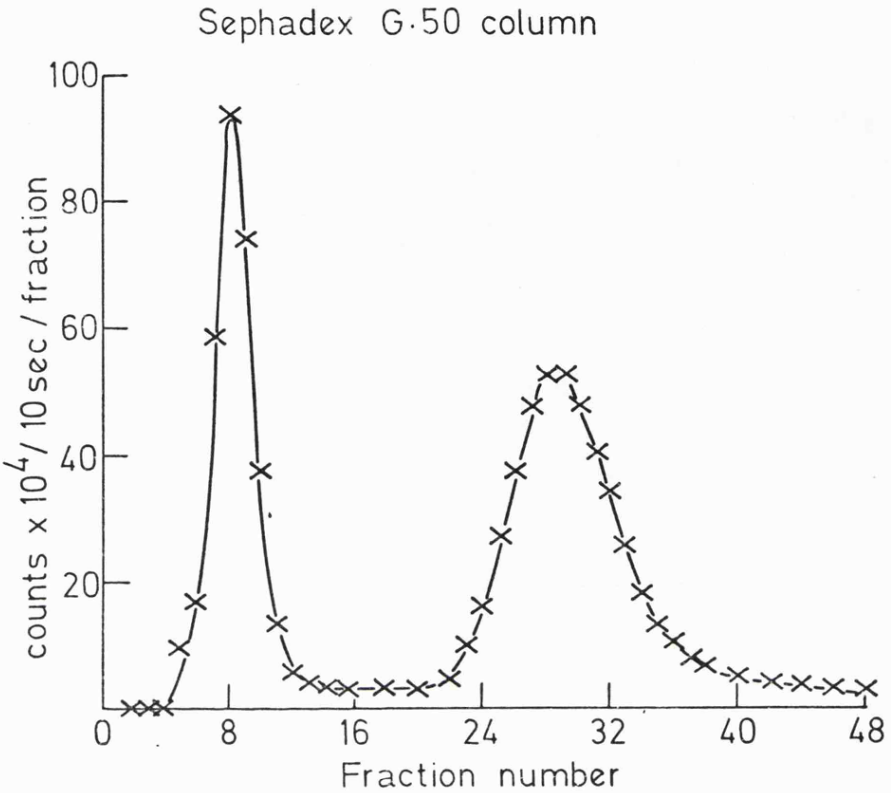
precipitation of A.A.O.-activity in the presence of control serum (N.R.S.). Similar experiments with mustard A.A.O. extracts were not successful, probably because of the difficulties in measuring small changes in A.A.O.-activity accurately using a spectrophotometric assay and cloudy enzyme solutions. It was necessary to use the radioactive binding assay to measure antibody titres with mustard A.A.O. samples (see Section 4.2.7). The immunotitration experiments did show that the antiserum was specific, using the Cucurbita samples.

4.2.6 Radioisotopic Labelling of Cucurbita A.A.O.: Samples of Cucurbita A.A.O. were iodinated with [^{125}I]-sodium iodide using the Chloramine T method generally employed to label polypeptide hormones from animals (Bolton,1977). The degree of labelling with this method was low, <1% radioactive label was incorporated into the A.A.O. protein (determined after separation of the free [^{125}I]-Na-iodide and labelled protein on a Sephadex G-50 column). A milder oxidant, sodium hypochlorite, was used for the iodination instead of Chloramine T. Optimum conditions for the iodination were determined empirically. Initially 1.0mCi [^{125}I]-iodide was used, but 0.5mCi was equally satisfactory, and also less expensive. The specific radioactivity was calculated from the recovery of radioactivity in the protein peak eluted from a Sephadex G-50 column, Fig.4.5 (used to separate the free [^{125}I] Na-iodide and labelled protein.) Values of 10-30μCi/μg. A.A.O. protein were obtained initially, this was improved to 70μCi/μg. A.A.O.protein, when freshly purchased [^{125}I]-Na-iodide and freshly dissolved Cucurbita A.A.O. were used. Freshly iodinated Cucurbita A.A.O. was partially purified by either cellulose chromatography or gel filtration (exclusion chromatography). Cellulose columns are used to purify iodinated polypeptide hormones in the laboratory where these iodinations were carried out (see acknowledgements); however with iodinated Cucurbita A.A.O. some 60-80% radioactivity passed directly through the column, this material is regarded as the 'damaged' material; only material that binds to the column and is subsequently eluted by solutions of increasing protein content (egg albumin or bovine serum

Figure 4.5

Elution profile of the reaction mixture for labelling Cucurbita A.A.O. on a Sephadex G-50 column.

The iodination reaction was carried out as described in Section 2.13.1, the iodination mixture was then passed through a Sephadex G-50 column, (1.25cm. diameter x 15.0cm. height) in 50mM Na-phosphate buffer, pH 7.0, in order to separate the [^{125}I]-labelled Cucurbita A.A.O. and free [^{125}I]-Na-iodide. The sample passed through the column was 1.0-2.0ml. (including washings from the reaction tube), once the void volume had been collected from the column (first 3-4 tubes), 20 drop fractions were collected. The radioactivity of the eluted fractions was measured in a Bioscint γ-counter ,10s. counts were sufficient (with the lead shield in place). Those fractions containing A.A.O. were pooled, ie. the first peak of radioactivity. All normal safety regulations for dealing with highly radioactive substances were observed at this stage.


Sephadex G·50 column
100
80
60
40
20
counts x 10^4 / 10 sec / fraction
0
8
16
24
32
40
48
Fraction number

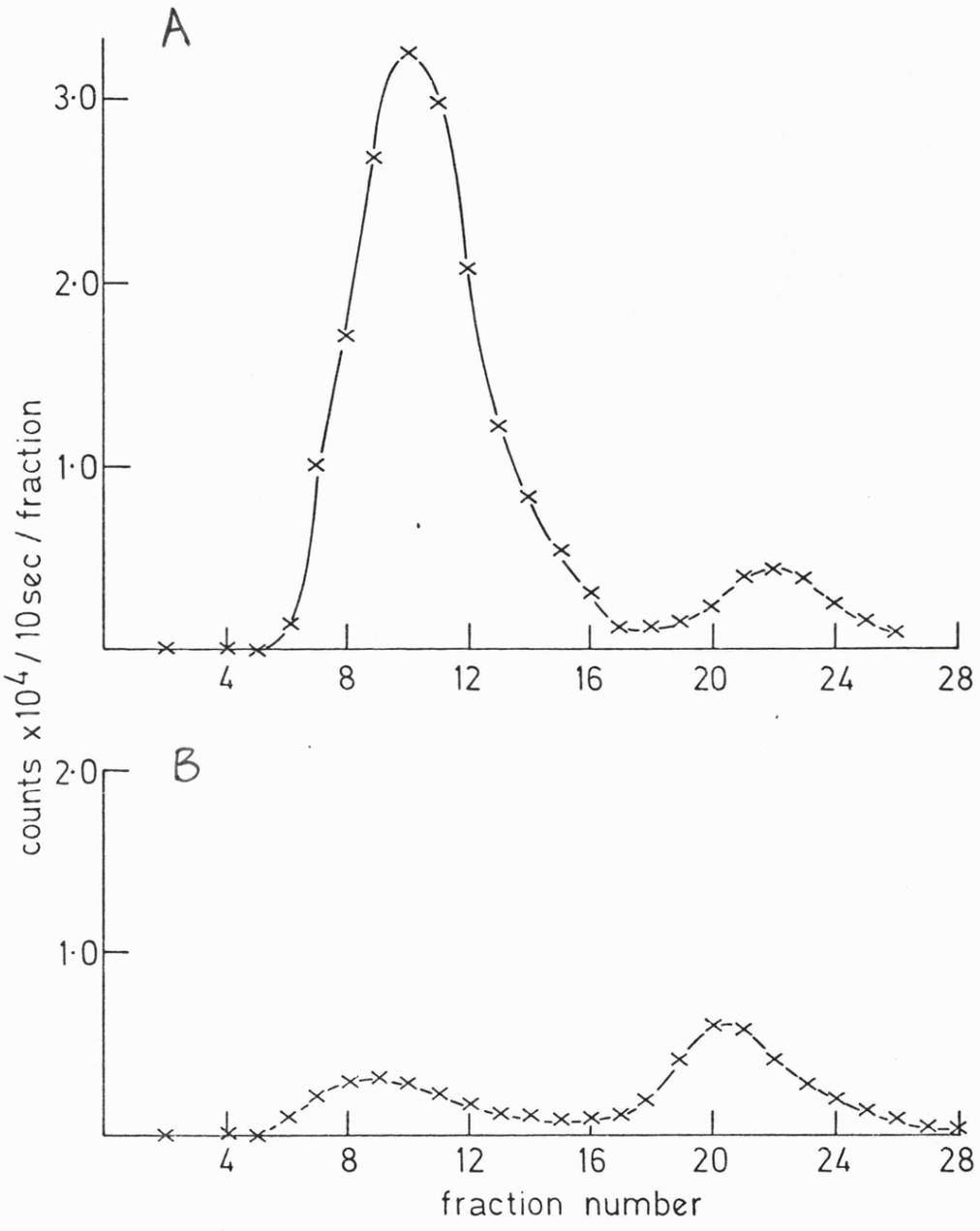
albumin) is used as undamaged labelled material. Using a Sephadex G-150 column (identical to the column used to determine MWs) 80-90% radioactivity eluted in a single peak, in an elution volume identical to that of a sample of unlabelled Cucurbita A.A.O. (Figure 4.6). This suggested that the cellulose column was not particularly suited to the purification of such a large polypeptide/protein as the A.A.O. enzyme molecule. The column may have been overloaded, but if this was the case, the method would still have been unsuitable, as an extremely large column would have been required, and this would have created a problem for disposal of the radioactive column. The Sephadex G-150 column was used in preference because good yields of [^{125}I]-labelled Cucurbita A.A.O. were obtained, and the amount of radioactive waste was kept to a minimum. The labelled enzyme (from the Sephadex G-50 column) was not particularly stable; after being kept at 4°C for 14 days, considerable damage/degradation had occurred. Figure 4.6B shows that the stored sample gave an elution profile on the Sephadex G-150 column with most of the remaining radioactivity in the second peak eluted, about 30% radioactivity was present in the peak corresponding to A.A.O.protein. A sample of the purified labelled enzyme (from the Sephadex G-150 column) appeared to be more stable, giving an elution profile on a second Sephadex G-150 column similar to the freshly prepared sample, but in test assays, stored samples gave a much higher non-specific binding (ie. binding with N.R.S.). The iodination and purification by Sephadex G-150 column were therefore carried out on the same day the assays were set up.

4.2.7 Radioimmunoassay: For an introduction to the technique of radioimmunoassay see Chard (1978). The basis of this technique is the competitive binding assay: a mixture of labelled and unlabelled antigen is added to a sample containing a limited amount of antiserum, after a suitable period of incubation some of the labelled and unlabelled antigen will have bound to the antiserum, but not all as the antibody is present in a limited amount; the bound and unbound antigen (both labelled and unlabelled) are then separated, and the amount of labelled antigen bound is inversely related to

Figure 4.6

Elution profile of [^{125}I]-labelled Cucurbita A.A.O. on a Sephadex G-150 column.

A sample (1.0ml.) of freshly prepared [^{125}I]-labelled Cucurbita A.A.O., from the Sephadex G-50 column (Figure 4.5) was passed through a Sephadex G-150 column (1.25cm. diameter x 21.0cm. height) in 50mM Na-phosphate buffer, pH 7.0. (A.). A sample of [^{125}I]-labelled Cucurbita A.A.O., which had been stored for 14 days at 4 °C after passing through the Sephadex G-50 column, was also passed through an identical column. (B.). The radioactivity of the eluted fractions was measured in a Bioscint γ-counter, 10s. counts were sufficient (with the lead shield in place). The first peak of radioactivity corresponds to the undamaged [^{125}I]-labelled Cucurbita A.A.O.. All normal safety regulations for dealing with radioactive substances were observed throughout.


A
3·0
2·0
1·0
B
2·0
1·0
4
8
12
16
20
24
28
counts x10⁴ / 10 sec / fraction
fraction number

the amount of unlabelled antigen present in the sample used. In order to carry out a radioimmunoassay it is therefore necessary to select a suitable antiserum volume and dilution, and volume of labelled antigen. The periods of incubation at each step may be varied, and the order of addition of the samples may be important. The labelled antigen may be added after an initial incubation with unlabelled antigen or the two may be added at the same time. Finally a method for separating the bound and unbound antigen must be found.

A. Binding Assays: Mixtures of [^{125}I]-labelled Cucurbita A.A.O. and a range of dilutions of the anti-Cucurbita A.A.O. serum were incubated. (A standard period of 24h. was used for the initial incubations, and all incubation steps were carried out at 4°C.) To separate the bound and unbound antigen, the second antibody method was used, a second period of incubation was required, after which the mixture was centrifuged. The precipitate was collected, this contained the bound antigens, in this case only labelled Cucurbita A.A.O..The supernatant was decanted from the centrifuge tubes (radioactive waste) and the radioactivity in the tubes (ie. in the precipitate) counted by placing the tubes in the γ-counter. The second antibody used was RD 17, an anti-rabbit serum, from donkey, specially prepared for radioimmunoassays (Wellcome Reagents Ltd.). This test assay was used to decide upon a suitable antiserum dilution for the radioimmunoassay, the results are shown in Table 4.3. A dilution of 1:1,000 or 1:5,000 gave a reasonable % binding. Binding between 30 and 50% is considered optimum for most radioimmunoassays, allowing a reasonable degree of competitive binding in the presence of unlabelled antigen. Partial purification of the labelled Cucurbita A.A.O. (in this case using the cellulose column, which gave only low yields, but in sufficient quantities for these assays), reduced the amount of non-specific binding, (ie. binding to control serum, N.R.S.) and also gave a slightly higher % binding. As the iodination and purification methods were improved, the % binding obtained at a given antiserum dilution was increased. Table 4.4 shows the results of test binding assays for a range

Table 4.3

Binding Assays:with Cucurbita A.A.O. and anti-Cucurbita A.A.O. serum.

Serum from two different bleeds (taken on the dates indicated in the table), from rabbit 3, was diluted with P.B.S. (Na-phoshate buffer, 50mM, pH 7.0, containing 0.15M NaCl). The dilutions of antiserum (100µl. aliquots) were incubated with an equal volume of P.B.S. and an equal volume of [^{125}I]-labelled Cucurbita A.A.O., from the Sephadex G-50 column, or from a cellulose column (partially purified), for 24h. at 4°C. The bound and unbound antigen were then separated using the second antibody method (RD 17 added at 1:40 dilution in P.B.S. in an equal volume, 100µl. and incubated for a further 24h. at 4°C; 500µl. P.B.S. was then added and the mixture centrifuged, the supernatant decanted). The radioactivity in the precipitates was counted by placing the tubes in a γ-counter. % binding was calculated using the following equation:

$$\% \text{ binding} = \frac{(\text{counts in precipitate}) - (\text{counts in blank*})}{(\text{total counts per tube})}$$

(*blank assay tube contained no antiserum, 100 l. P.B.S. added instead.)

Antiserum dilution	% binding value bleed date: 20/10/78	: 18/01/79
Sample from G-50 column: (10,000counts/min/tube(100µl.)		
1:100	35	38
1:1,000	38	38
1:5,000	29	29
1:10,000	20	17
1:20,000	11	18
N.R.S. (1:1 dilution)	8	-
Sample from cellulose column: (7,500 counts/min/tube(100µl.)		
1:100	39	40
1:1,000	49	50
1:5,000	34	35
1:10,000	20	18
1:20,000	6	3
N.R.S. (1:1 dilution)	3	-

Table 4.4

Binding Assays:with Cucurbita A.A.O. and anti-Cucurbita A.A.O. serum.

Serum from a range of bleeds (taken on the dates indicated in the table), from rabbits 1 and 3 were diluted 1:1,000 and 1:5,000 with P.B.S., and these dilutions used for binding assays, as described for Figure 4.3.

Rabbit number	Bleed date	% binding value Serum dilution: 1:1,000	: 1:5,000
1	20/10/78	78	67
	31/10	76	72
	6/11	77	69
	13/11	76	61
3	20/10	79	34
	31/10	75	50
	3/11	75	49
	13/11	68	34
N.R.S. (1:1 dilution)		22	
Blank (P.B.S.)		= 0	
Total counts.		=100	

of bleeds from the immunized rabbits at the selected optimum dilution of antiserum. The % binding obtained under the same conditions of assay, were 70-80%, compared to 30-40%, previously. A lower dilution of the antiserum could therefore have been tried.

B. Standard Curve: Serial dilutions of unlabelled Cucurbita A.A.O. were prepared and incubated with the selected dilution of anti-Cucurbita A.A.O. serum, after an initial period of incubation (24h.) the labelled Cucurbita A.A.O. was added. During the period of the assay, equilibrium is reached between the binding of labelled and unlabelled antigen, Cucurbita A.A.O, to the antiserum, anti-Cucurbita A.A.O. serum. The bound antigens, consisting of both labelled and unlabelled Cucurbita A.A.O., were separated from the unbound antigen as before, using the second antibody method. There are disadvantages to the use of this method, mainly in that the length of the assay is increased, which may or may not be a problem (this may have contributed to the poor results when instead of unlabelled Cucurbita A.A.O. a crude extract from mustard cotyledons was used, see subsection D). The % binding values for a standard curve prepared in this manner are presented in Table 4.5. Two batches of sera from different bleeds from the immunized rabbits were compared. There was a 10-fold higher sensitivity with the second serum, R1 06/11/78, at a 1;5,000 dilution. This can be seen more clearly when the results are plotted. In a separate set of assays, the results shown in Figure 4.7 were obtained; % binding (ie. % total labelled antigen bound) was plotted against the antigen dilution used, in the case of a standard curve the amount of antigen is plotted, ie. amount of Cucurbita A.A.O. protein. A straight line plot is obtained if a logit b plot is used: logit b = ln (% binding /(100 - % binding)), see figure 4.7B. There are many different ways of plotting the results (see Chard,1978), but this method gave satisfactory results here. In most of the assays, the unlabelled antigen and antiserum were incubated for a period (24h.) before adding the labelled antigen. If instead the two were added together, the % binding obtained was higher. This indicated that the (competitive) binding by the unlabelled antigen was reduced, this is seen as a reduction in the

Table 4.5

Standard Curve:with Cucurbita A.A.O. and anti-Cucurbita A.A.O. serum.

For the standard curve, the same procedure as for the binding assays was used, but dilutions of unlabelled Cucurbita A.A.O. were prepared and incubated with the selected dilution of antiserum, then labelled Cucurbita A.A.O. was added. Second antibody was then added as for the binding assays. Dilutions of Cucurbita A.A.O. were prepared from a solution containing 10µg. A.A.O.protein/100µl. P.B.S.. Two batches of antiserum were compared;

A. from rabbit 3, bleed date 20/10/78 at a 1:1,000 dilution;

B. from rabbit 1, bleed date 6/11/78 at a 1:5,000 dilution.

Antiserum	Dilution of Cucurbita A.A.O. sample.	A.A.O.protein ng./tube(100µl.)	% binding value.	logit b value.*
A.	1:250	40	41.8	-0.33
	1:500	20	60.6	+0.43
	1:1,000	10	67.6	+0.74
	1:2,500	4	72.6	+0.97
B.	1:500	20	8.0	-2.44
	1:1,000	10	9.7	-2.23
	1:2,000	5	39.2	-0.43
	1:4,000	2.5	55.4	+0.22
	1:10,000	1	67.1	+0.71

(* logit b values were determined for plotting the results in a linear form, see text.)

Figure 4.7

Standard Curve:with Cucurbita A.A.O. and anti-Cucurbita A.A.O. serum.

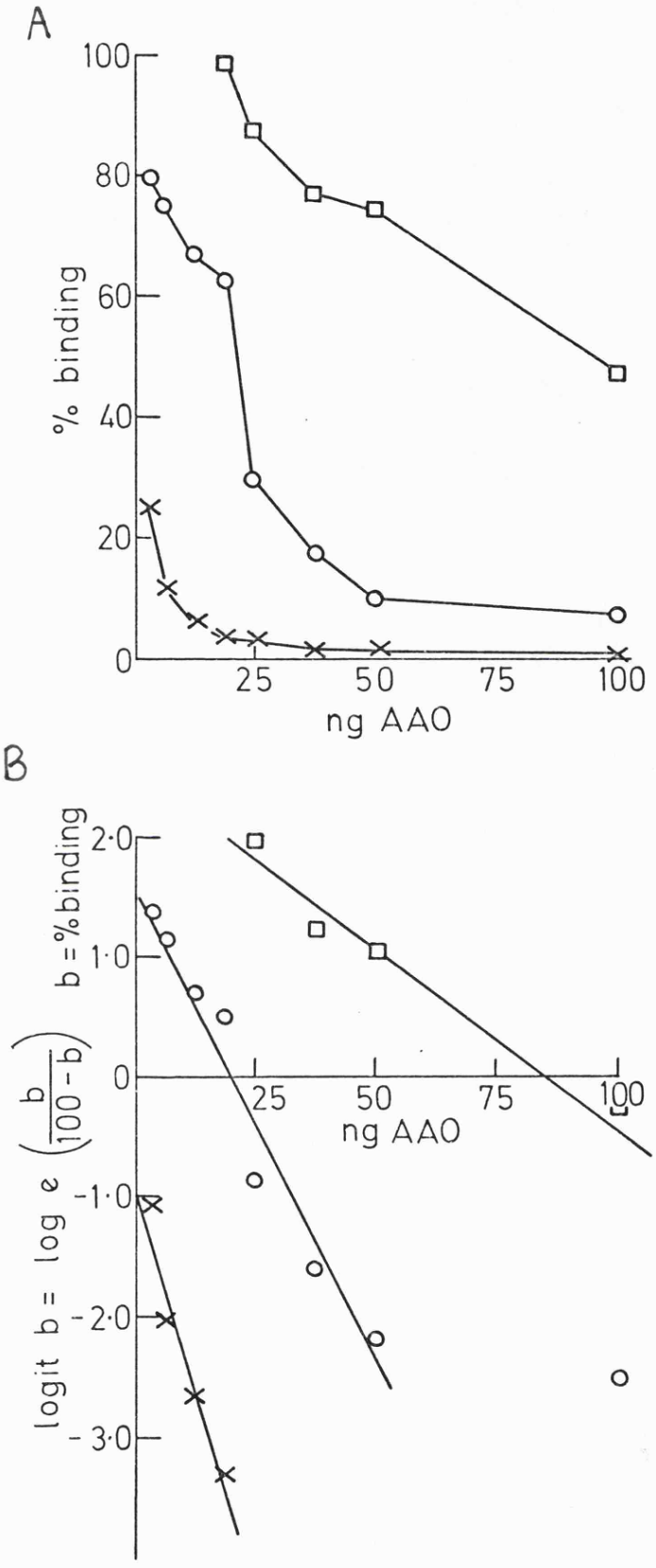
The standard curve is plotted in terms of the amount of Cucurbita A.A.O.-protein present in the dilution used,against the % binding obtained.(A.) A linear plot is obtained if a logit b plot is used; the results are plotted in terms of the amount of Cucurbita A.A.O.-protein against the logit b value (which is based on the % binding obtained.)(B.).

Two batches of antiserum were compared:

A. from rabbit 3, bleed date 20/10/78 at a 1:1,000 dilution (o),

B. from rabbit 1, bleed date 6/11/78 at a 1:5,000 dilution (×).

A standard curve was also prepared by mixing unlabelled and labelled antigen at the beginning of the assay, and incubating the mixture with antiserum A., instead of allowing an initial incubation with unlabelled antigen first. (□).


A
100
80
60
40
20
0
% binding
25
50
75
100
ng AAO
B
2·0
1·0
0
-1·0
-2·0
-3·0
b = % binding
logit b = log e (b / (100 - b))
25
50
75
100
ng AAO

sensitivity of the assay. (Figure 4.7). The linear portion of the logit b plot indicates the range (sensitivity) of the assay. If the labelled antigen was added first, there was negligible binding by the unlabelled antigen. These results clearly indicate that binding was biased towards the labelled-Cucurbita A.A.O. (this probably contributed to the poor results with extracts of mustard cotyledons, see subsection D).

C. Measurement of A.A.O. protein in plant extracts: Dilutions of plant tissue extracts containing A.A.O. were incubated with the anti-Cucurbita A.A.O. serum. After an initial incubation period (24h.) the labelled antigen, [^{125}I]-labelled Cucurbita A.A.O. was added, and the assay continued as for the standard curve assays. As mentioned before, the % binding is a measure of the amount of labelled antigen bound, therefore the higher the value for the % binding , the lower the amount of A.A.O. protein in the unknowns, ie. in the plant tissue extracts.

D. A.A.O Levels in Mustard Seedlings: The radioimmunoassay was carried out as for the standard curve, but using a range of dilutions of extracts from mustard cotyledons. The initial results were poor, so the period of the initial incubation, ie. in the presence of unknown and antiserum , was increased, 2 and 4 day incubations were tried (the other incubation periods were not changed). The results are given in Table 4.6: there was no significant difference in the % binding values for the two extracts used, one from dark-grown mustard and the other from far-red-treated mustard, although there was a 6-fold difference in the measurable A.A.O.-activity (Table 4.7). The ability of mustard A.A.O. to compete with binding by the [^{125}I]-labelled Cucurbita A.A.O. was poor compared to that of unlabelled Cucurbita A.A.O., the range of % binding values with Cucurbita A.A.O. was between 10 and 70% using the more sensitive of the two batches of antiserum, compared to 47-62% using mustard cotyledon extracts (with a 4 day initial incubation period). Attempts to improve the degree of competitive binding, and thereby improve the sensitivity of the assay for mustard extracts,by using freshly prepared extracts, or using partially purified mustard extracts

Table 4.6

Radioimmunoassay to measure A.A.O. levels in extracts from mustard cotyledons.

Crude supernatant extracts of mustard cotyledons were prepared, taken from seedlings which had been given 60h. dark or 36h. dark + 24h. far-red light. The extracts were diluted 1:5 and 1:50 with P.B.S, and the assay carried out as for the standard curve except the initial incubation period (ie. with the extract and antiserum)was either 2 or 4 days. For comparison a few dilutions of Cucurbita A.A.O. were also assayed with a 4 day initial incubation.

Initial incubation period.	Sample light treatment	Dilution of sample.	% binding value.
Antiserum: R3 20/10/78, 1:1,000 dilution-			
2 days	Mustard cotyledons		
	dark	1:5	59.8
		1:50	63.6
	far-red	1:5	61.4
		1:50	66.2
4 days	dark	1:5	57.3
		1:50	64.2
	far-red	1:5	56.6
		1:50	65.7
	Cucurbita A.A.O.	1:250	41.8
		1:500	60.6
		1:2,500	72.6
Antiserum: R1 6/11/78, 1:5,000 dilution-			
2 days	Mustard cotyledons		
	dark	1:5	42.4
		1:50	56.3
	far-red	1:5	40.2
		1:50	56.5
4 days	dark	1:5	47.4
		1:50	61.4
	far-red	1:5	47.5
		1:50	62.1
	Cucurbita A.A.O.	1:1,000	9.7
		1:2,000	39.2
		1:10,000	67.1

Table 4.7

A.A.O.-activity in Mustard Seedling extracts.

Crude supernatant extracts were prepared from mustard seedlings between 0 and 84h-old, grown in continuous darkness or given a far-red light treatment. The extracts were prepared using 100mM citrate-phosphate buffer, pH 5.0, and assayed for A.A.O.-activity using the spectrophotometric assay(Section 2.5.5)

Age of seedling-light treatment	A.A.O.-activity nmoles ascorbate oxidised/min /cotyledon pair.	/radicle(+ hypocotyl).
0h. dry seed	2.3	
36h. dark	18.0	13.5
60h. dark*	22.0*	-
84h. dark	28.4	4.3
36h. dark + 6h. far-red	33.3	24.1
36h. dark + 24h. far-red*	128.7*	44.2
36h. dark + 48h. far-red	141.5	13.6
36h. dark +24h. far-red + 24h. dark	86.6	6.3

(* extracts used in radioimmunoassay experiment.)

(eg. by passing extracts through a Sephadex G-150 column), or by concentrating extracts (eg. by ammonium sulphate fractionation or by freeze-drying) were no more successful. The most likely reason for this apart from the immunological difference between Cucurbita A.A.O. and mustard A.A.O., was the high degree of binding by labelled Cucurbita A.A.O. used in the assay. The presence of other proteins in the extracts may have contributed to the poor results, but is unlikely following the results obtained with crude extracts from pumpkin seedlings (see subsection F). The results that were obtained suggested that there was no difference in the amount of A.A.O.-protein in cotyledons from dark-grown or far-red treated mustard seedlings. This can be interpreted in terms of a pool of inactive enzyme molecules in dark-grown cotyledons, which is activated following a far-red light treatment. As the radioimmunoassay for mustard extracts required further improvements before carrying out assays on extracts taken from mustard seedlings over a wider range of ages (0-84h.), it is not possible to present a more detailed set of results.

E. A.A.O. Levels in Gherkin Seedlings: As mustard extracts were not particularly successful, gherkin extracts were tried, it was expected that as gherkin is closely related to Cucurbita sp. that there would be a higher degree of similarity and therefore cross-reaction, with Cucurbita A.A.O. and the anti-Cucurbita A.A.O. serum. This did not appear to be the case; the results obtained with extracts of gherkin seedlings were comparable with those obtained with mustard. This may have been partly due to the lower A.A.O.-activity in the extracts used,(taken from 4 day-old dark-grown gherkin seedlings), although concentrating the samples by freeze-drying before assaying did not improve the results.

F. A.A.O. Levels in Pumpkin Seedlings: The standard curve obtained with Cucurbita A.A.O. was good, therefore the assay should have been suitable for assaying extracts from Cucurbita sp., for this reason pumpkin was chosen. The results were much more successful. Table 4.8 shows the % binding values obtained with an extract from 3 day-old dark-grown pumpkin

Table 4.8

Radioimmunoassay to measure A.A.O. levels in extracts from pumpkin cotyledons.

A crude supernatant extract was prepared from 3 day-old dark-grown pumpkin seedlings using 100mM citrate-phosphate buffer,pH 5.0. The extracts were diluted in P.B.S. as indicated in the table, and the assay carried out as for the standard curve except the initial incubation period was 3 days.

Antiserum	Dilution of pumpkin extract.	% binding value.	logit b value.
R3 20/10/78			
1:1,000 dilution	1:1	17.0	-1.58
	1:10	50.7	+0.04
	1:100	68.7	+0.78
	1:1,000	65.4	+0.77
R1 6/11/78			
1:5,000 dilution	1:1	9.9	-2.20
	1:10	13.3	-1.87
	1:100	52.4	+0.09
	1:1,000	59.8	+0.40

seedlings and two different batches of anti-Cucurbita A.A.O. serum. A.A.O. levels in pumpkin also undergo changes during germination and following light treatment; using the radioimmunoassay to measure the amounts of A.A.O. protein present, it was possible to carry out a similar investigation on the changes in A.A.O.-activity in pumpkin seedlings. Pumpkin seeds were germinated under standard conditions of temperature (25°C) either in darkness or in white light and extracts made from sections of the seedlings as they developed, these extracts were assayed for A.A.O.-activity using the enzyme assay, and for A.A.O. protein using the radioimmunoassay. The results have been plotted in Figure 4.8 as % binding curves for dilutions of each extract. A series of roughly parallel curves was obtained: % binding increases with increasing dilution of the pumpkin extract, ie. as the concentration of A.A.O.protein declines. The curves were plotted in groups for ease of comparison. In order to measure the absolute amounts of A.A.O. protein present in unknowns, the % binding curves obtained must be parallel to that of the standard curve. The standard curve obtained with Cucurbita A.A.O. is also shown in the figure (dotted line) and is slightly steeper than the curves with pumpkin seedling extracts, when the same scale of dilution is used. A logit b plot was therefore not much use for comparing the pumpkin extract curves with the Cucurbita A.A.O. curve. It was possible to obtain relative values for the amounts of A.A.O. protein in the pumpkin extracts, for those extracts giving parallel curves. The curves for extracts from dry seeds (0h.) and 48h.-old dark-grown cotyledons were unusual in that the % binding did not appear to fall towards zero as in most of the other cases. One possible reason for this was the presence of substances in these extracts which competed for binding, or inhibited the binding by any A.A.O. present in the pumpkin extracts at low dilutions (these extracts would be rich in storage proteins compared to the other extracts). It was necessary to extrapolate some of the other curves, where the amount of A.A.O.protein present was high (alternatively a few more dilutions could have been tried). The changes in A.A.O.-activity in developing pumpkin seedlings are shown in

Figure 4.8

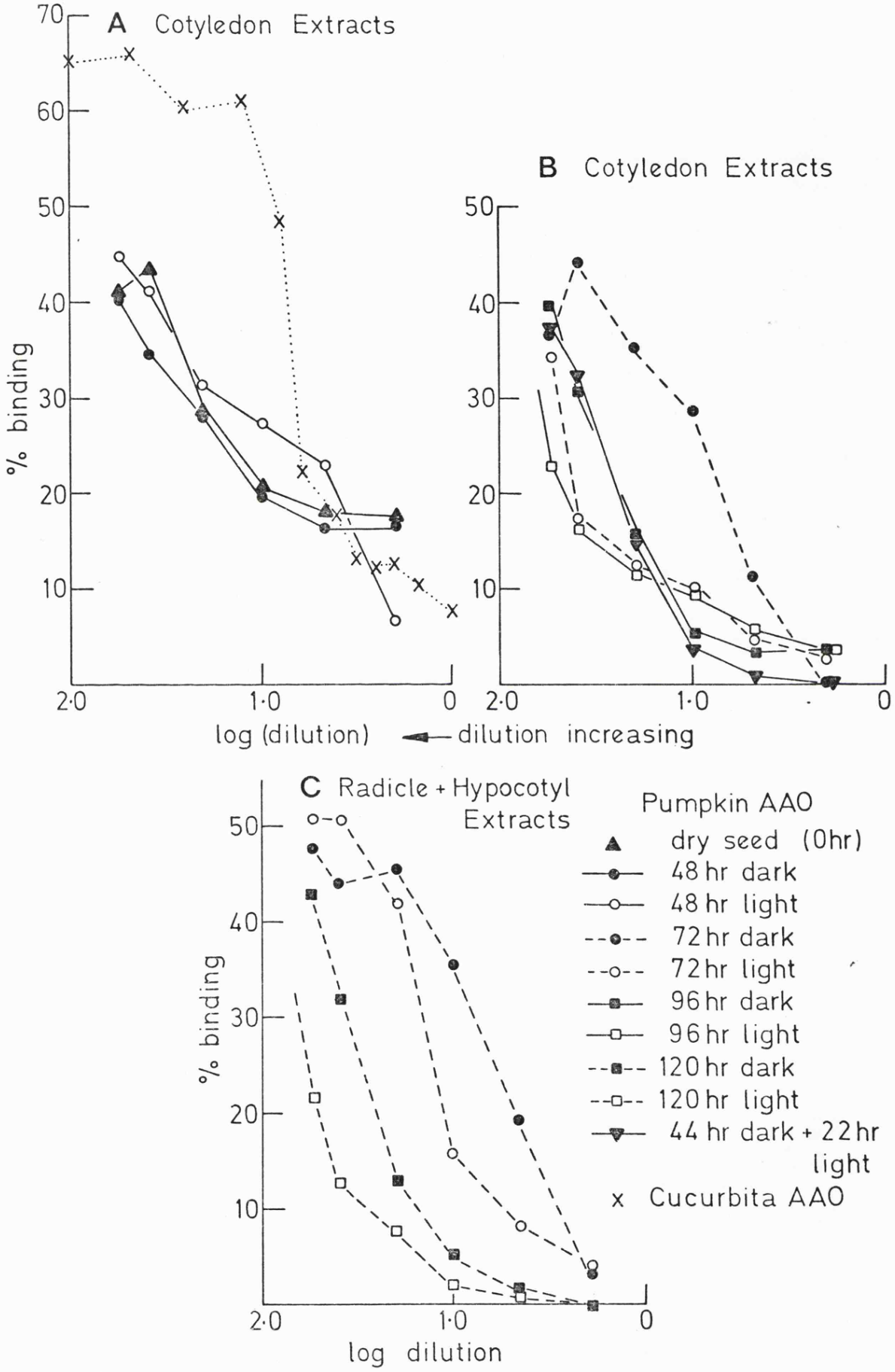
Radioimmunoassay to measure A.A.O. levels in extracts from pumpkin seedlings.

Crude supernatant extracts were prepared from pumpkin seedlings between 0 and 120h.-old, grown in continuous darkness or continuous white light, or given a white light treatment after 44h. dark. The extracts were prepared using 100mM citrate-phosphate buffer,pH 5.0, and dilutions for the radioimmunoassay made using P.B.S. (between 1:2 and 1:60).The assay was carried out as for the standard curve with an initial incubation period of 24h..% binding curves were plotted, in groups :

A. Cotyledon extracts 0-48h.-old and Cucurbita A.A.O.(100µg./100µl., with dilutions between 1:200 and 1:20,000)

B. Cotyledon extracts 72-120h.-old.

C. Radicle + Hypocotyl extracts 72-120h.-old.


A Cotyledon Extracts
B Cotyledon Extracts
% binding
70
60
50
40
30
20
10
2·0
1·0
0
log (dilution)
dilution increasing
C Radicle + Hypocotyl Extracts
% binding
log dilution
Pumpkin AAO
dry seed (0hr)
48 hr dark
48 hr light
72 hr dark
72 hr light
96 hr dark
96 hr light
120 hr dark
120 hr light
44 hr dark + 22 hr light
x Cucurbita AAO

in Table 4.9. The amounts of A.A.O.protein in the extracts has been estimated from the % binding curves in Figure 4.8, using the value of 30% binding as a base for the calculations. The dilution of extract giving 30% binding is a measure of the amount of A.A.O. protein present: the lower the dilution at which 30% binding is obtained, the higher the amount of A.A.O.protein. The specific activity values are therefore based on an arbitary scale, but do give an indication of the amounts of A.A.O.-activity and A.A.O. protein present, see Table 4.10. The results have also been plotted, Figure 4.9. The increase in A.A.O.-activity in light-grown cotyledon extracts is accompanied by an increase in the specific activity, this suggests that there is little change in the amount of A.A.O. protein during this period, and that the increase in enzyme activity arises from enzyme molecules already present. In dark-grown cotyledon extracts the specific activity also increases during the initial increase in A.A.O.-activity (48-72h.), then remains constant during the next increase, suggesting that activation of previously inactive enzyme molecules occurs first, then synthesis of more enzyme molecules takes place. There is a discrepancy between the increase in specific activity that occurs between 48 and 72h. compared to the increase in A.A.O.-activity in dark or in light; if the increase in specific activity occurring in the dark is sufficient to account for the increase in enzyme activity, the increase in enzyme activity occurring in the light must arise partly via activation and partly via de novo synthesis. This seems likely as there is also a slight increase in specific activity in the light before this. The increase in A.A.O.-activity occurring in the cotyledons therefore arises via activation of previously inactive enzyme molecules initially, then more enzyme molecules are also synthesised, these processes occur sooner in the presence of white light, compared to the dark. In the radicles, which are not readily distinguishable until 72h. the increase in A.A.O.-activity that follows is accompanied by a constant specific activity, therefore it seems likely that the enzyme activity arises from an increase in the number of enzyme molecules present, either by de novo synthesis of enzyme, or

Table 4.9

A.A.O.-activity in Pumpkin Seedling extracts.

Crude supernatant extracts were prepared from pumpkin seedlings between 0 and 120h.-old, grown in continuous darkness or continuous white light, or given a white light treatment after 44h. dark. The extracts were prepared using 100mM citrate-phosphate buffer, pH 5.0, and assayed for A.A.O.-activity using the spectrophotometric assay (Section 2.5.5).

Age of seedling-light treatment	A.A.O.-activity nmoles ascorbate oxidised/min. /cotyledon pair.	/radicle + hypocotyl.
0h. dry seed	4.5	
24h. dark	11.0	
20h. light	10.0	
48h. dark	41.0	
44h. light	121	
44h. dark + 5h. light	159	
72h. dark	242	93
44h. dark + 20h. light	511	123
72h. light	1097	224
96h. dark	796	222
96h. light	2835	424
120h. dark	1412	665
120h. light	1882	1470

Table 4.10

Radioimmunoassay to measure A.A.O. levels in extracts from pumpkin seedlings.

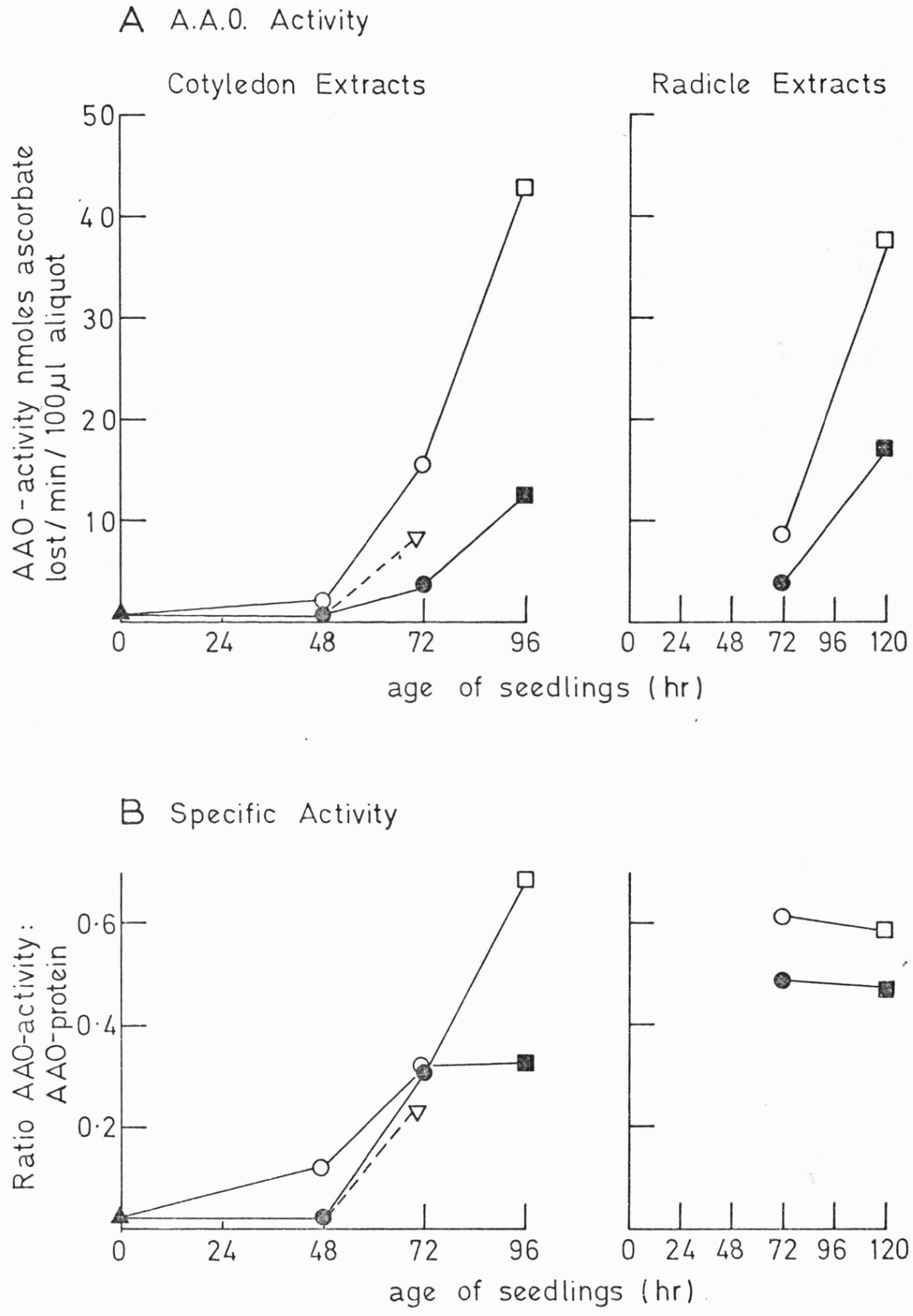
The data in this table are taken from the radioimmunoassay experiment as described in Figure 4.8. The amount of A.A.O. protein in the extracts was estimated from the dilution of extract required to give 30% binding in the assay. The dilution factor was taken as as arbitary unit of A.A.O. protein content; the specific activity is given in terms of A.A.O.-activity (in 100µl. sample, undiluted extract) : A.A.O. protein (dilution factor).

Sample - light treatment.	A.A.O.-activity nmoles ascorbate oxidised/min/100µl.	Dilution of extract giving 30% binding log.value	converted	Specific activity.
Cotyledon pairs:				
0h. dry seed	0.54	1.33	21.4	0.025
48h. dark	0.61	1.48	30.2	0.02
48h. light	1.92	1.22	16.6	0.12
72h. dark	3.67	1.06	11.5	0.32
44h. dark + 20h. light	8.5	1.56	36.3	0.23
72h. light	15.7	1.70	50.1	0.31
96h. dark	12.5	1.58	38.0	0.33
96h. light	42.6	1.79	61.6	0.69
Radicles (+ hypocotyls):				
72h. dark	3.7	0.88	7.6	0.49
72h. light	8.8	1.16	14.5	0.61
120h. dark	17.3	1.57	37.1	0.47
120h. light	38.7	1.82	66.1	0.59

Figure 4.9

Radioimmunoassay to measure A.A.O. levels in extracts from pumpkin seedlings.

The data in this figure are taken from the radioimmunoassay experiment described in Figure 4.8 and Tables 4.9 and 4.10. and shows the time course for the increase in A.A.O.-activity in germinating pumpkin seeds/seedlings (A.) and the specific activity of A.A.O.(B.).
The symbols used correspond to those used in Figure 4.8, closed symbols for dark-extracts and open symbols for light-extracts.


A A.A.O. Activity
Cotyledon Extracts
Radicle Extracts
AAO-activity nmoles ascorbate lost/min/100µl aliquot
50
40
30
20
10
0
24
48
72
96
120
age of seedlings (hr)
B Specific Activity
Ratio AAO-activity : AAO-protein
0·6
0·4
0·2
0
24
48
72
96
120
age of seedlings (hr)

by modification of enzyme molecules which were not previously recognized by the antiserum. As the increase in A.A.O.-activity occurring in the cotyledons after 72h. is probably largely due to de novo synthesis of the enzyme, it is reasonable to assume that this mechanism is responsible for the increases in A.A.O.-activity in the radicles.

4.2.8 Summary: The radioimmunoassay was most successful when the sample unknowns were closely related to that of the standard curve and the labelled antigen used, ie. Cucurbita A.A.O. The A.A.O. enzyme molecule in pumpkins may not be identical to that of Cucurbita A.A.O., the chances of immunological differences occurring between iso-functional molecules or macromolecules increases as the MW increases, therefore the degree of cross-reaction for protein molecules from different sources will be less than between smaller polypepetides, eg. animal hormones for which the radioimmunoassay technique is most widely used. Nor is it surprising that for such a sensitive assay, that minor differences, as would appear to be responsible for the poor results with mustard and gherkin A.A.O. extracts, are emphasised. The assay could be improved for these extracts by the preparation of an homogeneous antiserum, which only recognises one type of immunological determinant, preferably one that is identical to that on the labelled antigen used in the assay, ie. by selecting antibodies that recognize both A.A.O. molecules equally well.

The results obtained with mustard cotyledon extracts suggest that although the A.A.O.-activity increases following far-red light treatment, there is little change in the amount of A.A.O. protein. Such results need verification, either using an improved radioimmunoassay or another technique such as rocket immunoelectrophoresis to measure A.A.O. levels. The results with extracts from pumpkin seedlings suggest that the initial increase in A.A.O.-activity arises without a significant increase in the amount of A.A.O. protein both in the light and in the dark, but then more enzyme molecules are synthesised, and this occurs sooner in the light than in the dark, in agreement with the rate of change of A.A.O.-activity under the two treatments.

By the time the radicles emerge from the seed coat, the increase in A.A.O.-activity in the cotyledons, in both the dark and the light, is probably due to synthesis of new enzyme molecules (activation mechanisms may also be operative, but may be masked by rapid activation), and the same mechanism for increasing A.A.O.-activity extends to the radicles.

The radioimmunoassay technique is therefore quite suitable for measuring enzyme levels in plants, provided a sample of the enzyme is available in a purified form and can be labelled for use in the assay.

SECTION 5 DISCUSSION

5.1 DISCUSSION OF P.A.L. RESULTS

Several preliminary studies were carried out before proceeding with the main issue of the immunological studies, because it was necessary to establish the validity of using plant tissue extracts for the immunological assays. As a quantitative assay of enzyme protein levels, immunotitration was used; this technique relies on measurements of enzyme activity in the presence of a range of antiserum concentrations, and therefore requires extracts containing 'stable' enzyme. Initially, the prime concern was to purify P.A.L.; potatoes were chosen as the source of P.A.L., as the enzyme from this tissue is relatively stable, and large amounts are easily extracted. The next step was to raise antibodies against the enzyme, preferably mono-specific antibodies, then to check the antibody specificity before using the antiserum to measure P.A.L. protein.

P.A.L.-activity in extracts from incubated potato tuber discs, was stable, provided suitable stabilizing agents were included in the extraction medium. Extracts were prepared from discs aged for up to 42h., in either darkness or white light; the measurable P.A.L.-activity rose (after a lag of a few hours) and then declined during this period, but the total protein content remained unchanged. The concentration of chlorogenic acid, the main phenolic compound formed in potatoes, would have changed during this period, but the stability of P.A.L. in the extracts was not significantly affected. A comparison of extraction buffers and stability tests indicated that a combination of additional reagents (mercaptoethanol and D-isoascorbate) was suitable, optimizing yields and stability, when P.A.L. levels were at a peak; the same conditions were used for all extractions.

P.A.L.-activity in mustard cotyledons and gherkin hypocotyls was low in non-stimulated tissue. In mustard cotyledons large quantities of storage protein were present, the protein content fell as the seedlings aged, and was also affected by the far-red light treatment (Hacker,1967), therefore

the specific activity of P.A.L. (in terms of protein content) increased as the seedlings developed, even in the dark, but to a greater extent during far-red light treatment. The stability tests and the results of the P.A.L. assay when long assay periods (90min.) were used, indicated that P.A.L. from cotyledons of dark-grown mustard seedlings was less stable; this may have been caused by proteins or other compounds in the dark extracts, which were not present in, or differed from those in extracts from far-red treated seedlings. P.A.L.-activity in hypocotyls and radicles of mustard seedlings was less sensitive to far-red light, and the stability of P.A.L. in the extracts was improved by desalting. Desalting appeared to have the opposite effect with cotyledon extracts, particularly dark extracts. The stability of P.A.L.-activity was in some way related to the level of P.A.L.-activity present and the sensitivity to far-red light treatment. In extracts of gherkin hypocotyls P.A.L.-activity was less stable in desalted extracts, whereas P.A.L.-activity in gherkin cotyledon extracts was particularly stable in both crude and desalted extracts. This correlates with the differences in the effect of a continuous blue (or white) light treatment in the two sections of the seedling; in hypocotyls the increase in P.A.L.-activity is only transitory, in cotyledons the stimulated level of P.A.L.-activity is maintained for a longer period. The stability of P.A.L.-activity in desalted hypocotyl extracts was improved by adding D-isoascorbate, an anti-oxidant reagent. D-isoascorbate alone,was also effective in retaining P.A.L.-activity in unstable, desalted mustard extracts. The differences in behaviour (stability) of extracts from different parts of the seedling is to be expected if the mechanisms responsible for controlling P.A.L. levels in the various tissues is different (Grill,1968; Bellini et al.,1970; Dittes et al.,1971). Seedling extracts were not suitable for prolonged storage; as the losses in P.A.L.-activity following freezing and thawing could be reduced by adding 'stabilizing agents', but not prevented. Partial purification of extracts prior to storage may have been an improvement (as in the case of potato P.A.L. extracts), but was not tried. Whether the stability of P.A.L.

in extracts has any physiological significance, is difficult to assess, but the the possible effects of compartmentation and other 'stabilizing' mechanisms in whole cells should be considered.

The purification procedure for potato P.A.L. was based on conventional purification techniques, but differed from that used by Havir and Hanson, (1968a,1970) as there have since been several improvements in the types of ion-exchange gels and exclusion gels available; both time and labour can be saved by taking advantage of these gels, the gels require less preparation and often have a higher resolution than gels previously used (such as calcium phosphate). The greatest problem was the salt fractionation step, this is the only way of concentrating samples containing large quantities of protein. Following column chromatography steps, the protein content of the samples containing enzyme activity was greatly reduced, and ultrafiltration was possible as a means of concentrating the samples. Ammonium sulphate is widely used for salt fractionation, and in many cases does not affect the enzyme/protein activity adversely, but for P.A.L., considerable losses of enzyme activity occurred (some of the 'lost' P.A.L.-activity was recovered by desalting to remove the salt, but this was not very satisfactory). Fortunately an alternative was found, tri-sodium citrate; although not as selective as ammonium sulphate over the effective protein precipitation range, (ie. not suitable for step-wise fractionation), as concentration step, was far more successful than ammonium sulphate,for P.A.L.. The final step in the purification, polyacrylamide gel electrophoresis, ensures that an homogeneous enzyme preparation is obtained (especially if the procedure is repeated). Recovery of P.A.L. from the gels was not successful, this was attributed to technical difficulties, and the fact that only small samples could be separated with the size of apparatus available. (Enzymes are often unstable if excessive dilution of samples occurs, and this may have been a problem.) Fortunately this did not prevent the possibility of obtaining a purified P.A.L. sample, as sections of the gel containing P.A.L.-activity, could be used directly for the immunization. The improvement of existing

techniques and the development of new ones, such as immunoaffinity chromatography, could mean that future purifications will be more successful, and a wider range of plants used as sources of the enzyme. The purification of P.A.L. from far-red treated mustard cotyledons has recently been reported (Gupta and Acton,1979), the extraction medium used, contained D-isoascorbate and presumably increased the stability of P.A.L. extracted.

The properties of P.A.L. from the tissues investigated, potato tuber discs, mustard cotyledons, gherkin hypocotyls and tobacco cell cultures, were compared. The MW values obtained by gel exclusion chromatography for potato and tobacco were very similar; slightly smaller MWs were obtained for gherkin, mustard and Rhodotorula P.A.L.. Differences may reflect differences in the shape of the enzyme molecules, as the above method is based on values obtained for globular proteins, and separates on this basis. It has been suggested that high MW forms of the enzyme may have a regulatory role (Camm and Towers,1977), but no aggregation was observed here, for mustard P.A.L.(Schopfer,1971), or for potato P.A.L.(Havir and Hanson,1968a). In the recent purification of mustard P.A.L.(Gupta and Acton,1979), the MW obtained was lower than that obtained here, or previously reported (Schopfer,1971), but this may have occurred during the purification; as yet it is not possible to decide which is the correct MW. The pH optima of P.A.L.-activity from dark- and light-treated potato tuber and mustard cotyledons were compared: for potato P.A.L. there was no significant difference between the two treatments, however for mustard (with a slightly higher pH optimum than potato) there appeared to be a narrow pH range for P.A.L.-activity from dark extracts. Desalted extracts were used but the difference could be attributed to the presence of other compounds. Whether pH changes within the cell occur in the pH range measured is not known, but it is possible that the dark levels of P.A.L.-activity measured here were higher than would normally occur at a physiological pH ($pH \leq 7.0$). Alternatively, localized pH changes within the cell may affect P.A.L.-activity (and stability) in mustard, (Smith and Raven,1979; Flügge et al.,1980). A further difference

between potato and mustard P.A.L. was observed when Km values were measured. Negative co-operativity was obtained using concentrated potato tuber disc extracts (in agreement with Havir and Hanson,1968b; Lamb and Rubery,1976c), but not with crude supernatant extracts assayed at pH 7.5. Mustard P.A.L. did not show negative co-operativity either (using crude desalted extracts, and in agreement with the results of Gupta and Acton,1979, using purified mustard P.A.L.), but Km values for P.A.L. from dark-treated cotyledons and hypocotyls were twice those obtained for far-red treated or radicle extracts. The effect of temperature was not determined, the pH optima curves being obtained under the same assay conditions as for all experiments (37°C). Tanaka and Uritani (1977a) noticed that sweet potato P.A.L. only showed negative co-operativity at much lower temperatures (10°C). Further investigations into the properties of P.A.L. from mustard seedlings, and a comparison of P.A.L. in dark and far-red extracts would be interesting, and may be important to our understanding of regulatory mechanisms in plants.

Plant tissue extracts of potato tuber discs, mustard seedlings, gherkin seedlings and tobacco cells in culture, in which P.A.L.-activity was stable for a sufficient period to allow the use of the immunological assay (immunotitration), and therefore suitable for immunological studies were obtained. The specificity of the anti-potato P.A.L. serum was demonstrated with potato P.A.L. by immunoelectrophoresis, a single precipitin line co-incident with the position of P.A.L.-activity being obtained. The antiserum was not absolutely specific as two precipitin lines were obtained on double diffusion plates, and P.A.L. was not the only plant protein present in the washed immunoprecipitates, but it is unlikely that the 'contaminant' interfered with the immunotitration results with potato P.A.L. extracts. The antiserum cross-reacted with P.A.L. from mustard cotyledons, two precipitin lines were detectable on double-diffusion plates, and with gherkin and tobacco P.A.L.; a single precipitin line being obtained with the latter two. There was no cross-reaction with Rhodotorula P.A.L.. P.A.L. protein levels were determined by immunotitration, and from this the specific activity of the

enzyme was estimated. In all the immunological tests, control serum (N.R.S.) failed to give a response (ie. no precipitin lines, no immunoprecipitation and no inhibition or activation of P.A.L.-activity).

The roles of de novo enzyme synthesis and activation of previously inactive enzyme were inferred from the specific activity estimates. A constant specific activity, ie. where the P.A.L.-activity is directly proportional to the amount of P.A.L. protein, indicates that immunologically detectable P.A.L. increases with increasing P.A.L.-activity (and decreases with decreasing P.A.L.-activity), and provided it can be assumed that all the P.A.L. present is detected, then de novo synthesis of P.A.L. protein is probably responsible for the increases in P.A.L.-activity. Decreases in P.A.L.-activity would be due to a decrease in the rate of de novo synthesis of the enzyme with an increase in the rate of removal of the enzyme, ie. a change in the turnover pattern. A changing value for specific activity is evidence for the existence of two populations of P.A.L., one active and the other inactive, or two populations of P.A.L. with different activities. The specific activity would then depend on the ratio of the two P.A.L. populations. Assuming that both populations of P.A.L. are detected equally well, an increase in P.A.L.-activity could be due to a change in the proportion of active (or most active) to inactive (or least active) enzyme molecules. An increase in specific activity is brought about either by an increase in overall activity or a decrease in enzyme protein (or a combination of both), while a decrease in specific activity is brought about either by a decrease in overall activity or an increase in enzyme protein (or again a combination of both).

In potato tuber discs, a constant specific activity was obtained for P.A.L. from discs incubated for 2-24h. after slicing, for both the dark and the light incubations. Therefore in agreement with density-labelling results (Sachar et al.,1972; Lamb and Merritt,1979), the P.A.L.-activity increase appears to be due to de novo synthesis of enzyme protein. For tissue aged for 42h., P.A.L.-activity had declined to a steady state level (Zucker,1968),

and the specific activity in the dark-incubated discs remained constant, and could therefore be due to a decrease in P.A.L. protein, probably due to an increase in the rate of removal of the enzyme molecules (in agreement with Lamb et al.,1979). In the light-incubated discs, the specific activity increased by 2-fold during this period, due to a sharp fall in the amount of immunologically detectable P.A.L protein (but not such a sharp fall in measurable P.A.L.-activity). A similar increase in specific activity occurred between 0 and 2h. after slicing (in both the dark and the light), but there was no significant decrease in P.A.L. protein; therefore the increase in P.A.L.-activity at this stage appeared to be due to an increase in activity of existing enzyme molecules. Both these increases in specific activity, reflect an increase in the P.A.L.-activity of the existing population of enzyme molecules,which could be explained in terms of changes in the kinetic properties of the enzyme molecules, rather than requiring the existence of a population of inactive enzyme molecules. Density-labelling experiments (Lamb et al.,1979) suggested that the decline in P.A.L.-activity in potato tuber discs was due to a decrease in the rate of synthesis and an increased rate of removal of enzyme, in the absence of inactivation. The immunotitration results for the light-incubated discs could be accomodated within this suggestion if one allows for a light-induced increase in activity of each enzyme molecule, occurring as the overall (total) enzyme activity declines. In freshly sliced discs, there could also be a rapid (<2h.) 2-fold increase in the activity of each enzyme molecule, which is then followed by the synthesis of new enzyme molecules. The respiration pattern of freshly sliced potato tuber discs is also anomalous (Theologis and Laties, 1978), differing from aged discs and from whole tuber in the pathways of respiration that predominate (Laties,1964; Solomos,1977). The T.C.A. cycle is relatively inoperative in freshly sliced discs, but develops during aging, and is associated with suberin synthesis at the wound site (Lang,1970). P.A.L.-activity is also highest at the wound surface, although not directly correlated with suberization (Borchert,1978). An immediate change in the

biochemical pathways appears to occur upon wounding (or slicing), which the tissue then attempts to rectify as the discs age. Returning the discs to the tuber environment immediately after slicing delays the wounding effect (Smith and Rubery,1979). It is therefore likely that immediate changes occur in P.A.L. before the rise in enzyme synthesis becomes apparent. The decline in P.A.L.-activity occurring in the presence of white light may arise via specific light-induced effects upon the mechanism of P.A.L. control, also involving changes in the kinetic properties of the enzyme. The results can also be explained in terms of two distinct pools of P.A.L.: in this case the initial pool of P.A.L. molecules contains both active and inactive P.A.L. molecules. The initial increase in P.A.L.-activity could involve activation of some enzyme molecules, this is then followed by a period of increased synthesis of the enzyme, both active and inactive pools of P.A.L. increasing, and with a greater increase occurring in the presence of white light. The increase in P.A.L.-activity still correlates with an increase in P.A.L. protein but includes an increase in the pool of inactive P.A.L. also. When the P.A.L.-activity begins to decline the rate of synthesis declines and there is an increase in removal of the enzyme pools. The difference between dark and light-incubated discs arises via a more rapid removal of inactive enzyme molecules in the light, hence the increase in specific activity.

In sweet potato tubers, immunological techniques revealed that synthesis of enzyme molecules was responsible for the increase in P.A.L.-activity following slicing (Tanaka and Uritani,1976). However subsequent experiments incorporating radio-isotopic labelling with the immunprecipitation technique showed that the rate of synthesis did not decline although the P.A.L.-activity began to decrease (Tanaka and Uritani,1977b). This was attributed to the development of an inactivating system (Tanaka et al.,1977), the inactivation was vital to the process of degradation. In potato tuber discs a similar mechanism may exist, or develop, as originally postulated by Zucker,(1968), with inactive enzyme being degraded more rapidly than active enzyme molecules, but with active enzyme molecules still being synthesised,

albiet at a slower rate. Durst (1976) postulated that the changes in P.A.L.-activity in Jerusalem arctichoke tuber tissues, were regulated via an activator whose synthesis was linked to the size of the cinnamic acid pool. When the activity of cinnamic acid hydroxylase, the next enzyme in the pathway was limiting, cinnamic acid tended to accumulate and this led to inhibition of P.A.L.-activity. In potato tuber discs incubated in the light, P.A.L.-activity but not cinnamic acid hydroxylase, is stimulated (Camm and Towers, 1973; Lamb and Rubery,1976c), therefore an accumulation of cinnamic acid may occur in the light, and this may stimulate inactivation of P.A.L. in the presence of light. As the P.A.L.-activity stabilizes at a steady-state level in older discs, the rate of inactivation and degradation will also decrease, and a decrease in the specific activity, back to that obtained throughout the earlier part of the incubation (ie. from 2h. onwards), might occur. Further measurements of P.A.L. levels in incubated potato tuber discs are required in order to check this and to identify the point at which the increase in P.A.L. specific activity begins.

The light-stimulated increase in P.A.L. level in potato tuber discs is not controlled via phytochrome, but may involve the blue-light photoreceptor as blue light also stimulates P.A.L. levels. Initially light appears to have no effect, as indicated from the immunotitration results, and the results obtained when an initial dark period was given prior to the commencement of the light treatment; the initial increase in P.A.L.-activity and that occurring in the dark is due to the 'wounding effect'. The light-sensitive period appears to be restricted to a period 4-10h. after slicing. The experiments carried out with submerged discs indicated that a reduction in oxygen may affect P.A.L. levels, via a reduction in the activity of cinnamic acid hydroxylase and/or changes in the respiratory activity. The light effect appeared to be a separate phenomenon specifically related to P.A.L.-activity, but may be influenced by the activity of other enzymes via product repression. Submersion of the discs during incubation in the presence of white light did not inhibit the P.A.L.-activity to such a great extent,

compared to the absence of light. The stimulated level of P.A.L. in the presence of light appears to be less sensitive to product repression; this may reflect differences in the kinetic properties of the light-stimulated enzyme or may be a result of the rate of enzyme turnover occurring in the light. Obviously measurement of P.A.L. levels in submerged discs would be extremely useful to these investigations, helping to separate the light-induced effect from the wounding effect. Measurements of the rates of P.A.L. synthesis and degradation could also be carried out using a combination of radio-isotopic labelling and immunoprecipitation.

Immunotitration experiments were also carried out with extracts of mustard seedling tissue. Both double-diffusion and immunotitration suggested that the amount of P.A.L. protein in cotyledons remained unchanged following far-red light treatment, although the P.A.L.-activity increased. The simplest explanation, would be that the increase in P.A.L.-activity was due to activation of previously inactive enzyme molecules present. If this was the case, it would also be expected that P.A.L. serves an important role in photomorphogenesis, otherwise the presence of a pool of inactive enzyme molecules would appear to be rather extravagent. It has been claimed that phenolic compounds may be important regulatory compounds involved in development and growth (Russell and Galston,1967; Kefeli and Kutaček,1976; Koch and Wilson,1977; Stonier et al.,1979; Shairpov,1979; Tissut et al.,1980), and these compounds may be involved in phytochrome-mediated growth responses, especially as P.A.L. levels are also mediated via phytochrome.

The specific activity of P.A.L. in radicles from dark-grown mustard seedlings and in hypocotyls from far-red treated mustard seedlings was the same,(being slightly less than that in cotyledons from far-red treated seedlings),this suggests that P.A.L.-levels in these organs are under a different control mechanism than P.A.L. in cotyledons. It may be that specific phenolic compounds are involved in the regulation of the development within the cotyledons at this stage. Far-red light does not affect P.A.L. levels in the hypocotyls to the same extent, and this may be correlated with

differences in the far-red-induced responses in cotyledons and hypocotyls. As the specific activity in the hypocotyl and radicle extracts was near to that of far-red treated cotyledons it may be inferred that there is little inactive P.A.L. present in hypocotyls and radicles, but more experiments studying P.A.L. levels in all organs of dark- and far-red-treated mustard seedlings are needed before any conclusions can be drawn. These results are open to criticism as the specificity of the antiserum in precipitating P.A.L. from mustard extracts was not fully verified, and at this stage it is not possible to rule out the suggestion that other factors were responsible for the failure of the antiserum to precipitate P.A.L. as efficiently from dark cotyledon extracts. Similar experiments have recently been carried out on P.A.L. levels in mustard cotyledons (Acton, personal communication). The results obtained with several immunological techniques, were conflicting; double-diffusion tests suggested that the amount of P.A.L. protein in both dark and far-red treated cotyledons was the same (in agreement with the results given above), but their immunotitration experiments indicated that P.A.L. protein content was proportional to the amount of measurable P.A.L.-activity (conflicting with the results given above). While the second method is likely to provide the more convincing results, these are dependent on the specificity of the antiserum. The antiserum used in these experiments was raised against P.A.L. purified from far-red treated cotyledons; one of the problems encountered was a decrease in the amount of antiserum required with higher P.A.L.-activities, this effect tends to suggest that the conditions used in the immunotitration experiment were in need of improvement. Unfortunately the main issue, regarding the mechanism behind changes in P.A.L. levels in mustard cotyledons remains a contentious issue. Density-labelling experiments have proved inadequate for studies on this enzyme in mustard, due to the fast turnover rate (Acton and Schopfer, 1975). Hopefully further immunological studies, and the use of more refined techniques (not dependent on measuring P.A.L.-activity), preferably in combination with radio-isotopic labelling, will clarify the situation.

Finally, a brief look at P.A.L. levels in gherkin hypocotyls and tobacco cell cultures using immunotitration indicated that the increase in P.A.L.-activity was due to de novo synthesis of the enzyme. The problems encountered in the immunotitration experiments with second antibody were demonstrated for gherkin extracts; at higher antiserum volumes and in the presence of second antibody (which was added to ensure complete immunoprecipitation), the P.A.L.-activity in the supernatants was higher than expected. This suggested that either, the enzyme-antibody complexes were dissociating, or that conformational changes in the P.A.L. molecules were produced, which resulted in a higher than expected P.A.L.-activities.

P.A.L.-activity in gherkin cotyledons was also stimulated by light treatment, but did not then decline (over a 24h. period); this may be partly explained by the stimulation of cinnamic acid hydroxylase that also occurs in the cotyledons, but not in the hypocotyls (Billett and Smith,1980). P.A.L. levels in gherkin hypocotyls and cotyledons should be compared.

In tobacco cell cultures P.A.L.-activity increased within 24h. from subculturing the cells on to fresh medium; the subsequent pattern of P.A.L.-activity was then dependent on the balance of plant growth substances in the medium. This has been shown previously for tobacco cell cultures (Kubci and Yamada,1978) and also for cultures of rose (Davies,1972), and bean (Haddon and Northcote,1975; Bevan and Northcote,1979). The results of immunotitration experiments showed that in the stock medium (auxin and cytokinin level 10-fold higher than the experimental medium), where the P.A.L.-activity remained at a fairly high level for 4 days before declining, the specific activity remained fairly constant throughout. This is most simply explained in terms of changes in the rate of P.A.L. turnover, ie. synthesis and degradation. In the experimental medium, P.A.L. levels declined after this initial peak in activity, but showed a definite second peak 8-10 days after the transfer. The specific activity of P.A.L. during the first peak was similar to that obtained in the stock medium, but the second peak and subsequent decline in P.A.L. gave a lower specific activity. This could be

due to the appearance of, or the accumulation of a pool of inactive enzyme molecules or degradation products. In parsley cell suspension cultures, increases in P.A.L.-activity were attributed to increased rates of enzyme synthesis, due to an increase in the amount of m.R.N.A. coding for the enzyme (Hahlbrock and Schröder,1975). In older cultures, the correlation between the amount of translatable m.R.N.A. and measurable P.A.L.-activity tends to collapse, and this was attributed to degradation of the m.R.N.A. (Schröder,1977). An alternative explanation would be that the effects of nitrogen starvation are becoming apparent in the older cultures, and this upsets the type of regulation previously in operation. In cultures of Pinus elliotii (Lau et al.,1980), the results of inhibitor studies have suggested that activation mechanisms are operative. In Phaseolus vulgaris cultures (Dudley and Northcote,1979) there was no increase in the amount of m.R.N.A. coding for P.A.L. in tissue with stimulated levels of P.A.L.-activity, indicating that m.R.N.A. was not limiting, and it was suggested that m.R.N.A. was stored for a while before enzyme synthesis began.

Attempts to find a coherent theory to fit all the available evidence, is hampered by the volume of information and experimental results relating to P.A.L. levels in a wide range of plants. It seems likely that de novo synthesis of the enzyme occurs in all cases, but whether changes in the rate of enzyme turnover, or activation are responsible for the increases in P.A.L. activity may depend upon the tissue, ie. the section of plant and not just the plant species. A combination of two or more mechanisms operating within the plant at appropriate times, dependent upon the requirements of sections of the plant, would seem to be the most likely system.

5.2 DISCUSSION OF A.A.O. RESULTS

The phytochrome control of A.A.O. levels in mustard was first demonstrated by VanPoucke et al.(1969), using a manometric assay to measure enzyme activity. Studies on A.A.O. were facilitated by the development of a simple spectrophotometric assay (Drumm et al.,1972), enabling density-labelling experiments to be carried out. The results from two laboratories both suggested that the increase in A.A.O.-activity was due to de novo synthesis of the enzyme (Attridge,1974; Acton et al.,1974). The role of ascorbic acid in the plant is unclear, although it has been implicated in the photosynthetic electron transport chain (Elstner and Kramer,1973; Epel et al.,1973) and in the development of cyanide-insensitive respiration (Arrigoni et al,1976; Arrigoni et al.,1977). It would therefore appear to be involved in the energy-coupling processes within the cell, and may be important in development. The interpretation of density-labelling experiments has been questioned recently (Lamb and Rubery,1976); the technique is not capable of detecting inactive enzyme, so immunological techniques have been applied to the study of A.A.O. levels.

A.A.O. was readily available, and an antiserum against the enzyme was successfully raised in rabbits. A quantitative assay technique which required only small volumes of antiserum was desired, so radioimmunoassay was chosen. This technique is particularly sensitive and ideal for measuring low levels of antigen, and has only recently been applied to measuring plant growth substances (Walton et al.,1979; Weiler,1980) and secondary products (Weiler and Zenk,1976; Trewner and Zenk,1978; Mansell and Weiler,1980), as well as larger macromolecules including enzymes (Iki et al.,1978; Sexton et al.,1980; Tucker et al.,1981) and even phytochrome (Hunt and Pratt,1979), in plant tissue extracts.

Before proceeding with the radioimmunoassays, the specificity of the antiserum was checked by double-diffusion tests and immunotitration with the commercial preparation of A.A.O., which was used as the antigen to raise

the antiserum. Mustard seedling extracts also cross-reacted with the antiserum, and on double-diffusion plates showed partial immunological identity with the commercial A.A.O. preparation from Cucurbita sp.. A standard curve for the radioimmunoassay was prepared using Cucurbita A.A.O., and a linear plot (logit b plot, see Section 4.2.7.B) obtained. By substituting the dilution of Cucurbita A.A.O. for samples of the plant extract containing an unknown amount of A.A.O. protein, the radioimmunoassay was applied to mustard cotyledon extracts from dark-grown and far-red-treated tissue. The mustard extracts did not appear to cross-react well with the antiserum in the assay. There are several factors which may have contributed to this, such as the need to use a second antibody, which prolongs the period required for the assay, and the presence of large amounts of protein in the extracts; factors which may have emphasized the differences between the Cucurbita and mustard A.A.O.. The results that were obtained however, indicated that there was no difference in the amount of A.A.O. protein in the extracts from the two treatments, although there was a 5-6-fold difference in the measurable A.A.O.-activity. As discussed in Section 5.1 for P.A.L. results, the simplest interpretation of these results is that the increase in enzyme activity arises via activation, and that the pool of A.A.O. present in dark-grown cotyledons consists of mostly inactive enzyme molecules. However, this is not the only possibility. Similar results have recently been obtained for A.A.O. levels in mustard, using rocket immunoeletrophoresis to measure A.A.O. protein (Newbury and Smith,1981). It has been suggested that these results may be reconciled with density-labelling evidence, if the changes in A.A.O.-activity are attributed to de novo synthesis of the enzyme, but which is masked by a large pool of inactive A.A.O., not directly involved in the response,(large enough to hide any increase in A.A.O. protein). As absolute levels of A.A.O. in mustard have not been measured yet (only relative amounts have been indicated by both sets of experiments), it is not possible to decide which mechanisms is operating. If the latter mechanism is found to be correct, then it will be necessary to study the role of the

A.A.O. in the plant to explain the need for a large pool of inactive enzyme molecules, and to see whether the excess protein is eventually degraded and made available to other biochemical processes in the cell.

The radioimmunoassay was found to be much more successful with extracts of plants more closely related to Cucurbita . For this purpose pumpkin seedlings were chosen (Surprisingly gherkin was only as successful as mustard, but this may have been because A.A.O. levels were particularly low in the extracts tried.) When estimates of specific activity for A.A.O. levels in pumpkin cotyledons were compared with the increases in A.A.O.-activity, a large increase in specific activity was shown to accompany the increased activity. In pumpkin radicles the specific activity remained constant although the enzyme activity increased. It would therefore appear that in the cotyledons activation of existing inactive enzyme molecules occurs, but in the radicles the increase in activity is due to de novo synthesis,or involves modification of existing, but immunologically unrecognisable, enzyme molecules. Closer examination of the cotyledon results reveals that in the dark, the initial increase in A.A.O.-activity is accompanied by an increase in specific activity, but the further increase in A.A.O.-activity (after 72h.) is not. In the light, a similar pattern occurs, only beginning sooner than in the dark, but then a second increase in specific activity occurs. This may indicate the operation of two mechanisms, ie. activation and de novo synthesis in the light, compared to a switch over from activation to synthesis in the dark. Whether this response is a phytochrome-mediated response or not, was not determined but the enzyme appears to be involved in the light responses occurring during germination of the seedlings.

5.3 GENERAL DISCUSSION

The photocontrol of enzyme levels in plants was the subject of these investigations; by using immunological techniques it was hoped to be able to distinguish between the two main types of mechanisms, generally regarded as being responsible for changes in enzyme activity, namely de novo synthesis and activation. In potato tuber discs P.A.L.-activity increases shortly after slicing of the tuber, in both the dark and the light; in both cases it was found that de novo synthesis was responsible for the increased level of activity, with the light-stimulated level of activity being due to a faster rate of synthesis. Synthesis was implied by the increase in immunologically recognisable P.A.L. protein. Similarly the subsequent decrease in P.A.L.-activity was accompanied by a decrease in the amount of P.A.L. protein, with the rate of removal of the enzyme being faster in the light-incubated discs. However there was also evidence which indicated that the P.A.L.-activity could increase by up to 2-fold without an increase in the amount of P.A.L. protein; this occurred during the first 2h., whether the discs were incubated in darkness or in the light, and again during the decline in overall activity for the light-incubated discs. This was taken as evidence for 'activation' of either the enzyme population or individual enzyme molecules. Activation of the existing enzyme pool requires that part of that pool consists of inactive enzyme molecules, and this possibility was not favoured as there appeared to be a good correlation between enzyme activity and enzyme protein for the main increase in P.A.L.-activity. The alternative was that the properties of the enzyme molecules changed, giving twice the amount of measurable enzyme activity. The fact that potato P.A.L. shows some negative co-operativity, which is more evident at pH 8.8, than at pH 7.5 may be relevant, and suggests that the kinetic properties of this enzyme are capable of change. The properties of P.A.L. from peas appears to be dependent on whether the plants were grown in darkness or in light (Attridge and Smith,1973a). Other enzymes are also known to be affected by

the light treatment received by the plant, such as phosphoenolpyruvate carboxylase in sugar cane (Goatly and Smith,1974). An example of changes in kinetic properties is shown for phosphofructokinase,between the preclimacteric and climacteric,in banana; the substrate concentration giving half the maximum enzyme activity decreases while the enzyme activity increases (Salminen and Young,1975). Phosphofructokinase also shows negative co-operativity, and is important in controlling the influx of substrates into the glycolytic pathway (Dixon and ApRees,1980). The conditions of extraction may also affect the kinetic properties of enzymes (Bahr and Jensen,1974; VanSteveninck,1975). Without further studies it is not possible to decide whether some form of allosteric mechanism is involved in controlling P.A.L.-activity, as well as changes in the rates of enzyme turnover.

In mustard cotyledons, the increase in P.A.L.-activity following far-red light treatment, was not accompanied by an increase in P.A.L. protein, and so the results were in favour of the activation of existing enzyme molecules. There was also a marked difference in the stability of P.A.L. from dark-grown and far-red treated tissue, which correlated with the degree of stimulation by the light treatment of the P.A.L.-activity. This is similar to the results obtained with nitrate reductase, where the ammonium-induced enzyme appears to be less stable than the nitrate-induced enzyme (Mehta and Sriroastava,1980). In view of the results obtained previously concerning P.A.L. levels in mustard cotyledons, (discussed in the Introduction, Section 1), a more detailed study is necessary before this issue can be settled.

In gherkin hypocotyls and tobacco cell cultures the immunological results indicated that P.A.L.-activity increased as a result of de novo synthesis of the enzyme. There is strong evidence in support of de novo synthesis of P.A.L. in UV-irradiated parsley cell cultures (Schroder,1977). In tobacco cell cultures a second peak of P.A.L.-activity was obtained when lower auxin and cytokinin concentrations were present in the fresh medium, and it was likely that a pool of inactive P.A.L. molecules had accumulated following the first peak of P.A.L.-activity, and that this contributed to the second

peak of P.A.L.-activity. It would appear that an inactivating mechanism develops, although the inactivated enzyme molecules may not necessarily be involved in any subsequent increase in enzyme activity. It is thought that an inactivating system also develops in sweet potato tuber discs (Tanaka et al.,1977) and radish cotyledons (Huault and Klein,1979). In gherkin hypocotyls, cold treatment of the seedlings, or excision leads to an increase in P.A.L.-activity (Engelsma,1968,1970); and it was suggested that an inactivating system was involved, but was not operative at low temperatures In red cabbage P.A.L.-activity does not decline,indicating that a P.A.L. inactivating system is not operative (Engelsma,1970a).Product repression has since been found to be important in controlling P.A.L.-activity in gherkin hypocotyls (Engelsma,1974; Johnson et al.,1975; Billett and Smith, 1980), and also in Jerusalem artichoke tubers (Durst,1976) and potato tuber discs (Lamb and Rubery,1976b). The level of cinnamic acid which has accumulated in the tissue may control the inactivating system.

The immunological studies on A.A.O. levels were also interpreted in terms of both de novo synthesis of enzyme and activation of existing enzyme molecules,for pumpkin seedlings, although in pumpkin radicles only synthesis appeared to occur. In mustard cotyledons, the results were the same as those obtained for P.A.L. levels, and were in favour of activation mechanisms being operative. These results are surprising as it was thought that A.A.O. levels in mustard cotyledons were altered by changes in the rate of de novo synthesis (Attridge,1974;Acton et al.,1974). Unfortunately it was not possible to measure absolute levels of A.A.O. protein in mustard, nor of P.A.L. protein in the same tissue, in these investigations, but antiserum against enzymes from mustard would enable this to be done, and help to solve this problem.

The two main mechanisms involved in regulating enzyme levels in plants would appear to be synthesis/degradation and activation/inactivation. The exact points of control however have still not been established. De novo synthesis of the enzyme may be immediate if the m.R.N.A. coding for the

enzyme has been transcribed already and is stored within the cell, or may not occur until the m.R.N.A. has been freshly transcribed (in which case a lag phase would be observed before the enzyme activity increased). The newly synthesised enzyme molecules may be active, or may be synthesised in an inactive form; in the latter case, these may be activated immediately or require modification before the activity could be expressed. The stability of the enzyme may be another potential control point, and this may be dependent on the presence of other factors, or conditions within the cell. It is also possible that localized effects within the cell may occur and this may lead to a confusing picture, when attempting to explain results in simple terms.

P.A.L. is usually extracted as a soluble enzyme, but it has been found in association with organelles (eg. spinach chlorplasts, Nishizawa et al.,1979) and with membrane material, and it was suggested that it may be part of an enzyme complex (Czichi and Kindl,1975,1977). In the purification of P.A.L. from maize, Havir (1979) measured appreciable amounts of carbohydrate associated with the P.A.L., and attempts to remove the polysaccharide component resulted in losses of P.A.L.-activity, possibly indicating that this was an integral part of the enzyme *in vivo*. In *Xanthium* the level of P.A.L.-activity appears to be dependent on photosynthesis, and is stimulated in the presence of an exogenous supply of carbohydrate (Zucker,1969). A similar effect was reported using strawberry leaf discs (Creasy,1968), and sunflower (Creasy et al.,1974). Estimates of P.A.L.-activity using an *in vivo* assay for P.A.L. (Amrhein and Gödeke,1976), indicated that P.A.L. levels in leaf discs of buckwheat were stimulated whether the discs were incubated in water or on sucrose solution, whereas the *in vitro* assay only showed stimulation in the sucrose-incubated discs.It was suggested that the presence of sugars, reduced the inhibition of P.A.L., and that possibly a specific inhibitor was involved. It would appear that in these tissues P.A.L.-activity is closely linked to photosynthesis, and other examples of only white light being effective may be explained in these terms.

One of the greatest problems in photomorphogenesis has been relating phytochrome-mediated responses to the level of phytochrome in the plant. In several cases, it has been necessary to postulate the presence of two or more pools of phytochrome (Schopfer and Mohr,1972; Tong and Schopfer,1978). Wagner and Mohr (1966), explained the differences in the degree and timing of phytochrome-mediated responses in terms of the state of primary differentiation of the tissue, upon which the subsequent pattern of secondary differentiation was dependent. There appear to be two types of photocontrol,'photomodulation', which covers those processes that are affected immediately, and'photodetermination', which covers those processes where the response is initiated early on, but is not apparent until later (Schopfer,1972a;Steinitz et al.,1976). An understanding of the primary reaction of phytochrome would be invaluable in sorting out the apparent diversity of phytochrome-mediated responses. Not all photoresponses are controlled via phytochrome, many blue light and UV-light effects have been reported, as well as white light effects. The blue- and UV-light effects may be regulated via cryptochrome, the blue-absorbing photoreceptor; it has also been suggested that there is a link between these and the phytochrome-interactions (Drumm and Mohr,1978; Kochhar et al.,1981).

The success of plants lies in their ability to utilize the radiant energy of the sun, and to cope with a range of environmental conditions. One of the advantages of eucaryotes over unicellular organisms, is the mechanism of protein turnover, this allows new enzyme molecules to be synthesised continuously, without necessarily increasing the size of the enzyme pool. Not only does this save valuable resources from being tied up in one form within the cell, but also allows interaction of different pathways within the cell and vital co-ordination of cellular processes. As a large part of this investigation was concerned with P.A.L. levels it is worth considering the role of secondary plant products in the plant. The products and intermediates of secondary metabolism must form an integral part of the overall metabolism, as the formation of these products requires a supply of suitable substrates.

P.A.L. is the first enzyme in the pathway leading to the phenylpropanoid compounds, this pathway branches from primary metabolism at L-phenylalanine, a major aromatic amino acid also required for protein synthesis. It is therefore not surprising that the two pathways are in competition, and in some cases where P.A.L. does not appear to be limiting, it is the level of L-phenylalanine that is the rate-limiting step, as has been suggested for flavonoid biosynthesis (Creasy,1971). Many of the secondary plant products form coloured pigments which are important in flowering and pollination mechanisms; indeed phenolic compounds have been implicated in the detection of daylength (Engelsma,1979, and in flowering (Zucker et al.,1965). Phenolic compounds are also involved in interactions with plant growth substances (Tanaka and Uritani,1979; Miller,1978; Baranov,1979), which may be important in modulating the control of growth by these substances, and processes such as lignification (Margio and Boudet,1980). Phenolic compounds have also been implicated in responses to infection (Rathmell,1973; Henderson and Friend,1977). The importance of secondary compounds has been recognized, but the task of unravelling the biochemical processes involved is even more daunting than tackling those mechanisms underlying photomorphogenesis.

It is often difficult to decide whether the results of in vitro experiments have any physiological significance, because of the disruption of the internal structure of the cell and the loss of compartmentation, and the possibility that the subject of the investigations may be modified during the extraction. If it becomes possible to predict what will happen to a plant grown under specified conditions, then it is likely that we are on the right track, otherwise it is difficult to be sure. To quote P.Valery, (Mackay,1977), "Science means simply the aggregate of the recipes that are always successful. All the rest is literature".

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ABSTRACT

"Immunological Studies on the Regulation of Enzyme Levels in Plants."

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Phenylalanine-ammonia-lyase, P.A.L. (E.C. 4.3.1.5) was purified from white light-incubated potato tuber discs, and antibodies against the purified P.A.L., raised in rabbits. Immunological techniques, based on the ability of the anti-potato P.A.L. serum to form immunocomplexes specifically with P.A.L., were used to measure P.A.L. protein levels in plant tissue extracts. Plants in which P.A.L.-activity appears to be under photocontrol were investigated. In potato tuber discs, the changes in measurable P.A.L.-activity were accompanied by changes in the amount of immunologically detectable P.A.L. protein; however, there was no apparent increase in P.A.L. protein for the phytochrome-mediated increase in P.A.L.-activity in mustard cotyledons. P.A.L. levels in gherkin hypocotyls and tobacco cell suspension cultures were also investigated. The results were discussed in terms of the roles of de novo enzyme synthesis, activation/inactivation and allosteric mechanisms, in the photocontrol of P.A.L. levels in these plants.

Antibodies were also raised against a commercially available, purified preparation of ascorbic acid oxidase, A.A.O. (E.C. 1.10.3.3), from Cucurbita sp.. A radioimmunoassay was developed to measure A.A.O. levels in plant tissue extracts. Changes in A.A.O.-activity in germinating pumpkin seeds, in the presence and absence of white light, were followed, and the amounts of A.A.O. protein determined. Activation or modification mechanisms and de novo enzyme synthesis were involved in the increase in A.A.O.-activity. A.A.O. levels in mustard cotyledons were also investigated.

The results indicate that the photocontrol of enzyme levels operates via several mechanisms, either acting together or in a co-ordinated manner, within each plant; allowing the activity of specific enzymes to be modulated by a range of environmental factors, including light, wounding or infection and level of nutrients, and also by the developmental stage of the plant.