Analytical Determination of Ribulose-1,5-Bisphosphate Carboxylase / Oxygenase Using Monoclonal Antibodies

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ABSTRACT

Monoclonal antibodies produced against a partially purified ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC/O-ase) from *Skeletonema costatum* were used to study the enzyme.

To establish that the antibodies were monospecific for the enzyme in a crude plant extract, a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted on the crude plant extract and the separated proteins were electrotransferred onto nitrocellulose paper (Western blot) where they were available for immunodetection. It was found that all the protein bands seen on the gel were successfully transfered onto the nitrocellulose paper, but the only band that immunostained was the one corresponding to the large subunit (LS) of RuBPC/O-ase. It was concluded that the antibody was monospecific for the LS of RuBPC/O-ase.

An attempt was made to immunopurify RuBPC/O-ase from a crude extract using a column of CN-Br activated sepharose 4B onto which the monoclonal antibodies were immobilized. This was not successful due to the fact that the antibodies had too high an affinity for the enzyme. This conclusion was reached after repeated failure to elute the enzyme off the column. Its presence on the column was shown by boiling the sepharose beads in SDS sample buffer and perfoming an SDS-PAGE and a Western blot on the soluble phase which contained

the denatured protein. After failing to immunopurify, another method of obtaining a pure enzyme from a crude extract was used. This involved ammonium sulphate precipitation at 50% followed by sucrose density gradient ultracentrifugation. The resulting enzyme was electrophoretically pure when visualized by staining with Coomassie brilliant blue. An immunoquantitation assay which involved a modified Enzyme Linked Immuno Sorbent Assay (ELISA), was worked out using pure spinach RuBPC/O-ase (Sigma). The ascites solution was titrated to determine an appropiate antibody dilution (limiting dilution) to be used in the assay. A standard curve was successfully constructed showing a linear relationship between the logarithm of the enzyme concentration and the light absorbance at 490nm by the reaction solution. The successful construction of a standard curve indicated that unknown enzyme concentrations in crude plant extracts can be determined by extrapolating from the absorbance on the standard curve if the extracts are reacted with the antibody on the ELISA.

Since the antibody had been found to be monospecific for the LS, it was assumed that it would readily cross-react with enzymes from other photosynthetic species. This would enable unlimited use of the quantitative assay. When cross-reactivity was assayed for on the Western blot, it was non-existent. The antibody stained only the enzyme from *Skeletonema costatum*, the antigen donor. When a dot immunobinding assay of crude extracts was conducted, extensive cross-reactivity was found, but this method could not identify the cross-reacting molecule as being the RuBPC/O-ase. To resolve this problem of identity without using the Western Blot, an SDS-denatured pea (*Pisum sativum*) extract was separated by gel permeation chromatography using a TSK 3000

column. The RuBPC/O-ase fraction (as indicated by SDS-PAGE) was examined for antibody reactivity using a dot immunobinding assay. The pea enzyme cross-reacted with the antibody. Cross-reactivity had already been shown on the ELISA with pure spinach RuBPC/O-ase, which had also been used for assaying for positivity when cloning the hybridoma. It was concluded that the antibody was cross-reacting between species and failure to detect cross-reactivity on the Western Blot was a consequence of physical artifacts during the transfer.

Monoclonal antibodies against RuBPC/O-ase, having been shown to be monospecific for the LS of RuBPC/O-ase, are hereby presented as a convenient tool for qualitative and quantitative determination of RuBPC/O-ase in soluble plant extracts.

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Chapter I

INTRODUCTION

General History of RuBPC/O-ase

The existence of RuBPC/O-ase was first recognized as early as 1947 by Wildman and Bonner when they observed a major protein in green leaves and called it Fraction 1 protein because of its homogeneity. In 1956, Weissbach *et al.* purified the enzyme and showed that it formed PGA from CO_2 and ribulose bisphosphate (RuBP). The physical properties, including sedimentation coefficients, were identical to the Fraction 1 protein of Wildman and Bonner (1974). RuBPC/O-ase often comprises more than 50% of the soluble leaf protein, which probably makes it the most abundant protein in nature (Kung 1976).

Many reviews on the properties of RuBPC/O-ase have been written, (Akazawa, 1970; Buchanan and Schurman, 1973; Kung, 1976; McFadden, 1973; McFadden and Tabita, 1974; Siegel *et al.* ,1972; Jensen and Bahr, 1971; Lorimer,1981; Miziorko and Lorimer,1983) so that now much is known concerning its biosynthesis and its genetic regulation by both nuclear and chloroplast genomes. It has been used as a genetic marker to probe many nucleocytoplasmic relationships in plants and has been used as a tool to follow the evolution of autotrophy (McFadden,1973; McFadden and Tabita,1974; Takabe and Akazawa,1975).

Role of RuBPC/O-ase in Photosynthesis

All organisms with the ability to assimilate carbon dioxide to form sugars do so through the action of RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE / OXYGENASE (RuBPC/O-ase). Even in C4 plants where CO_2 is initially fixed as a four-carbon acid, decarboxylation of this acid has to take place, releasing the CO_2 to be fixed by RuBPC/O-ase.

Summarizing the parameters influencing photosynthetic rates, Boardman (1977) stated that the capacity of light saturated photosynthesis should be independent of the efficiency of light absorption and the primary photochemistry. It should be influenced by one or more of the dark steps of photosynthesis (Gaastra,1959) which include : the resistance to CO_2 diffusion at the stomates; the rate of diffusion of CO_2 from the cell wall to the chloroplast; the carboxylation reaction and possibly other reactions of the carbon reduction cycle; and the rate of photosynthetic electron transport and photophosphorylation.

Curves relating photosynthetic rates to light intensity in plants, particularly C3 plants, show that the photosynthetic rate approaches a limiting value at high radiation (Black,1973; Gaastra,1959) with CO_2 becoming a limiting factor under these conditions. It has thus been suggested that under saturating light intensities, the physical resistance to CO_2 diffusion in the mesophyll tissue determines the photosynthetic rate (Gaastra,1959). But when Holmgren *et al.* (1965) measured the stomatal resistance of a number of plant species with differing light-saturated rates of photosynthesis, they found that the minimal stomatal resistance for CO_2 at ambient CO_2 concentration varied widely from an average of 0.72 sec/cm for *Helianthus annus*, a sun species, to 21.0 sec/cm for *Ciraea lutetiana*, a shade

species. All the species were grown under identical artificial light conditions. The mesophyll resistance was much lower in *Helianthus* (2.45 sec/cm) than in *Lamium galeobdolan* a shade plant (14.3 sec/cm). Photosynthesis by leaves of *C. lutetiana* showed non-linear dependence on CO_2 concentration, whereas a linear dependence was observed for the sun species. It would seem, therefore, that photosynthesis in the shade plants was limited by an interaction of limiting factors, whereas in the sun plants the transfer of CO_2 appeared to be a dominant factor.

On measuring the diurnal courses of the rate of CO_2 exchange and the stomatal resistence for an *Alocasia* plant on the forest floor of the Queensland forest (shade plant), Bjorkman *et al.*,1972 found that there was little variation in CO_2 concentration on the forest floor. The average concentration was 360 ppm with a minimum of 330 ppm and a maximum of 390 ppm. There was no apparent diurnal trend. Stomatal conductance was very low in the morning and the evening. Its diurnal fluctuation corresponded with those of light and CO_2 uptake, suggesting that the stomatal aperture was regulated by light intensity. They concluded that the stomatal resistance imposed only a small limitation on photosynthetic rate by the rain forest species on the forest floor.

Bjorkman *et al.* (1972) further observed that at all light intensities below saturation, the leaves adjusted their stomatal apertures so that the resistance was well below the minimum needed to sustain the measured photosynthetic rate of CO_2 uptake. They found that even during periods of relatively bright sunflecks, when CO_2 uptake was light-saturated for part of the time, stomatal resistance did not impose more than a small restriction on rate the of light-saturated photosynthesis. They thus concluded that the low capacity for light-saturated CO_2

uptake by the shade plants could not be attributed to restrictions to CO_2 diffusion from the external air to intercellular spaces. This conclusion was consistent with the report of Mache and Loiseaux (1973) that CO_2 fixation by isolated chloroplasts of the shade plant *Marchantia polymorpha* was saturated at the same light intensity as CO_2 fixation by intact thalli. Growth of the thalli was also saturated at the same light intensity.

Goodchild *et al.* (1972) showed that levels of soluble protein are much higher in sun species than in shade plants, both on the basis of chlorophyll or that of fresh weight. Bjorkman (1968) found that the low content of soluble protein in shade plants resulted in a lower carboxylase activity, which correlated with the amount of RuBPC/O-ase, the major soluble protein of a leaf. The activity of RuBPC/O-ase in extracts from five sun plants averaged tenfold higher than the activity of five shade plants when expressed on the basis of leaf area, fourfold higher on the basis of fresh weight of tissue, and sixfold higher on a chlorophyll basis. The RuBPC/O-ase activity of sun and shade plants paralleled their rates of CO_2 uptake, suggesting that the low amount of the enzyme in shade plants is at least partly responsible for their low light-saturated photosynthetic rate.

Where photosynthetic product demand was increased by partial defoliation or shading, increased photosynthetic rates were observed within days (Thorne and Koller,1974; Wareing *et al.*,1968). The levels of RuBPC/O-ase increased (Wareing,1968) suggesting that under saturating light conditions in the field the photosynthetic rates were limited by the levels of the RuBPC/O-ase.

The total soluble protein per unit leaf area was observed to decrease with decreasing light intensity (Andreeva and Avdeeva,1970; Bjorkman *et al.*,1972;

Blenkinsop and Dale,1974). RuBPC/O-ase declined in shade leaves, but to a greater extent than total soluble protein (Andreeva and Avdeeva,1970; Blenkinsop and Dale,1974). RuBPC/O-ase activity in extracts of *Atriplex* leaves grown at different light intensities also showed a greater decline with light intensities than soluble protein (Bjorkman et al.,1972). The differences in light-saturated rates of CO_2 uptake reflected the levels of RuBPC/O-ase in *Atriplex* (Bjorkman *et al.*,1972). On shading of barley (Blenkinsop and Dale,1974) and bean (Crookston *et al.*,1975), the enzyme activity was reduced to a greater extent than photosynthetic activity. In clones of *Solidago* (Bjorkman,1968) and in soybeans grown under artificial light (Bowes *et al.*, 1972), there was a good correlation between light-saturated rate of photosynthesis and RuBPC/O-ase activity. Bjorkman(1976) found the same Km for RuBPC/O-ase isolated from various clones of *Solidago* at different light intensities, so that differences in carboxylase activity could not be attributed to a modification of the enzyme with light intensity during growth.

Shading of bean plants resulted in a 70% decrease in glycolate oxidase and malate dehydrogenase, as well as in RuBPC/O-ase (Crookston *et al.*,1975).

Most observations on the kinetic properties of RuBPC/O-ase suggest that the amount of enzyme and the degree to which the enzyme is activated under given field conditions should be considered as regulating CO_2 assimilation in saturating light.

The Genetics of RuBPC/O-ase

The Large Subunit (LS)

The most common form of RuBPC/O-ase is composed of eight large (catalytic) and eight small subunits of unknown function (Rutner and Lane,1967). A genetic analysis of the mode of inheritance of the large subunit in the genus *Nicotiana* provided the first evidence that the large subunit (LS) is encoded in the chloroplast DNA (Sakano *et al.*,1974; Chan and Wildman,1972). The LS gene is present as a single copy per chloroplast DNA molecule (Bedbrook *et al.*,1979; Rochaix and Malone, 1979). A chloroplast contains from 10 to 100-fold more DNA than can be accounted for by a single chloroplast DNA molecule (Miziorko and Lorimer,1983). Thus, each chloroplast contains, depending on the species, from 10 to 100 copies of the LS gene. In addition, since leaf cells may contain, depending on the species, from 10 to 200 chloroplasts per cell (Possingham,1980), it follows that there may be several thousand copies of the gene for the LS per cell.

The basis for the genetic analysis of the large and small subunits in the genus *Nicotiana* was the variation in the isoelectric focusing patterns of carboxymethylated subunits (Kung,1976; Wildman,1979). This technique also revealed apparent charge heterogeneity in the composition of the large and small subunits. Chemical analyses of the isoelectric variants among large subunits failed to detect any differences among them, and Gray *et al.*,(1978) concluded that the variants result from the modification of a single gene product. This conclusion was strenghtened by the findings of Coen *et al.*,1977; Bedbrook *etal.*,1979; Bottomely and Whitfield,1979; Rochaix and Malone,1979; Spreitzer and Mets,1980; Dron *et al.*,1983, that one gene for the LS appears to be present per chloroplast

chromosome. A report by O'connell and Brady (1982) indicated that the charge heterogeneity arose artifactually during the alkylation procedure that preceedes isoelectric focusing.

So the majority of evidence indicates that the LS is homogeneous, the product of a single gene.

The Small Subunit (SS)

The situation with the SS is more complex. Genetic analysis in the genus *Nicotiana* first demonstrated that the SS was inherited biparentally, and the gene was encoded with nuclear DNA (Kawashima and Wildman,1972). Now it is known that the mRNA for the precursor of the SS is transcribed in the nucleus and translated on cytoplasmic ribosomes (Dobberstein *et al.*,1978; Highfield and Ellis,1978; Cashmore *et al.*,1978), to yield a precursor of molecular weight 4000-6000 Daltons larger than the mature polypeptide.

It would appear that a multigene family encodes the SS. In pea there are seven copies per haploid nuclear genome (Coruzzi *et al.*,1983). Two cDNA clones from two different varieties of the pea (*Pisum sativum*) have been isolated and sequenced (Bedbrook *et al.*,1980; Coruzzi *et al.*,1983). There are ten nucleotide differences that translate into eight differences in the amino acid sequence of the mature polypeptide without altering its charge. While these differences might be due to the different varieties of pea, they may also reflect the presence of several species of SS mRNA (Coruzzi *et al.*, 1983).

Isoelectric focusing has revealed the presence of charge variants in the mature SS from pea (Takruri *et al.*,1981). However, no heterogeneity was observed in the amino acid sequence (Takruri *et al.*,1981). The charge variants were

attributed to the partial loss of the N-terminal methionine followed by cyclization of the glutamine residue so exposed. This same mechanism has been used to account for the occurrence of two charge variants of the spinach SS (Martin,1979). A considerable amount of proof for the heterogeneity of the SS was obtained for spinach (Martin,1979) and tobacco (Gray *et al.*,1978; Strobaek *et al.*,1976) small subunits by amino acid sequencing and tryptic peptide fingerprint analysis. In the spinach SS there is a tyrosine-proline substitution at residue 97 (Martin,1979). The tyr-pro substitution does not account for the occurrence of charge variants which are probably experimental artifacts. The occurrence of charge variants in the SS from *Nicotiana tabacum* is based on defined amino acid substitutions (Strobaek *et al.*,1976). Heterogeneity has been detected at three sites: tyr/ile at 6, asn/gly at 7, his/arg at 48 (Strobaek *et al.*,1976). These substitutions constitute strong evidence that the SS is encoded by more than one gene within the nuclear DNA. Assuming that each gene for the SS is equally transcribed and translated, the final assembled heteroenzyme would represent a population of different molecules. Two different SS genes would generate nine different heteroenzyme molecules, three different SS genes would generate fourty-five different heteroenzyme molecules, and so on (Hirai,1977). So elctrophoretically homogeneous preparations of purified enzyme might well represent a complex population of molecules. Wildman (1979) pointed out that the electrically neutral substitutions would be difficult to detect.

Physicochemical Properties of RuBPC/O-ase

The Native Enzyme

RuBPC/O-ase, as found in all photosynthetic organisms,is a protein of high molecular weight and exists as a spherical aggregate of subunits (Jensen and Bahr,1977). Higher plants (Kawashima and Wildman,1970; Siegel et al., 1972), green algae (Anderson *et al.*,1968; Givan and Criddle,1972), the hydrogen bacteria (Kuehn and McFadden,1969), some of the blue-green algae (Anderson *et al.*,1968; Kieras and Haselkorn,1968), and *Thiorhodaceae* (Akazawa *et al.*,1972; Tabita and McFadden, 1976) exhibit molecular weights around 550 000 Daltons. The enzymes from *Thiobacillus dentrificans* (McFadden and Denend,1972), *Chlorobium thiosulfatorium* (Tabita *et al.*,1974), and *Rhodopseudomonas palustris* and *R. sphaeroides* (Anderson *et al.*,1968), are of an intermediate size of about 360 000 Daltons.

The only example of an enzyme with a small size is from *Rhodospirillum rubrum* of 120 000 Daltons (Anderson *et al.*,1968; Tabita and McFadden,1974). While these three ranges may be enough to describe the molecular size of RuBPC/O-ase in nature (Anderson et al.,1968; McFadden, 1973; McFadden and Tabita 1974), there are some reported discrepancies (Akazawa *et al.*,1970). The enzyme from several blue-green algae (Tabita *et al.*,1976) and *Thiobacillus intermedius* (Purohit *et al.*,1976) have been considered to have molecular weights of about 540 000 Daltons.

Subunit Composition

In 1965, Haselkorn *et al.* observed the complex quaternary structure of Chinese cabbage RuBPC/O-ase by electron microscopy and proposed that the enzyme was a cube of about 120 Angstroms per edge having 24 subunits. Later, evidence for two types of subunits, having distinct sizes and amino acid compositions was found (Kawashima,1969; Moon and Thompson,1969; Rutner and Lane,1967; Sugiyama and Akazawa,1967; Sugiyama *et al.*, 1971). The larger of the two subunits has a molecular weight of 51 000 to 58 000 while the smaller subunit is about 12 000 to 18 000 Daltons. The holoenzyme dissociates into its subunits under a variety of denaturing conditions such as urea, sodium dodecyl sulphate (SDS), p-mercuribenzoate, and extreme pH (Akazawa,1970; Kawashima and Wildman,1970; Nishimura *et al.*,1973).

The LS is coded for by the choroplast genome; its mRNA is present in the chloroplast RNA, and it is synthesized on chloroplast ribosomes. The SS seems to be coded for by the nuclear genome; its mRNA is present in the cytoplasmic ribosomes (Blair and Ellis,1973; Criddle *et al.*,1970; Gooding *et al.*,1973; Hartley *et al.*,1975 and Kung,1976).

By combining the information from X-ray diffraction, electron microscopy, optical diffraction and crystal density, Baker *et al.* (1975) proposed that the most likely crystalline tobacco RuBPC/O-ase would be eight large and eight small subunits (L8S8). In agreement, physical and chemical studies have provided good evidence for eight copies of the large subunit (Kawashima *et al.*,1971; Nishimura and Akazawa,1973; Rutner,1970), and there are a maximum of eight binding sites for RuBP in the enzyme molecule (Siegel and Lane,1973; Wishnick *et al.*,1970).

From their immunological observations, Gray and Kekwick (1974) suggested that the small subunits might be buried within the protein with the large subunits on the surface. They found that the determinant groups detected on the isolated small subunits were unavailable for binding at the surface of the native enzyme.

Bacterial RuBPC/O-ases contrast with the enzyme from higher plants in that there is substantial variability in both the reported native molecular weights and the subunit composition of their proteins (McFadden,1980). *Rhodospirillum rubrum* is composed of only two large subunits (Tabita and McFadden,1974), and *Chlorobium thiosulfatophilum* of six large subunits (Tabita *et al.*,1974). In *Rhodopseudomonas sphaeroids* two forms of the enzyme with L8S8 and L6 structures have been isolated (Gibson and Tabita,1977). However, failure to detect the SS must be interpreted with caution (Miziorko and Lorimer,1983). *Thiobacillus intermedius* (Purohit *et al.*, 1976) and the blue-green algae *Anabaena cylindrica* (Tabita *et al.*,1976) were reported to lack the SS. Reinvestigation of this finding by other workers (Bowman and Cholett,1980; Takabe,1977; and Okabe and Codd,1980) demonstrated its presence. Both Codd and Stewart (1977) and Abel (1981) have demonstrated the ease with which small subunits can be stripped from the native enzyme by mild acid treatment, leaving a core of large subunits intact. Even in the case of eukaryotic protein (Miziorko and Lorimer,1983), it is possible to generate oligomers of large subunits, which appear to be free of small subunits upon gel electrophoresis. Thus care should be taken that the SS is not artifactually lost during purification. Many bacterial RuBPC/O-ases have been reported to have an L6S6 structure (Lawlis *et al.*,1979; Taylor *et al.*,1980; Taylor and Dow, 1980). However, after the study of Andrews and Abel (1981), and Andrews *et al.*,(1982),

on the cyanobacterial enzyme from *Synechoccus* these reports must be viewed cautiously. When the molecular weight of the *Synechoccus* enzyme was determined by pore-gradient electrophoresis, gel filtration chromatography, and density gradient centrifugation, a value of Mr 430 000 was obtained (Andrews and Abel,1981). Since the protomer molecular weight was shown to be Mr 70 000, a hexameric L6S6 structure was the most likely. However, further investigation employing equilibrium sedimentation yielded a value of Mr 530 000, which is within the range for an L8S8 structure (Andrews *et al.* 1982). Electron microscopic studies further revealed a four-fold symmetry characteristic of an octameric L8S8 structure. With the exception of the well documented dimeric L2 structure of *R. rubrum* enzyme, Miziorko and Lorimer (1983) suggested that subunit compositions other than L8S8 should not be regarded as having been very well established.

Amino Acid Sequences

The Large Subunit (LS)

Akazawa *et al.* (1978) recognized that the large subunits from various RuBPC/O-ases are similar in amino acid composition. It has become possible to ascertain that the amino acid sequence is also highly conserved. The genes coding for this subunit in maize (McIntosh *et al.*,1980), spinach (Zurawski *et al.*,1981), *Chlamydomonas* (Dron *et al.*,1982) and *Synechococcus* (Reichelt and Delaney,1983) have been cloned and sequenced, allowing the amino acid sequence to be deduced. Comparing the sequences of the evolutionary most distant eukaryotic polypeptides (*Chlamydomonas* and *Zea mays*) reveals greater than 85% homology in a total of 475 residues. This very high degree of homology is also apparent in the sequence

of the barley enzymes's LS (Poulsen *et al.*,1979). There is complete sequence conservation between residues 169 and 220, and 321 and 340. These regions contain lysine residues at 175 and 335, which have been implicated in the catalytic process by affinity-labeling studies (Hartman *et al.*,1978), as well as the lysine at 201, which is associated with the activator CO_2 (Lorimer and Miziorko,1980; Lorimer,1981). Dron *et al.*,1983, have observed that the region 161-192 contains only neutral or basic amino acids, a feature that may be of functional importance. When a negative charge was introduced by a glycine/aspartate substitution at residue 171, the resulting protein was catalytically inactive (Dron *et al.*,1983).

In contrast to the homology that characterizes the primary structures of the LS from plant enzymes is the amino acid composition and sequence of *R. rubrum* enzyme. Akazawa *et al.* (1978) had suggested that the *R. rubrum* enzyme was much different in amino acid composition from the plant enzymes, which supported the idea that the prokaryotic and eukaryotic enzymes might not be very closely related in their evolution. Hartman *et al.* (1982) elucidated 70% of the primary structure of *R. rubrum* enzyme by sequencing cyanogen bromide fragments. Only 28% of the residues were found identical to those of the plant carboxylases. However, even though the overall homology is low, two regions stand out as being highly conserved. These two regions contain the target sites for the affinity labels 2-bromoacetylaminopentitol-1,5-bisphosphate (Fraij and Hartman,1982), and pyridoxal phosphate (Herndon *et al.*, 1982). The former selectively alkylates methionine 335, which is adjacent to lysine 334. In the spinach enzyme this lysine residue is derivatized by the affinity label 3-bromo-1,4-dihydroxy-2-butanone-1,4-bisphosphate (Hartman *et al.*,1978). When

this segment of the *R. rubrum* enzyme was compared with the corresponding segment of the spinach enzyme, eight residues out of thirteen were identical. More homology was found at the lysine modified by pyridoxal phosphate, residue 175, of the spinach enzyme. This residue lies within a seven residue segment that is identical in the two species. So it remains unproven that the *R. rubrum* enzyme may have evolved independently.

The Small Subunit (SS)

The analysis of relatedness performed by Takabe and Akazawa (1975) on various eukaryotic and prokaryotic RuBPC/O-ases indicated that the SS was less highly conserved than the LS. Highly conserved portions of the SS may provide clues to the function of the SS. The regions encompassing residues 3-5, 11-21, 38-45, 49-75 and 109-117 show much more homology than can be seen in the remainder of the molecule. Not much is known about the amino acid sequences of the SS from prokaryotic RuBPC/O-ases.

The amino acid sequence of the cytosolic precursor to the SS has been deduced by cloning procedures. On processing to the mature SS, a protein of 123 amino acids is produced (Dobberstein *et al.*,1977; Highfield and Ellis,1975; Cashmore *et al.*,1978). There is no apparent homology on comparison between the transit peptide sequences from higher plants and green algae (Bedbrook *et al.*,1980; Berry-Lowe *et al.*,1982; Schmidt *et al.*, 1979). The transit peptide for the pea SS is 11 residues shorter than the one for the *Chlmydomonas* SS. It has been observed that the transit polypeptides for the SS are characterized by basic amino acids. Schmidt *et al.* (1979) suggested that this feature is involved in the binding and passage of the SS precursor through the negatively charged chloroplast envelope membrane (Neuburger *et al.*,1977).

Putting together all the observations that have been made on RuBPC/O-ase subunit heterogeneity, it seems that during phylogenetic evolution of the enzyme, genetic information has been conserved in the chloroplasts for the LS, while genetic variability occurred with the nuclear coded small subunit.

Quaternary Structure of RuBPC/O-ase

Most of what is known of the quaternary structure of RuBPC/O-ase is largely based on electron microscopy and X-ray crystallographic studies on plant and bacterial enzymes which contain eight large and eight small subunits (L8S8). Eisenberg *et al.* (1978) studied the protein purified from tobacco and proposed a bilayer model which indicated that four large subunits are stacked over a layer of another four large subunits. There is a channel between the large subunits that was easily detected by electron microscopy of negatively stained protein samples. The crystallographic data indicated a fourfold axis of symmetry, coincident with the centre of the channel formed by eclipsed rings of the large subunits. The exact location of the small subunits of the tobacco enzyme was unclear, but the model showed them arranged in the same plane as the large subunits, situated peripherally in an array compatible with the fourfold symmetry.

Bowien *et al.* (1980) studied the enzyme from *Alcaligenes eutrophus*, a chemoautotrophic hydrogen bacterium. They found some similarities between the structure of this enzyme and the enzyme from higher plants. It had four large subunits involved in forming each of two stacked layers perpendicular to the fourfold rotational axis. This model differs from the one proposed by Eisenberg *et al.* (1978) because in this model the large subunits were suggested to be U-shaped

rather than globular. In addition, in this model the small subunits are thought to be above and and below the layers of the large subunits, instead of around the edge in the same plane as the large subunits.

Function of Subunits

The Large Subunit

The observation that the *R. rubrum* enzyme, which only contains large subunits (Tabita and McFadden,1974; Schloss *et al.*,1979), can undergo activation by CO_2 and Mg^{2+} via carbamate formation (Christeller and Laing,1978; Whitman *et al.*,1979; O'Leary *et al.*,1979), and can catalyze both carboxylation and oxygenation reactions (Ryan *et al.*,1974; McFadden,1974), suggests that all the amino acids that are involved in these processes are on the large subunit. Evidence has been found that even for the L8S8 type of enzyme the same is true. Lorimer and Miziorko (1980) and Lorimer (1981) demonstrated that the activator CO_2 was trapped on the LS of the plant enzyme and that the amino group of lysine 201 linked the activator CO_2 to the protein. Miziorko *et al.*(1982), using cobalt as an exchange-inert probe of the activator cation binding site, showed that the metal ion bound directly to the amino acids of the LS of the spinach enzyme. Nishimura *et al.* (1973) and Nishimura and Akazawa (1974) established that the LS contained the catalytic site in the spinach enzyme. The work of Hartman *et al.* (1978) with active-site directed irreversible inhibitors demonstrated that the LS contained the RuBP binding site. The genetic analyses of a catalytically inactive mutant of RuBPC/O-ases in *Chlamydomonas* (Spreitzer and Mets,1980) and of its revertants (Spreitzer *et al.*, 1982) confirmed the catalytic role of the LS.

The Small Subunit

The function of the small subunit remains unknown. It is usually stated that it has a regulatory function, but how it does this, has not been elucidated. This proposition relies only on an analogy with other enzymes which contain heterologous subunits. The fact that *R. rubrum* which has no SS, undergoes activation by CO_2 and Mg^{2+}, and catalyzes both carboxylation and oxygenation means that none of these processes necessarily depends on the presence of the SS. A difference has been observed though between the *R. rubrum* enzyme and the enzyme of higher plants. The higher plants enzyme has an affinity for CO_2 that is about tenfold greater than the *R. rubrum* enzyme (20 µM versus 300 µM) (Chisteller and Laing,1978; Yeoh *et al.*, 1980; Yeoh *et al.*,1981). This may suggest that the SS enhances the affinity of the enzyme for CO_2. But this is not necessarilly true because some L8S8 enzymes which have just as poor affinities for CO_2 are known: from *Anabaena* Km (CO_2)=293 µM; (Badger,1980); from *Synechoccus* Km(CO_2)=250 µM (Andrews and Abel,1981). It has been observed that RuBPC/O-ases with SS are generally more susceptible to inhibition (competition against RuBP) by 6-phosphogluconate than those without SS (McFadden,1980; Gibson and Tabita,1977; Tabita and McFadden,1972). This was assumed to indicate differences in active-state topology or variations in protein structure that limit the ability of the enzyme to assume a conformation that would permit tight binding of the inhibitor. 6-phosphogluconate binds to the catalytic site of the LS (Badger *et al.*,1981) and also stimulates the activation of the enzyme by CO_2 and Mg^{2+} even in *R. rubrum* (Christeller and Laing,1978). So the relationship between the different sensitivities toward inhibition by 6-phosphogluconate and presence or absence of the SS is unclear.

Andrews *et al.* (1982), working with the *Synechococcus* enzyme from which the SS had been partially stripped, observed that the remaining catalytic activity was exactly proportional to the SS content. When isolated SS were added to the depleted preparations, catalytic activity was reconstituted to levels comparable with that of the native enzyme. It was then inferred that only those large subunits that had small subunits associated with them were catalytically competent.

It is still not possible to assign a particular function to the SS, but the hypothesis that it helps to maintain the protein in a stable conformation capable of activation to a state of catalytical competence seems to account for all existing information.

Activation of the Enzyme

RuBPC/O-ase is a complex enzyme that needs to be activated. It has long been known that the activity of RuBPC/O-ase could be enhanced by preincubation with CO_2 and Mg^{2+} (Pon *et al.*,1963), but there was no known molecular mechanism to account for this phenomenon. Lorimer (1981) and Lorimer and Miziorko (1983) have suggested a mechanism for the activation process of RuBPC/O-ase by CO_2 and divalent metal ions (Me^{2+}). The reaction (Fig.1) involves the formation of a carbamate with the amino group of lysine 201 on the large catalytic subunit (Lorimer and Miziorko,1980; Lorimer,1981). This is followed by the binding of Me^{2+} to form the catalytically competent complex E-CO_2-Me^{2+}. It is this complex that catalyzes both carboxylation and oxygenation (Lorimer *et al.*,1976; Badger and Lorimer,1970). Carbamate formation is a readily reversible equilibrium process which involves CO_2 (rather than carbonate or bicarbonate) and the uncharged amine as reactants (Ewing *et al.*,1980).

The final position of equilibrium (activation state) of the enzyme depends on the concentration of CO_2 and H^+. Since the activation equilibrium is shifted to the right by Me^{2+}, it is thought that Me^{2+} interacts directly with carbamate (Lorimer *et al.*,1976).

Kinetic and spectroscopic studies (Lorimer *et al.*,1976; Miziorko and Mildvan,1974) established that addition of activator CO_2 preceeded the addition of Me^{2+}, that CO_2 is the active species in the activation reaction, and that the addition of activator CO_2 is the rate determining step. From the response of the activation reaction to pH, it was concluded that the activator CO_2 reacted with a group on the enzyme with a distinctly alkaline pK (Lorimer *et al.*,1976). Studies on competitive binding (Miziorko,1979) and kinetic turnover (Lorimer,1979) have clearly established that the molecule of CO_2 which participates in activation is not the same as that which becomes fixed during carboxylation.

Activation by CO_2 and Me^{2+} seems to be a property common to all RuBPC/O-ases, regardless of their origin. Also noticeable is the fact that activator CO_2 gets bound to a highly conserved region of the LS protein (residues 168-219). Perfect homology in this region is seen between the *Chlamydomonas* and spinach enzymes (Dron *et al.*,1982; Lorimer,1981). This highly conserved region also contains the lysine residue #175 which has been identified as being within the catalytic site (Hartman *et al.*,1978). The enzyme from *R. rubrum* is activated in the same manner, with the formation of a carbamate, but the residue involved has not been conclusively determined. It is implied from the work of Bowien and Gottschalk (1982), with the activation properties of the enzyme from *Alcaligenes*, that activation of the enzyme involves also a substantial change in either its shape and/or its density because of the observed change in its sedimentation coefficient.

The role of the Me^{2+} in the activation reaction is yet to be established. Currently it is accepted that Me^{2+} coordinates with and thus stabilizes the carbamate (Lorimer,1981; Lorimer and Miziorko,1981). This view is supported by the acidic nature of the amino acids adjacent to lysine 201 (Lorimer,1981). The presence of these is likely to make the formation of a carbamate thermodynamically unfavourable, since this introduces a negative charge to an already anionic region (Lorimer,1981). So Me^{2+}, coordinated to the carbamate, and possibly also to some of the adjacent acidic groups, would neutralize these negative charges.

Catalysis

RuBPC/O-ase catalyzes a dual reaction: (a) carboxylation of RuBP to give two molecules of Glycerate 3-phosphate; and (b) oxygenation of RuBP to form a molecule each of phosphoglycolate and glycerate 3-phosphate (Fig.2) The carboxylation reaction is the primary reaction of the photosynthetic carbon reduction cycle (C-3PCR) and the oxygenation reaction is the primary reaction of the photorespiratory carbon oxidation cycle (C-2PCO).

A Mechanism For Carboxylation

Lorimer and Miziorko (1980) and Pierce *et al.* (1980) have suggested a mechanism for the carboxylation reaction which seems to be in agreement with most of what is known (Fig.3).

The essence of the mechanism is the use of the carbamate-divalent metal ion complex to stabilize the transition state intermediate, 2-carboxy,3-ketaarabinitol 1,5-bisphosphate and the carbon acid-acid which is to form the "top" 3-glycerate.

CO_2 (the carboxylating species) is a linear symmetrical molecule in which the electrons are delocalized toward the oxygen atoms rendering the carbon atom electrophilic. Carbon #2 of RuBP, to which the CO_2 ultimately becomes covalently bound, is an electrophilic centre. So it would not be favourable for carboxylation without prior arrangement of the RuBP to a nucleophilic centre (Calvin,1954).

This chemical requirement is satisfied by the formation of an ene-diol due to the removal of a proton from C-3 of RuBP (step 1). The nature of the base that brings this about is not clearly known.

The binding of substrate CO_2 may actually preceed the formation of the ene-diol, since the kinetics indicate a random order of substrate addition. Step 2 results in the quinternary complex of Enzyme-RuBP-sub CO_2-Me^{2+}-actCO_2.

Me^{2+} contributes to the polarization of the carbon-oxygen of the substrate CO_2 facilitating the electrophilic attack on the C-2 of the ene-diol (step 3) which leads to the formation of the transition state complex.

Formation of top D-3-phosphoglycerate requires that the hydrolysis of the transition state complex be stereochemically directed. Pierce *et al.* (1980) have suggested a carbanion inversion mechanism for achieving this. The expulsion of bottom 3-phosphoglycerate (step 4) leaves behind the acid-acid derivative of top 3-phosphoglycerate with the carbamate-divalent metal ion providing stability. Addition of a proton to the top face of the acid-acid yields the top D-3-phosphoglycerate.

A Mechanism For Oxygenation

RuBPC/O-ase has long been known as a carboxylase, and only recently has it become apparent that it can oxidize RuBP in the presence of molecular oxygen to produce phosphoglycolate and phosphoglycerate. This oxygenase activity was first proposed by Ogren and Bowes in 1971, basing their proposition on their observation that oxygen inhibited CO_2 fixation by RuBPC/O-ase. The evidence for phosphoglycolate as a product was obtained indirectly by assaying for glycolate phenylhydrazine by Bowes and Ogren (1971). Andrews *et al.* (1973) and Lorimer *et al.* (1973) confirmed phosphoglycolate as a product and the fact that one mole of O_2 was consumed per mole of RuBP, using ^{14}C(U)RuBP. When ^{18}O. was supplied as molecular oxygen it was incorporated into one of the carboxyl groups of phosphoglycolate, but there was no label in PGA.

The mechanism on Fig.4 (Lorimer,1973) is the most consistent with the known facts of the oxygenation reaction.

The first step is the formation of the ene-diol(ate) due to the nucleophilic attack at the active site and subsequent rearrangement. Addition of the O_2 to the ene-diol(ate) and electron transfer (steps 2&3) creates the radical pair; i.e. the radical at C-2 of RuBP and the superoxide radical anion.

Step 4 involves spin inversion, and for this to occur it is postulated that the radical reactants are held in place by the "cage" effects of the catalytic site. The peroxide formed at C-2 of RuBP then undergoes degradation to the final products by the addition and elimination of a hydroxyl ion. This way only the carboxyl group of phosphoglycolate becomes labeled with an oxygen atom from $^{18}O_2$.

Summary of the main features of both mechanisms of catalysis

CO_2 (not HCO_3^- or CO_3^{--}) is the reactive species in the carboxylation (Badger,1980; Cooper *et al.*,1969). This requires that the C-2 of RuBP be converted from an electrophilic to a nucleophilic centre. Carbon-carbon bond cleavage occurs between C-2 and C-3 in both reactions (Mullhofer and Rose,1965; Pierce *et al.*,1980). Substrate CO_2 becomes the carboxyl group of top 3-glycerate.

The hydrogen atom on C-3 of RuBP is lost to the medium during carboxylation (Fiedler *et al.*,1967). The hydrogen atom on C-2 of top 3-P-glycerate is derived from water (Hurwitz *et al.*,1956; Mullhofer and Rose,1965).

The oxygen atom on C-2 of RuBP is retained during both reactions; it becomes the oxygen atom on C-2 of top 3-P-glycerate in carboxylation (Lorimer,1978; Sue and Knowles, 1978), or one of the two carboxyl oxygen atoms of P-glycolate in oxygenation (Pierce *et al.*,1980). The oxygen atom on C-3 of RuBP is retained during carboxylation to become one of the carboxyl oxygens of bottom 3-P-glycerate (Lorimer,1978; Sue and Knowles,1978).

Only one atom of molecular oxygen is incorporated into the carboxyl group of P-glycolate during oxygenation. 3-P-glycerate remains unlabelled (Lorimer *et al.*,1973).

Evidence for Carboxylation and Oxygenation of RuBP

This section summarizes the nature of evidence which indicates that the carboxylation of RuBP is the primary reaction for the photosynthetic carbon reduction cycle (C-3 PCR), and that the oxygenation of RuBP is the primary reaction for the photorespiratory carbon oxidation cycle (C-2 PCO). It should be

obvious from this section that for the mechanisms suggested for these reactions, there is good agreement between physiological evidence on the one hand and biochemical properties of the RuBPC/O-ase on the other.

The Carboxylation of RuBP as the Primary reaction of the C-3 PCR

(a). 3-Phosphoglycolate as the first detectable product

When photosynthesis was allowed to occur for very short periods in $^{14}CO_2$, 3-P-glycerate was the compound most heavily labelled (Calvin *et al.*,1951; Calvin and Benson,1948).

When the percentage of the total ^{14}C fixed into various intermediates was plotted versus time, the slope of the plot for 3-P-glycerate was negative and extrapolated back to values close to 100% at time zero (Calvin et al.,1951). When the distribution of the ^{14}C within the 3-P-glycerate was examined following a short pulse of $^{14}CO_2$, the ^{14}C was confined predominantly to the C-1 position (Bassham *et al.*,1950; Calvin and Massini, 1952). These results clearly established 3-P-glycerate as the first detectable product of photosynthetic CO_2 fixation.

(b). RuBP as the Immediate Precursor of 3-P-glycerate

Reciprocal changes in the pool sizes of RuBP and 3-P-glycerate were observed during light-dark transitions or as a result of suddenly lowering the concentration of CO_2 (Bassham *et al.*,1956; Calvin and Massini,1952; Wilson and Calvin,1955). These reciprocal changes gave compelling evidence for RuBP as the immediate precusor of 3-P-glycerate

(c). ^{13}C Values as Indicators of Carboxylation Reaction

Atmospheric CO_2 has a ^{13}C value of about -6.7%. . During photosynthesis, considerable discrimination against $^{13}CO_2$ occurs making the mean ^{13}C for C-3 plants -27.8%. (Troughton,1979). *In vitro* measurements of the fractionation which accompanies the carboxylation of RuBP yielded similar values (Christeller *et al.*,1976; Wong *et al.*,1979).

(d). Oxygen as Competitive Inhibitor for Carboxylation

Ogren and Bowes (1971) successfully demonstrated that O_2 was not only a competitive inhibitor for carboxylation,with respect to CO_2, but also a substrate for the formation of P-glycolate from RuBP.

The Oxygenation of RuBP as the primary reaction of the C-2 PCO

(a). 2-P-glycolate as the Immediate Precursor of glycolate

If the oxygenation of RuBP is the primary reaction of the C-2 PCO cycle, P-glycolate must be the immediate precursor of glycolate. A phosphatase is located within the chloroplast and is highly specific for converting P-glycolate into glycolate (Christeller and Tolbert,1978; Richardson and Tolbert,1961). Larsson (1974) demonstrated an accumulation of ^{14}C (from $^{14}CO_2$) in P-glycolate in response to fluoride, an inhibitor of phosphatases. Somerville and Ogren (1979) provided more compelling evidence when they studied *Aradopsis* mutants deficient in P-glycolate phosphatase. When incubated with $^{14}CO_2$ in the presence of an inhibitor of glycolate oxidase, ^{14}C accumulated in P-glycolate and not in glycolate as it did in the wild type. This result could only have come about if glycolate was exclusively derived from P-glycolate.

(b). Glycolate Synthesis from Sugar Monophosphates Needs ATP

Kirk and Heber (1976) hypothesized that if glycolate is synthesized by the oxygenation of RuBP, which is the only intermediate of the C-3 PCR cycle requiring ATP, then its formation from triose-P should be blocked by the inhibitor of photophosphorylation (carbonylcyanide-4-trifluoro-methoxy phenylhydrazine FCCP). They demonstrated this using intact isolated chloroplasts. Krause *et al.* (1977) extended these observations to include the inhibitors Dio-9 and arsenate. All of these inhibited glycolate synthesis from triose-P, even though each inhibits photophosphorylation by a different mechanism. Inhibition of RuBP synthesis because of the lack of ATP was the most straight forward explanation for the inhibition of glycolate synthesis by inhibitors of photophosphorylation.

(c). Incoporation of an Atom of Molecular Oxygen

Lorimer *et al.* (1973) showed that *in vitro* oxygenation of RuBP occured with the incorporation of an atom of molecular oxygen into the carboxyl group of P-glycolate. This allowed *in vivo* investigation of glycolate synthesis using $^{18}O_2$. From the results obtained with intact isolated chloroplasts, the observed relative enrichment of ^{18}O. in glycolate was 92% or more, regardless of the carbon source with which the glycolate was synthesized (Berry *et al.*,1978).

(d). Carbon Labelling Patterns

During photosynthesis with $^{14}CO_2$, carbon atoms #1 and #2 of all the sugar phosphates of the C-3 PCR cycle, including RuBP, become and remain labelled with ^{14}C. The oxygenation of RuBP is known to occur with carbon-carbon cleavage between carbon atoms #2 and #3 of RuBP, thus satisfying the requirement of uniform labelling (Pierce *et al.*,1980).

(e). Competition between Carbon dioxide and Oxygen

In both the chemosynthetic system (*Alcaligenes*) and the photosynthetic system (*Chlamydomonas*), glycolate synthesis and CO_2 fixation were shown to be competitive processes (Berry *et al.*,1976; King and Anderson,1980). This was explained by mutual competitive inhibition of RuBP carboxylation by O_2 and of RuBP oxygenation by CO_2 (Badger and Andrews, 1974).

(f). Double Labelling Experiments

It has been observed from double labelling experiments using ^3H_2O and $^{14}CO_2$ that the $^3H/^{14}C$ ratio is greater in glycolate than in P-glycolate (Peterson,1980; Plamondon and Bassham,1966). McDonough and Wood (1961) explained this by demonstrating that the reaction catalyzed by ribose-5-isomerase, the interconversion of ribose-5-P and ribulose-5-P, occurs with the incorporation of an atom of hydrogen from water into the C-1 position. In double labelling experiments it would be expected that RuBP would become labelled with ^3H at carbon #1 sooner than ^{14}C at carbon #1. The glycolate formed from this RuBP therefore would have a higher $^3H/^{14}C$ ratio than 3-P-glycerate as observed.

Purpose of Study

To understand the control of photosynthetic assimilation of CO_2, it is important to know the limiting steps of photosynthesis under various environmental conditions. According to the analysis of Jensen and Bahr (1977), the kinetic properties of RuBPC/O-ase suggest that the amount of enzyme, as well as the degree to which it is activated should be considered as regulators of the photosynthetic CO_2 assimilation.

RuBPC/O-ase is an essential enzyme for carbon assimilation in all photosynthetic organisms. Several studies have shown strong relationships between RuBPC/O-ase activity and photosynthetic rates. Wareing *et al.* (1968) correlated the increased photosynthetic rates of bean after partial defoliation with an increased RuBPC/O-ase activity. Bjorkman (1968) showed a close relationship between the activity of RuBPC/O-ase and light-saturated rates of photosynthesis in various shade and sun adapted plants. Variations in carboxylation efficiency and net photosynthesis in different genotypes of tomatoes were positively correlated with the quantity and properties of RuBPC/O-ase (Anderson *et al.*,1970).

In C3 plants, the activity of RuBPC/O-ase is thought to be rate-limiting for photosynthesis since increased CO_2 (above atmospheric levels) causes a substantial increase in the rate of photosynthesis. This may be due, in part, to the fact that O_2 inhibits the carboxylase function of the enzyme causing an increase in apparent Km for CO_2. C4 plants, however, usually are CO_2-saturated well below ambient CO_2 levels, the explanation being that the CO_2 concentrating mechanism in C4 photosynthesis provides a saturating level of CO_2 at the carboxylation site. From this theoretical consideration, Ku *et al.* (1985) suggested that a higher level of the protein in C3 plants may help compensate for its lower affinity for CO_2 in the presence of O_2, while C4 plants might be expected to require less RuBPC/O-ase than C3 plants.

It would, therefore, be in the interest of future photosynthetic research to be able to determine conclusively the concentration of RuBPC/O-ase in plants, and in this way gain more information about the effects of enzyme concentration on photosynthesis. To do this, a reliable assay method for the enzyme is required. The actual need and usefulness for such a method cannot be overemphasized.

RuBPC/O-ase is essential for photosynthesis and is probably the main target of many factors that affect photosynthesis. A quantitative analysis of high sensitivity and reliability could be used to study interaction between the environment, such as water stress, or light intensity and plant photosynthesis. Also, it would be possible to pinpoint factors that affect enzyme concentration, leading to an understanding of temporal variation in enzyme concentrations. RuBPC/O-ase makes up more than 50% of total soluble leaf protein. So the quantitative assay would also be a valuable tool for determining changes in protein synthesis in the leaf. Further, a comparison of RuBPC/O-ase concentration among plant groups could be carried out to determine the relative enzyme concentrations among C3, C4 and algal plants and to aid in understanding their autotrophic evolution.

The amount of RuBPC/O-ase in photosynthetic organisms has been determined by many workers with various methods which differed in their degrees of simplicity and precision. Yokota and Canvin (1985) determined the amount of RuBPC/O-ase by determining the concentration of binding sites in plant extracts by reacting the extracts with ^{14}C carboxypentitol bisphosphate (CPBP) and precipitating the resultant RuBPC/O-ase-(^{14}C)CPBP complex with a combination of Polyethylene glycol-400 and $MgCl_2$. Plots of the relationship between concentrations of (^{14}C)CPBP in the reaction mixture and the precipitated (^{14}C)CPBP gave a straight line and the concentration of the binding sites was estimated by extrapolation to zero (^{14}C)CPBP. In this method it was observed that specific binding of CPBP to RuBPC/O-ase and non-specific binding of CPBP to bulk proteins increased linearly with protein concentration, and that

non-specific binding was dependent on the type of leaf extract and the characteristics of the PEG-400. This would cause inconsistency and would reduce reliability of measurement because the number of RuBPC/O-ase binding sites was calculated from the amount of reacted (precipitated) CPBP. Total binding could be corrected for non-specific binding, but the accuracy of this correction could not be verified. There was also a possibility that RuBP and carboxyribitol bisphosphate were interfering with the binding of CPBP to RuBPC/O-ase by competitive inhibition.

In other cases RuBPC/O-ase concentration has been determined by isolating a radioactively labeled enzyme (usually the LS) by polyacrylamide gel electrophoresis, and then resolubilizing the exised protein band and counting radioactivity (by Liquid Scintillation Counting) of ^{14}C (Hobson *et al.*,1985) or ^{35}S and ^{3}H (Verling and Key,1985), or determining the UV absorption of the protein solution (Phelong and Brady,1979).

The problem with these methods is the inaccuracy of PAGE as an isolating technique, especially if it is only done one dimensionally. If other macromolecules of the same molecular weight as the LS of RuBPC/O-ase are present at the same position, then these methods would overestimate the enzyme concentration (Hobson *et al.*,1985). Verling and Key (1985) stated that since other proteins do migrate in close proximity with the LS of RuBPC/O-ase, even on two dimensional electrophoresis, quantification of the LS from the gel would be an overestimation and that best estimates would be obtained using immunological techniques with monospecific LS antibodies.

In this respect, immunological attempts have been made to quantitate RuBPC/O-ase in extracts of photosynthetic organisms using antisera against partially purified RuBPC/O-ase. In 1979, Collatz *et al.* did a radioimmune assay where the antiserum was radiolabelled and incubated with the extracts and the product of reaction exposed to scintilation counting.

Ku *et al.* (1979) immunoprecipitated RuBPC/O-ase in plant extracts using anti-RuBPC/O-ase antisera, and then measured the amount of protein in the precipitates according to Lowry *et al.* (1951).

The fact that these immunological methods utilized antisera renders their reliability questionable. The problem with conventional antisera is that it is expected to be very heterogeneous, containing products of many different cell lines (Goding,1980). It is therefore difficult to verify that all the antibodies produced in response to purified RuBPC/O-ase are against RuBPC/O-ase and not against some contaminant. Since the development of methods for the production of monoclonal antibodies by Kohler and Milstein (1975), the limitation imposed by the use of polyclonal antisera can be overcome. If an antibody has been produced by a monoclonal cell line, it follows that it is monospecific (Goding,1980; Kennet *et al.*,1980). So even if the RuBPC/O-ase sample used for immunization is impure, one has only to determine that the resultant antibody binds RuBPC/O-ase.

The primary objective of this study was to develop a sensitive and convenient quantitative assay using monoclonal antibodies, which could be used to determine the concentration of RuBPC/O-ase in the extract of any photosynthetic organism.

As well as their inherent monospecificity, monoclonal antibodies have other advantages over conventional antisera. They can be produced in unlimited

quantities *in vitro* and *in vivo* (Cordonnier *et al.*,1983). They can be selected for certain characteristics such as high affinity for the antigen (Cordonnier *et al.*,1983).

As a standard reagent, monoclonal antibodies are clearly superior to conventional antisera. Polyclonal antisera varies within and among animals. The animals are mortal and therefore a regular supply of both the animal and purified immunizing material is essential. On the other hand, a single monoclonal antibody can be used throughout a variety of samples (Campbell,1984).

Monoclonal antibodies were chosen for this study as the most appropriate tool for studying RuBPC/O-ase which, because of its complexity, is generally difficult to purify to homogeneity and is also susceptible to proteolysis. Also, antibodies raised to RuBPC/O-ase from one species may cross-react with enzyme from a wide variety of species (Gray and Kekwick 1974; McFadden and Tabita,1974; Gray and Kekwick,1976), which would make it possible to use one set of antibodies to quantify the enzyme from a variety of species.

A monoclonal cell line that is capable of producing antibodies against RuBPC/O-ase was established by the method of Kohler and Milstein (1975), as revised by Campbell (1984). Supernatant from this culture contained antibodies which could also be acquired through *in vivo* propagation in Balb C mice to get higher titres. As a first step the monospecificity of the antibodies had to be established to show that in a plant extract the antibodies would recognize only RuBPC/O-ase as the antigen. Determination of monospecificity was not entirely necessary since the hybridoma had been cloned thrice, but this step was carried out as a precaution to strengthen reliability of the assay and also to determine

which subunit of the enzyme carried the epitope. For this procedure, Western Blotting was used.

Following this, an attempt was made to immunopurify RuBPC/O-ase from a crude extract of *Skeletonema costatum*. For this step, Affinity Chromatography was employed, and to establish the degree of purity of the product, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used.

Purification of the enzyme using sucrose gradient centrifugation was also carried out to enable comparison between this technique and immunopurification. Again SDS-PAGE was used to assess purity of enzyme. After these preliminary studies, the next step was the development of the quantititave assay, which remained the main objective of the entire study. The assay had as its basis the well known Enzyme Linked Immuno Sorbent Assay (ELISA) using commercial spinach RuBPC/O-ase (Sigma) as standard.

The ability of the antibody to cross-react with enzymes from other plant species was determined using the dot immunobinding assay to show that the assay could be used with a variety of plant species.

The Quantitative Assay

In 1959, Yalow and Berson used insulin labeled with radioactive iodine to develop a quantitative immunological method for the determination of insulin amounts in human plasma. This method depended upon a competition between insulin labeled with radioactive iodine and unlabeled insulin for a fixed and limited number of specific binding sites on the antibody to insulin.This type of assay, the radioimmunoassay, combines the ability to detect very low levels of radioactivity

with high specificity of the antibody, forming the basis of a very versatile analytical tool. The rapid and wide acceptance of radioimmunoassay as a routine clinical tool and in research can be attributed to (1) the general applicability of the method, (2) the selectivity and specificity of the method, (3) the sensitivity of a radioactive isotope as the label in radioimmunoassay and (4) the ease of perfoming the test. There are however disadvantages in using radioimmunoassay. These include (1) the relatively short half-life of gamma-ray emmiting isotopes, (2) the health hazards involved in perfoming the test, (3) radiation structural damage on the labeled molecules, and (4) the need to separate antibody bound labeled and unlabeled antigens from unbound ones and therefore the difficulty in automating radioimmunoassays.

Engvall and Perlman (1971) and Van Weemen and Schuurs (1971) independently introduced the use of enzymes as another category of sensitive and even more versatile labels for immunoassays. This was to be known as the ELISA, for Enzyme Linked ImmunoSorbent Assay. According to Ngo and Lenhoff (1985) the advantages of enzyme labeled systems for immunoassays are (1) long term stability of most enzyme labels, (2) their concentrations can be determined by using commonly available laboratory instruments, (3) no radiation hazards, (4) the possibility of developing rapid immunoassays that can be automated, (5) the development of qualitative visual tests for different populations of antigens, and (6) the possibility of amplifying the detection signal. The limitations imposed by using enzyme labeled systems include (1) limited sensitivity of some systems, (2) carcinogenicity and instability of some substrates, (3) interference by some endogenous enzymes and other substances found in biological fluids, (4) long assay

time for some enzyme linked immunoassays and (5) procedures for measuring enzyme activity can be more complicated than determining the radioactivity of an isotope.

Ngo and Lenhoff (1985) conluded that enzyme labels are particularly sensitive and versatile because they are protein molecules endowed with an efficient catalytic power. The presence of minute amount of enzyme can be detected and quantified by measuring the products of the reaction catalyzed by the enzyme. Also, there are numerous functional groups in an enzyme molecule such as the amino, sulfhydryl, carboxyl, carboxamide and tyrosyl groups that are available for covalent linking to ligand molecules.

These considerations led to the Enzyme Linked ImmunoSorbent Assay being chosen for the quantification of RuBPC/O-ase.

Chapter II

MATERIALS AND METHODS

Plant Material and Growth Conditions

Cultures of the euryhaline alga, *Skeletonema costatum*, were propagated in both 2-litre and 5-litre flasks in a growth chamber with a photoperiod of 12:12 L:D. The cells were placed in f/20 enriched seawater medium (Guillard and Ryther, 1961) and grown well into exponential phase. The cultures were exposed to an irradiance provided by high output, cool-white flourescent bulbs. The temperature was held at 15°C which has been shown to provide an optimal environment for *Skeletonema costatum*. Culture volumes were harvested and fresh medium added once every two days. Harvesting was carried out using the continuous centrifuge system at 5000 rpm. The concentrated cells were stored in freezing conditions until there was adequate volume for experiments.

Leaves of *Hedera helix* and *Lonicera ciliosa* were collected from the University of Victoria campus. Pure spinach RuBPC/O-ase was purchased from Sigma Chemical Company, St. Louis, Missouri.

Soluble Protein Extraction (cell disruption)

The cell suspension was further concentrated by centrifuging at 10000 rpm for 10 min. The packed cells were treated with three cycles of freezing at -80°C (dry ice in acetone), thawing at room temperature, and sonication on ice, which ruptured all cells and organelles. Cell disruption could be assessed by examination of extracts under the microscope.

Leaves from higher plants were ground in a blender in ice-cold soluble protein extraction buffer (see App.G).

Monoclonal Antibodies

The antibodies were used after ammonium sulphate precipitation of ascites fluid. (see App.B for Ascites production and purification).

For non-relevant controls, monoclonal antibodies prepared against sea urchin embryonic tissue were used. These antibodies did not react with RuBPC/O-ase.

Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis SDS-PAGE

This is a method for separating complex mixtures of polypeptides according to their molecular size (Laemmli, 1976)

Running Procedure:

The gel slab is prepared between two plates. One of the plates is made of glass and is rectangular, measuring 8x10 cm. The second plate is the same size but with a notch 0.9 cm deep and 8.2 cm wide cut from one of the long edges. This second plate is made of a heat dissipating material, which is white. The two plates are placed together, with the notch at the top and three 7-mm wide plastic

spacers separating them along the two sides and along the bottom. The thickness of the spacers determines the thickness of the gel. The plates are held together using ordinary foldback office clips. A small amount of vacuum grease is placed at the junction of the plastic spacers to avoid leakage. The acrylamide solution is poured between the plates to a level of about 1.5 cm from the notch. It is then gently overlayered with butanol that has been saturated with water. The gel sandwich is then allowed to stand at room temperature and polymerization allowed to take place for one hour. The clips at the bottom of the plates are big enough to hold the unit in an upright position during polymerization.

Before pouring the upper gel, the butanol and the unpolymerized gel layer are removed and the surface of the gel is rinsed with upper gel buffer. Excess fluid is blotted with filter paper with care to avoid touching the gel. The comb is inserted to a depth sufficient to leave a space of about 5 mm between the surface of the lower gel and the fingers of the comb. This gel is allowed to polymerize for only 30 min because a longer time will result in an equilibration of pH between the stacking and separating gels, reducing the stacking efficiency of the upper gel. After the stacking gel has set, the bottom plastic spacer is removed and the unit is attached to the electrophoresis apparatus so that the notch on the white plate is next to and lines up with the notch on the upper buffer chamber. A liquid tight seal is provided by vacuum grease along the contacting edges of the gel unit and the upper buffer chamber. Electrode buffer (0.025 M Tris-base, 0,192 M glycine, 0.1% SDS) is then placed in the lower buffer chamber covering about 2 cm of the bottom edge of the gel. To eliminate air bubbles from the lower surface of the gel, the whole unit may be tilted while pouring the running buffer. Enough running

buffer is then placed in the upper chamber to flow across the notch. The comb is then removed leaving sample wells filled with running buffer. Samples which contain a polypeptide complex of interest are boiled for 3 min in the sample buffer (App. C) and cooled on ice to room temperature, and then applied with Eppendorf pipets. Up to 50 µl of a sample may be added to each well by allowing it to run down between the glass plates to the bottom of the sample well.

The electrodes are then connected to a power supply, with the anode to the lower chamber, and the cathode to the upper chamber. Electrophoresis is allowed to run at a constant current (8 mA for 30 min, and then 12 mA) at room temperature until the marker dye, bromophenol blue, approaches the bottom of the gel, which takes about 60 min.

Staining

At the end of the run, the gel sandwich is removed from the electrophoresis apparatus. The two side spacers are removed and the plates are pried apart with a spatula while running a stream of water between them. The gel is removed from the plate into a fingerbowl containing the staining solution (0.05% Coomassie brilliant blue in methanol:acetic acid:water, 5:1:5) at room temperature. The staining procedure is preferrebly done on a shaking water bath.

Destaining

After staining, the dye is removed and replaced with destaining solution (7% acetic acid in water,v/v).

Molecular Weight Determination

To obtain an estimate of the molecular size of the various protein bands after electrophoresis, protein standards with well-characterized polypeptide molecular weights are routinely run on the same slab gel. The molecular weight of an unknown protein may be estimated by comparing its mobility to the those of known standards run on the same gel. Studies of the hydrodynamic properties of protein-SDS complexes indicate that these are rodlike particles, with the length of the rod varying in a certain manner with the molecular weight (Laemmli,1976). Since the apparent free mobilities of protein-SDS complexes are nearly constant in the SDS-polyacrylamide gel system, the molecular size of such complexes, over a specific range, may be determined from plots of relative mobility versus the logarithm of the molecular weight. On a single slab gel, proteins are exposed to identical conditions of pH, temperature, current, and voltage gradient, so that direct comparison of molecular sizes is possible. The distance of each molecular weight standard from the top of the gel is measured and plotted, on semilogarithmic paper, against its respective molecular weight. The molecular weight of an unknown protein is then interpolated from its migration distance.

Electrophoretic Transfer (Western Blot)

(Towbin *et. al.* 1979).

This is a method which combines the high resolution of SDS-PAGE for separating polypeptides with the high specificity of monospecific antibodies to identify antigens. In this method a protein mixture is first resolved by SDS-PAGE analysis. This is followed by the elctrophoretic transfer of the polypeptides from

the polyacrylamide gel onto nitrocellulose sheets, where they are more accessible to antibody than inside a polyacrylamide gel.

Procedure

The gel is run as usual except that on removal from the plates it is not placed in the staining solution. Instead it is placed in a bowl that is half filled with transfer buffer (25 mM Tris-base, 192 mM glycine, 20% methanol, in water) where assembly for the transfer is performed to avoid trapping air bubbles.

The gel slab is placed on a sheet of nitrocellulose and supported by four sheets of wet Whatman 3 MM filter paper on both sides. The sandwich is placed between two 0.5-cm thick scouring pads (Scotch-Brite) which are, in turn, supported on both sides by two rigid plexiglass plates with holes to allow free access of electrolyte to the gel sandwich and the flow of current through the system.

The whole sandwich is placed between the electrodes of an electrophoretic transfer chamber containing the transfer buffer with the nitrocellulose sheet facing the anode (+) for SDS gels.

The electrodes of the transfer chamber consist of two stainless steel wire mesh sheets placed 3.5 cm apart. A constant voltage of 4 V/cm is applied for 1-2 hr. Transfer is done with cooling by immersing the transfer chamber in ice water. At the end of the run the gel is stained in 0.05% Coomassie brilliant blue dissolved in methanol: acetic acid: water (5:1:5) for 1 hr and destained in 7% acetic acid to assess the efficiency of transfer.

The part of the nitrocellulose paper that corresponds to the marker protein lane is stained with the amido black stain for 10 min. and then destained in the destaining solution until all background has been removed. This also helps to

indicate the extent of transfer. The rest of the nitrocellulose sheet is incubated with 5% fish skin gelatin (or 5% milk powder) in the washing buffer (20 mM Tris-HCl, 150 mM NaCl, 1% BSA buffer) for 1 hr at 37°C to saturate protein binding sites on the nitrocellulose. The membrane is then incubated for 2 hr at 37°C (or overnight at 4°C) with a dilution of 1 in 100 of the primary antibody diluted in the blocking solution (5% milk powder).

The membrane is washed once by shaking for 10 min in the washing buffer, then thrice in 0.1% Tween-20 in washing buffer, and again for the last time in washing buffer. It is further incubated for 15 min at 37°C with the blocking solution. The membrane is then incubated for 2 hr at room temperature (or overnight at 4°C) with a 1 in 500 dilution (by blocking solution) of the horseradish peroxidase conjugated secondary antibody. At the end of this incubation the membrane is washed as extensively as before and blown dry. To visualize the antigen-antibody sites, the membrane is shaken in the developer, a 20 ml solution of 3 mg/ml 4-Chloro-1-naphthol in methanol,mixed with 100 ml of Tris-saline (2.4 g Tris-base; 29.2 g NaCl in a litre of water), and 600 µl of 3% hydrogen peroxide. To stop development after dark bands appear, the membrane is removed from the developing solution and washed in distilled water. It is then blotted dry between layers of filter paper.

Affinity Chromatography

(Wilchek and Hexter, 1976).

Affinity chromatography is a type of adsorption chromatography in which the molecule to be purified is specifically and reversibly adsorbed by a complementary

binding substance (ligand) immobilized on an insoluble support (matrix). Purification is usually of the order of several thousand-fold and recoveries of active material are generally high. Affinity chromatography also has a concentrating effect which enables large volumes to be conveniently processed. The high selectivities of the separations derive from the natural specificities of the interacting molecules. It was for these reasons that an attempt was made to utilize the monoclonal anti-RuBPC/O-ase to purify the enzyme from a crude plant extract.

The matrix that was used to immobilize the monoclonal antibodies was cyanogen bromide activated Sepharose 4B (CNBr-activated Sepharose 4B from Bio-Rad). According to the the Bio-Rad manual, this complex is produced by the reaction of Sepharose 4B with cyanogen bromide. The active product is freeze-dried in the presence of additives to preserve the bead form of the gel. 1 g of freeze-dried material swells to give about 3.5 ml swollen gel. The reaction of cyanogen bromide with Sepharose results in a reactive product to which proteins, nucleic acids, or other biopolymers can be coupled , under mild conditions, via primary amino groups. Cyanogen bromide reacts with hydroxyl groups on Sepharose and it converts these groups to imidocarbonate groups, which react with nucleophiles. These may not be the only reactive groups, however, since there may be some cyanate ester groups present on the activated Sepharose. The activated groups react with primary amino groups of the ligand to form isourea linkages. The activation procedure also cross-links Sepharose and thus enhances its chemical stability.

Swelling and washing the gel

1 g of freeze-dried CNBr-activated sepharose 4B powder is swollen for 15 min in 1 mM HCl and washed on a sintered glass filter with the same solution. A total of 200 ml is added in several aliquots, the supernatant being sucked off between successive additions. The gel is then washed with 5 ml of coupling buffer (0.1 M $NaHCO_3$,0.1 M, pH 8.3) containing 0.5 M NaCl, and immediately transferred to the ascites fluid (containing approximately 10 mg of anti-RuBPC/O-ase protein).

Coupling the ligand

The mixture containing the antibodies (ligand) and the swollen gel (matrix) is rotated end over end for 2 hr at room temperature (or overnight at 4°C).

Blocking excess active groups

A number of residual active groups will remain on the gel after coupling. These groups are blocked by adding the gel-antibody suspension to an excess of a small primary amine (e.g. ethanolamine, glycine or glutamic acid) at pH 8.0 with 0.5 M NaCl.

Washing the product

To remove excess uncoupled antibody that remained after coupling, the gel is washed alternately with acetate buffer (0.1 M, pH 4) and coupling buffer (pH 8.3) each containing 0.5 M NaCl. This wash ensures that no free antibody remains ionically bound to the immobilized antibodies.

Storage of column

Suspensions are stored at 4°C in phosphate buffered saline (PBS) (pH 7.4) (see App.E).

Sample application

The soluble plant extract that has been dialyzed against PBS (pH 7.4) is applied to the column at a flow rate of 2 ml/hr and the column is washed with 35 ml of PBS (pH 7.4) to remove unbound substances.

Elution

Elution is effected with 35 ml of a glycine/HCl buffer (0.1 M glycine adjusted to pH 2.5 with 0.2 M HCl). The pH of the eluent is immediately raised to 7.4-8.5 with solid Tris-base.

Regeneration of gel

The column is then washed with 35 ml of 0.1 M Tris/HCl buffer containing 0.5 M NaCl adjusted to pH 8.5. The column is further washed with 35 ml of 0.1 m sodium acetate buffer containing 0.5 m NaCl adjusted to pH 4.5. It is then equilibrated with PBS (pH 7.4).

Analysis of eluent

The purity of the eluent was determined by SDS-PAGE.

Enzyme Purification by Alternative Methods

To enable investigation of RuBPC/O-ase under simulated *in vivo* conditions, large quantities of pure enzyme would be required to produce *in vitro* systems providing a realistic analogy to the enzyme's state in the chloroplasts. This is why fast purification techniques yielding high quality enzyme are in demand. Some good techniques exist for the fractionation of protein mixtures, however, applicability of these to this specific situation could not be predicted and thus some had to be tested experimentally.

According to Bezkorovainy,1970, when several isolation procedures are considered, it is necessary to select those that will not alter the protein of interest either physically or chemically. For this reason it is preferable to conduct isolation experiments in the cold (4°C) and relatively mild pH (4-9). Bacterial contamination must be kept at a minimum to avoid the degradation of proteins by bacterial proteases. Foaming must be avoided since it causes surface denaturation of proteins.

After completion of the isolation, it is necessary to decide on the form in which the protein is to be stored. Many proteins are stored in the frozen state, others are stored as precipitates under a saturated solution of ammonium sulphate. Most frequently, protein preparations are dialyzed against distilled water and lyophilized (freeze-dried). Dialysis is a procedure in which a protein solution is placed in a semi-permeable bag (cellophane or acetylated cellulose), and the bag is immersed in a container of distilled water. The bag and the surrounding water are continuously agitated by some stirring mechanism. The small ions such as the various buffer salts and organic solvents pass through the

pores of the membrane, whereas the protein is retained in the bag. If the protein is water soluble, dialysis will eventually produce a salt-free aqueous protein solution, which can be freeze-dried under vacuum. If the protein is insoluble, it will precipitate in the bag when the salt is removed, and may be recovered by centrifugation and drying.

In most cases a single purification step will not suffice to isolate a specific protein from a complex protein mixture. Thus several consecutive fractionation operations must be carried out to obtain the protein in a reasonably homogeneous state.

In our case attempts were made to immunopurify RuBPC/O-ase utilizing monoclonal antibodies immobilized onto CNBr-activated Sepharose 4B on an affinity column (see section on Affinity Chromatography). These attempts were not successful due to the high affinity of the monoclonal antibodies for the RuBPC/O-ase that made elution of an enzyme of high quality and quantity difficult.

After this failure, purification of RuBPC/O-ase was carried out by ammonium sulphate precipitation and sucrose density gradient ultracentrifugation following the method of Paech and Dybing (1986).

Ammonium Sulphate Precipitation

Most proteins are precipitated by high salt concentrations, the amount of salt necessary to precipitate a protein being directly related to the solubility of the protein under the conditions being employed (pH, temperature). This method of precipitation is called salting-out. The precipitation of proteins by salts is based on the fact that a certain degree of water-protein interaction must be maintained

in order to keep the protein in solution. Addition of salts lowers the activity of water, and hence decreases the degree of water-protein interaction resulting in precipitation of the protein, (Berzkorovainy,1970).

Ammonium sulphate is the most popular salt used in fractionation of protein mixtures because of its high solubility in water. Sodium sulphate and phosphate salts can also be used for this purpose. There are several ways to mix the protein solution and the salt to attain the desired salt solution : (1) Solid salt is added to a protein solution with constant stirring. The disadvantage of using this method is that localized high salt concentrations are momentarily obtained resulting in coprecipitation of undesired proteins and denaturation. Volume changes are, however, minimal if this method is employed. (2) A saturated solution of the salt is added to the protein solution with constant mixing. Volume changes are large in this procedure. (3) The protein solution is dialyzed against solutions of different salt concentrations. This technique has the advantage of introducing the salt into the protein solution very gradually, so that coprecipitation and denaturation are minimal.

Preparation of proteins by the salting-out method requires a final exhaustive dialysis step to get rid of the salt from the protein.

In our case, the salting-out procedure was intended to partially purify the RuBPC/O-ase and also to concentrate it into a small volume to improve the efficiency of the sucrose density gradient ultracentrifugation since only very small volumes could be used in one run on the ultracentrifuge.

Ultracentrifugation

This is a method of separating molecules on the basis of their size, using a high gravitational force. For a given force field, a particular molecule will move at a particular velocity, and the ratio of this velocity to the applied centrifugal field is called the sedimentation constant of the molecule. It is analogous to the mobility of a molecule in electrophoresis. A mixture of molecules with different sedimentation constants will be partially separated by the force field, producing concentration gradients of the molecules.

Sedimentation in a Density Gradient

This method allows a separation of materials on the basis of their buoyant density. A high concentration of low molecular weight solute (sucrose), and a low concentration of macromolecules (proteins) are placed in a centrifuge cell and spun at a high angular velocity until buoyancy forces between the protein and the medium are minimal (equilibrium). The equilibrium distribution of the solute produces a concentration gradient in the solution, resulting in a continuously increasing density from the meniscus to the base of the tube. The initial concentration of the low molecular weight solute, the centrifugal field strength, and the length of the liquid column may be chosen such that at equilibrium the density gradient encompases the effective density of the macromolecular species.

Cell Disruption and Ammonium Sulphate Precipitation

Pelleted cells of *Skeletonema costatum* were resuspended in 3 ml of extraction buffer (100 mM Tris-HCl, 100 mM NaCl, 20, mM MgCl, 10 mM $NaHCO_3$, 1 mM EDTA, 5 mM dithiothreitol, 10 mM isoascorbate, 1 mM phenyl

methyl sulfonylflouride, 10 mM iodoacetimide, 20 mM 2-mercaptoethanol). The buffer was at 4°C. The cells were disrupted as usual (freezing, thawing, sonication). ATP was added (as a 0.5M solution adjusted to pH 7) to a final concentration of 5 mM and the extract was brought to 58°C (within 5 min) and maintained at this temperature for 9 min, and then chilled within 5 min to below 8°C in an ice/salt bath, and centrifuged for 10 min at 18 500 rpm.

This resulted in a solid pellet and a yellow supernatant that contained RuBPC/O-ase. The enzyme was precipitated with an equal volume of saturated ammonium sulphate solution (adjusted to pH 7), collected by centrifugation within 10 min, and resuspended in minimal extraction buffer.

Sucrose Density Gradient Ultracentrifugation

The ammonium sulphate precipitate that contained the RuBPC/O-ase was further purified on a sucrose density gradient by the method of Goldwaite and Bogorad (1971) and Taylor (1979). The ammonium sulphate precipitate was redissolved in minimal extraction buffer. 500 µl aliquots of this solution were layered on top of freshly prepared sucrose density gradients (step-type: 1.4, 1.0, 0.6, 0.3 M sucrose) in 4.0 ml cellulose nitrate centrifuge tubes. The samples were centrifuged at 50,000 rpm for 5 hours at 4°C in a Beckman L2-65B ultracentrifuge equipped with an SW 56 rotor. 30 sec fractions were collected from the top by pumping a 2 M sucrose solution (coloured with methyl red) through the bottom using a peristaltic pump. The fractions were read at 280 nm on a Beckman UV-Vis light Spectrophotometer. SDS-PAGE was used for qualitative analysis.

Enzyme Linked Immuno Sorbent Assay (ELISA)

The Enzyme Linked Immuno Sorbent Assay (ELISA), as first reported by Engvall and Perlman in 1971 (reviewed in Engvall and Pesce, 1982, and rewritten by A.M. Campbell, 1984) is a solid-phase assay system used to detect antigens with their specific antibodies. In this technique the antigen is bound onto the solid phase and incubated with the specific (monoclonal) antibody. A second antibody coupled to an enzyme is then used to detect the first antibody.

Nature of the Solid Phase

The chemical nature of the solid support may be glass, sepharose, cellulose, nitrocellulose paper, polyvinyl, or polysterene. The physical nature can be balls, rings, beads, discs or sheets of paper. In our case the solid phase was in the form of a 96-well polyvinyl plate made by Costar. The plates are coated with material called Immunolon which encourages binding of the antigen.

Attachment of Antigen

Most soluble protein and nucleic acid antigens are passively adsorbed onto the solid phase. The capacity of the various supports varies widely. In addition, the amount adsorbed depends on the diffusion coefficient of the protein, the surface area to volume ratio of the protein solution, and the time and temperature of the adsorption incubation (Campbell,1984). A volume of 100 µl of a solution containing 1-50 µg/ml of antigen per well is usually used for attachment. More protein can be bound, but the percentage bound falls as higher concentrations of proteins are used (Campbell,1984). It is important to note that since the binding is non-covalent, some protein leaches off the plate during subsequent incubation

with the antibody, and coated polyvinyl and polysterene seem to have provided maximum binding of protein compared to other solid supports (Campbell,1984).

Blocking of Remaining sites on Solid Support.

Since the solid support adsorbs protein non-specifically by hydrophobic interactions, it is evident that they will also adsorb the antibody if the plates are not fully saturated with antigen. Once the antigen has been bound, the remaining empty sites are blocked with either bovine serum albumin (1%,w/v), fish skin gelatin (0.5%,v/v), or milk (1%, w/v), dissolved in phosphate buffered saline. Tween-20 or Triton X-100 (0.01%) is also used in the buffer to reduce non-specific binding because it discourages the formation of further hydrophobic interactions between the solid support and the first and second antibodies.

The Nature of the Second Antibody.

The monoclonal antibody which binds to the antigen is detected with a second antibody directed against it. Covalently linked to this second antibody is an enzyme with a chromogenic substrate. Sheep, goat or rabbit antimouse antibodies linked to enzyme are thus used to detect murine antibodies.

Enzyme used in ELISA

The enzyme used for colour development with the second antibody was horseradish peroxidase. A variety of other enzymes could be used since the detection system only requires that the enzyme have a chromogenic substrate, and any enzyme which releases a hydrogen or hydroxyl ion can be used in conjunction with a pH indicator. The substrate for the horse radish peroxidase was O-phenylenediamine.

Procedure

Coating the Plates

Commercially available spinach RuBPC/O-ase (Sigma) was adjusted to 50 µl/ml in PBS. 50 µl of this solution was added to each well and incubated at 37°C for 2 hr and then overnight at 4°C. The next morning the coating antigen was removed from the plate and the plate was blocked.

Blocking the Plates

Blocking solution (0.5% rabbit albumin and 0.05% Tween-20, both in PBS) was added to each well (100 µl) and the plate was incubated for 1 hr at 37°C. The albumin was from the species in which the second antibody (enzyme linked) was made.

First Antibody

At the end of the blocking period, the solution was removed from the plate, which was washed three times with PBS-Tween (0.05% Tween-20 in PBS). 50 µl of ascites fluid diluted by blocking solution was added to each well and the plate was incubated for 1 hr at 37°C

At the end of this period the plate was washed thrice, with flicking , with PBS-Tween. The third wash was left for 10 min and washing was repeated twice more.

Second Antibody

The rabbit antimouse antibody was diluted 1/1000 in the blocking solution and 50 µl of this solution was added to each well. The plate was further incubated for 1 hr at 37°C. The plate was then washed as extensively as described before.

Enzyme Substrate

The enzyme substrate was made by mixing 10 ml of citric acid ammonia buffer (pH 5.5) with 10 mg of O-phenylnediamine and 0.1 ml of the hydrogen peroxide solution. 50 µl of this solution was added to each well and the plate was incubated for 15 min at room temperature, in the dark. Colour development was stopped by adding 50 µl of 4 N H_2SO_4 and the plate was read at 490 nm with a multiscanning spectrophotometer.

Blank wells recieve

1. Antigen
2. Blocking solution.
3. Secondary antibody
4. Substrate

Development of the Quantitative Assay for RuBPC/O-ase

Competitive Inhibition Assay

The basis of the proposed assay is the ELISA method outlined above. Slight modifications of this method have been made. Competitive binding or inhibition immunoassay combines the high sensitivity and specificity of antibodies with economic use of reagents and good precision.

In the basic method, a fixed amount of antigen competes with antigen that has been immobilized on a solid support, for a limiting amount of specific antibody. A standard curve is constructed using known amounts of the free-floating (not immobilized) antigen, and by reference to this, antigen concentration in unknown samples can be determined.

Preliminary Binding Assay (Titration of Ascites Fluid)

It is necessary to establish the correct antibody dilution for use in the competitive assay by carrying out a preliminary binding assay. A fixed amount of antigen bound to a solid support is incubated with various dilutions of antibody for 1 hr at 37°C. Unbound antibody is washed off and the amount of bound antibody is detected with a secondary antibody which is directed against the first and is conjugated to an enzyme that has a chromogenic substrate. The substrate is added to develop the colour and the plate is read in a spectrophotometer. A plot of absobance against antibody dilution is constructed and used to determine the dilution of antibody that binds 50% to 80% of the maximum that is bound (fig.8). This is the dilution that should be used in the competitive assay to ensure that the antibody is in limiting amounts.

Coating of Plates

As above

Blocking of Plates

As above

First Antibody

Proteins in ascites fluid were precipitated by ammonium sulphate, dialyzed against PBS and stored at freezing temperatures. Dilutions of this fluid starting from 1/50 to 1/102400 were made. 50 µl of each dilution was added to each of three wells in each of the 12 columns of a plate. The plates were incubated at 37°C for 1 hr and washed as above.

Second antibody

As above

Substrate

As above

The plates were read at 490 nm with a scanning spectrophotometer and a graph of absorbance against antibody dilution was made. From the lower part of the curve a suitable antibody dilution was chosen for the following procedures (fig.8).

Homologous Inhibition Assay (Construction of the Standard curve)

A standard curve enables the antigen concentration in unknown samples to be calculated. It is constructed by incubating the coating (immobilized) antigen with known concentrations of a free-floating antigen plus the limiting antibody concentration, all at the same time. This enables competition for the limiting amount of antibody between the immobilized and the free-floating antigen. The

free-floating antigen-antibody complex is washed off, and the enzyme-conjugated second antibody is added onto the immobilized antigen-antibody complex.

The chromogenic substrate is added and the absorbance of the solution is determined. Absorbance is plotted on a linear scale against antigen concentration on a logarithmic scale.

The linear part of the curve is used for determining antigen concentration in unknown samples.

Coating of Plates

As above

Blocking of Plates

As above

First Antibody (Competing antigen + First Antibody)

Dilutions of the standard (spinach RuBPC/O-ase) competing antigen starting from a concentration 50 times higher than that used for coating were made. 50 µl of this solution was added to the wells, the highest concentration added to the first three wells of the first column, and so on until the lowest concentration was added to the first three wells of the last column.

Then immediately, 50 µl of the limiting antibody concentration was added into each well and the plates incubated for 1 hr at 37°C

Second Antibody

As above

Substrate

As above.

Controls received:

1. Coating Antigen + PBS + 2nd antibody
2. Coating Antigen + antigen dilution + 2nd antibody
3. Antigen + 1st antibody + 2nd antibody

After reading the absorbance values from the spectrophotometer, a curve was constructed by plotting the log of antigen concentration (competitor) against absorbance. This is the standard curve to determine enzyme concentrations in unknown samples.

Dot Immunobinding Assay for Cross-reactivity of Antibody

The purpose of this procedure was to determine the ability of the monoclonal antibody to cross-react with enzymes from other plant species. The result of the Western Blot indicated that the epitope was on the large subunit (LS) (see Results and Discussion). So it was assumed that the antibody would react with enzymes from a wide variety of plants because the LS is quite homologous even in unrelated species (see Genetics of enzyme above). It was important at this point to establish cross-reactivity before declaring that the immunoquantitative assay could be used with all photosynthetic species.

Assaying Procedure:

Approximately 1.5 by 1.5 cm squares were ruled out on a sheet of nitrocellulose paper using a soft pencil. The paper was washed in distilled water and dried at room temperature. Serial dilutions of enzyme preparations were made from *Skeltonema costatum*, Spinach RuBPC/O-ase (Sigma), and leaves of two higher plant species (*Hedera helix*, *Lonicera ciliosa*) collected from the University of Victoria campus. All RuBPC/O-ase preparations were made in their native and denatured (SDS plus 2-mercaptoethanol) states. 2 µl of each enzyme preparation was added onto each square using a micropipet. Each species had a distinct row of squares assigned to it and the dots on each row were applied in increasing dilutions. The negative control rows received dilutions of BSA. The paper was then incubated in 5% milk powder dissolved in the Tris-Saline-BSA buffer (see App.D) for 1 hr at 37°C to block excess reactive sites. After this time the paper was gently air dried in preparation for the next step. A 1/20 dilution of the $(NH_4)_2SO_4$ precipitated proteins from ascites fluid was prepared and 2 µl of this was pipeted onto each antigen dot. Negative control dots received ascites fluid containing antibodies prepared against sea urchin embryonic tissue. The paper was incubated for 1 hr at room temperature and then washed once with Tris-Saline-BSA buffer, thrice with 0.1% Tween-20 in the same buffer, and then once again in the buffer. The paper was incubated for a further 15 min in the blocking solution. Then the paper was incubated for 1 hr at room temperature with horse radish peroxidase conjugated rabbit antimouse IgG diluted 1 in 100 with the Tris-Saline-BSA buffer. At the end of the hour the paper was extensively washed as before.

To develop the colour, a 20 ml solution of 3 mg/ml of 4-Chloro-1-naphthol (Sigma) dissolved in methanol and stored in the dark was mixed with 100 ml of Tris-Saline and 600 µl of 3% H_2O_2. Blue colouration was observed 5-15 min later.

The paper was then washed with distilled water and stored in the dark.

Confirmation of Cross-reactivity

While the dot immunobinding assay had fairly well established the ability of the monoclonal antibody to cross-react with extracts from different plant species, it lacked the ability to show the identity of the reacting protein. To remove this doubt, the following was done:

Soluble protein extractions were done on pea (*Pisum sativum*) leaves and Sea urchin gonad tissue (negative control). A 50% ammonium sulphate precipitation was carried out on the soluble extracts to concentrate the protein and partially purify the RuBPC/O-ase in the pea extract. 50% ammonium sulphate had already been established as the appropriate percentage to precipitate most of the RuBPC/O-ase.

The resulting precipitates were redissolved in minimal extraction buffer and dialyzed against distilled water to eliminate the ammonium sulphate. The protein solutions were then mixed with SDS sample buffer (see App.C) without the glycerol and the bromophenol blue. The denatured proteins were separated on a gel permeation TSK 3000 column (HPLC). SDS-PAGE was used to identify the RuBPC/O-ase fraction which was then tested for antibody reactivity using the dot immunobinding assay. The negative controls were the sea urchin protein and non-relevant monoclonal antibody that was prepared against sea urchin embryonic tissue.

Chapter III

RESULTS AND DISCUSSION

Monospecificity of Antibody

Electrophoretic Transfer and Immunostaining (Western Blot)

A cell extract had been run on an SDS-PAGE and subsequently electro-transferred onto a nitrocellulose membrane where the proteins could be immunostained (Western Blot). Amido black stained marker strips had been saved for molecular weight determination. The mouse anti-RuBPC/O-ase monoclonal antibody stained only one band which corresponded to the large subunit of RuBPC/O-ase (55 000 d). The irrelevant antibody did not react, as was expected (Fig.5). However, only the *Skeletonema Costatum* enzyme was stained and not the spinach enzyme or any of the other two higher plants (*Hedera helix* and *Lonicera ciliosa*). It was found in other assays (ELISA, Dot Immunobinding Assay) (results to be shown) that enzymes from these sources do react with the antibody. These results can be explained if the other enzyme types have epitopes that are destroyed during transfer. The result of this experiment showed that the antibody is monospecific to the LS of RuBPC/O-ase and nothing else in the crude plant extract.

Further,if the enzyme used for immunization was the native form, then the results agree with the suggestion of Gray and Kekwick (1974) that the small

subunits might be buried within the protein with the large subunits on the surface and are thus antigenically inactive. Thus, immunization with the native enzyme produces a similar response to that when immunizing with large subunits.

However, Weir (1973) has suggested that an immunizing molecule may be broken into its subunits by the digestive enzymes of phagocytic cells, resulting in a division of the antibody-producing cell lines between the different subunits. In this case we would expect after immunizing with native RuBPC/O-ase, that the resultant antibodies would be divided into anti-LS and anti-SS. Since the principle of monoclonal antibody production is based on cloning from a single cell, the final culture of hybridomas would either be anti-LS or anti-SS, not both. The fact that in this study the anti-LS cell line was the final culture could be a consequence of chance. However if on assaying for positivity during cloning, the native enzyme was used, then only the cell line producing antibodies that could recognize an epitope on the surface of the native enzyme could be maintained in culture. This reiterates the suggestion of Gray and Kekwick (1974), that assaying with the native enzyme was analogous to assaying with the LS.

Immunopurification

The attempt to immunopurify RuBPC/O-ase from the crude extract was unsuccessful. Different eluting procedures including low pH, high pH, and polyethylene glycol were not able to elute enough enzyme from the column even to visualize on SDS-PAGE. I concluded that the reason for failure was the high affinity of the antibody for the enzyme.

Assessment of Column:

The optical density of the antibody solution was measured before and after reaction with the Sepharose beads and the decrease in the optical density after reaction was an indication of antibody binding onto the column. When all elution procedures had failed, the sepharose was removed from the column and was boiled in SDS sample buffer and electrophoresed on SDS-PAGE. Antibody bands (heavy chain, 45kd and light chain, 28kd) could be seen, another indication of successful antibody immobilization on beads. There was also a faint band where the enzyme band was expected 55kd (LS) (Fig.6). The electrophoresed proteins were transferred onto nitrocellulose paper (Western Blot) and immunostained for firm identification. A control paper was stained with non-relevant monoclonal antibodies. As was expected, both experimental and control membranes showed the antibody bands. This was further proof of the presence of antibody on the column. The experimental paper which was stained with anti-RuBPC/O-ase had the antibody bands stained darker. On this paper there was also a faint band at about 55kd which would be the LS of RuBPC/O-ase, suggesting that the enzyme did bind to the column but could not be eluted (Fig.6).

Ammonium Sulphate Precipitation and Gradient Ultracentrifugation

A combination of procedures has led to the isolation of electrophoretically pure RuBPC/O-ase from pelleted cells of *Skeletonema costatum* (Fig.7). Fractionation with ammonium sulphate precipitated some, but not all of the green material in cell extracts. Removal of membranous material prior to gradient ultracentrifugation was important. If not removed, the resulting pellet often

would not stick onto the vertical wall of the centrifuge tubes after ultracentrifugation, thus interfering with the fractionation of the gradient. The heat stability of RuBPC/O-ase (Bowes and Ogren, 1972) made possible the heat denaturation of extraneous material (Jakoby *et al.*,1956; Weissbach *et al.*,1956). The presence of high levels of thiols (20 mM 2-mercaptoethanol and 10 mM dithiothreitol) and the protease inhibitors (phenyl methyl sulfonyl flouride and iodoacetimide) was essential for inhibiting proteolytic activity during the isolation procedure (Paech and Dybing, 1986).

To maximize the yield of purified RuBPC/O-ase in a single run in the centrifuge, the enzyme extract was concentrated by ammonium sulphate precipitation, which also served as a preliminary fractionation step, prior to centrifugation. Enzyme yields were always low because only small amounts of cells were available (about 2 g pelleted cells of *Skeletonema costatum*). Some material was further lost throughout the procedures. Thus, the immunopurification procedure was attractive because affinity chromatography concentrates the product (Wilchek and Hexter,1976). Also, immunopurification is faster and reduces the extent of proteolysis.

The Quantitative Assay

Titration of the Ascites Fluid

A curve relating absorbance to antibody dilution was derived from experimental results (Fig.8). From the lower part of the curve, an antibody dilution of 1/1600 was chosen as the most appropriate dilution for the Equilibrium Inhibition Assay. An antibody dilution at this region of the curve was chosen

because linearity is required for the Equilibrium Inhibition Assay and is favoured by antibody dilutions below saturation.

Equilibrium Inhibition Assay

Plotting the log of "antigen-added-as-competitor" versus absorbance gave a straight line, which becomes the standard curve (Fig.9). On the curve each point represents an average of three replicates. Absorbance was linearly related to the logarithm of antigen concentration at high competitor concentration, and as the competitor concentration decreased the curve became non-linear. As the competitor concentration approached zero, the curve reached a plateau. This discrepancy could be easily overcome by decreasing the antibody concentration below which a plateau cannot be attained because of low competitor concentration. This occurs because the antibody concentration becomes comparably high with less competing antigen, and thus is not limiting, a requirement for inhibition. Also, replication of the curve was difficult although conditions were kept constant. Thus a new curve should be constructed for each assay.

For adequate replication, the two most sensitive stages occur after incubation with primary, and secondary antibodies where extensive washing and flicking is involved. These activities could easily vary among individuals and days. Also, the efficiency of protein binding on plates depends on the time and temperature of incubation. It was not possible to avoid variability among plates, but it was relatively easy to maintain uniformity within plates because wells are fairly uniform on each plate, which increases the precision of the assay. The large number of wells per plate makes the assay very efficient because more than ten duplicated samples can be assayed at once. There is also room for controls.

Cross-Reactivity

Dot Immunobinding Assay

All the dots that received plant extract and the anti-RuBPC/O-ase showed a purple colour after incubation with the enzyme substrate (see Fig.10). This result meant that the antibody is capable of recognizing extracts from different, unrelated species, the antibody cross-reacts. The control dots that received BSA as the antigen had no colour change as expected since the antibody is not supposed to react with BSA. The non-relevant antibody control dots also showed no colour change. This ability of the antibody to cross-react with various plant extracts was an essential observation because the main objective of this study was to develop a quantitative assay utilizing an antibody which would enable quantification of the enzyme in extracts from photosynthetic organisms regardless of their phylogenetic position. So, had this antibody failed to cross-react, this goal would be deemed impossible. The assumption had been made earlier in this study that the antibody would cross-react because many reseachers had found that antibodies prepared against RuBPC/O-ase in one species do cross-react with enzymes from other species. This assumption was further strengthened by the fact that on producing the antibody a spinach enzyme was used for assaying for positivity at each cloning stage even though the immunizing antigen had come from *Skeletonema costatum*, a diatom. Just this fact should have been enough to convince us that the antibody was cross-reacting had it not been for the result of the Western Blot. A total of four trials of the Western Blot showed the antibody reacting very well with the LS of the enzyme from *Skeletonema costatum*, but not at all with the enzyme from three higher plants that were run at the same time

(Fig.5). For this reason a dot immunobinding assay, including native and denatured (boiled in SDS-buffer) extracts from four different plant sources, was carried out to conclusively determine cross-reactivity. The results from this showed the antibody to be cross-reacting (Fig.10), even with denatured forms of all the extracts. So the only possible explanation for the ambiguity of the Western Blot is that during transfer the epitope on other plant enzymes is probably destroyed or altered.

Confirmation For Cross-Reactivity

(HPLC Purification and Dot Immunobinding assay).

Fig.13 contains results showing the reaction of the different protein samples with the monoclonal antibody against RuBPC/O-ase. There was a positive reaction with the sample that was pure pea RuBPC/O-ase as indicated by the HPLC chromatograms (Fig.11), and SDS-PAGE (Fig.12). There was no reaction with the samples that contained no RuBPC/O-ase, nor with the samples that were fractionated from the sea urchin extract. There was no reaction with the non-relevant antibody.

The result of this experiment gave more definite proof that the antibody is cross-reacting with enzyme from different plant species other than the *Skeletonema costatum*. The dot immunobinding assay discussed previously did show cross-reactivity, but that assay was done on crude plant extracts. So while it showed cross-reactivity, it did not indicate the nature of the molecule that was being recognized by the antibody in the crude and heterogeneous extracts. This experiment, on the other hand, shows the identity of the HPLC purified RuBPC/O-ase on the SDS-PAGE and the reaction of this with the antibody on the dot immunobinding assay (see Figs. 11,12 and 13).

From this result, the monoclonal antibody prepared against RuBPC/O-ase from *Skeletonema costatum*, has been shown to cross-react with RuBPC/O-ase from other plant species (spinach, pea, and *Hedera helix*). This is the cross-reactivity that had to be established before I could be claime that the immunoquantitative assay can be used with all photosynthetic plants.

Chapter IV

CONCLUDING REMARKS

Development of the analytical method was difficult but was justified because of its research potential. I have shown by the Western Blot that the monoclonal antibody is monospecific for RuBPC/O-ase and that the epitope recognized by the antibody is on the large subunit of the enzyme. This enables the antibody to cross-react with enzymes from different plants as was demonstrated by the dot immunobinding assay. Thus the method provides a means to determine RuBPC/O-ase concentrations in any plant extract, no matter how crude and heterogeneous, as long as the enzyme is in solution. Preparation of plant material only requires cell disruption and centrifugation prior to analysis. This eliminates long purification procedures, which may result in proteolytic breakdown of enzymes. Also, use of monoclonal antibodies rather than conventional antisera, which are often used (Collatz *et al.*(1979), Ku *et al.* (1979), Perchorowicz *et al.* (1981), and Tingey and Andersen, (1986), gives superior analytical capabilities.

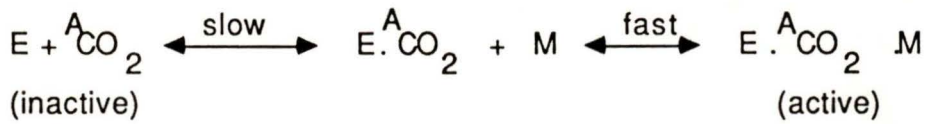
Chapter V

SUMMARY

The main contribution from this study is a simple method for the immunoquantitation of RuBPC/O-ase in soluble plant extracts. Because RuBPC/O-ase is so important to photosynthesis, the value of this study in photosynthetic research is very evident and exemplified by observations made by other workers: High photosynthetic rates corresponded to total concentrations of RuBPC/O-ase per mg of chlorophyll in C3 winter annuals, *Camissonia and Geraea spp* in Death Valley (Seemann *et al.*,1980; Mooney *et al.*,1976). Investigations of higher photosynthetic rates in polyploid tall fescue (*Festuca arundinaceae*), Schreb and alfalfa (*Medicago sativa*) implicated higher leaf levels of RuBPC/O-ase as the major contributing factor (Joseph *et al.*,1981; Molin *et al.*,1982; Meyers *et al.*,1982). Perchorowitz *et al.*(1981) demonstrated a close correlation between activation state of RuBPC/O-ase *in vivo* (which affects the concentration of catalytically available active sites) and photosynthetic rates of leaves of intact wheat (*Triticum aestivium* L) seedlings and corn (*Zea mays* L) varieties. In the corn study, RuBPC/O-ase was fully activated *in vivo* prior to extraction (Baer and Schrader, 1985), so photosynthetic rates corresponded directly to the amount of leaf enzyme. This study, therefore has made available a method in which the concentration of the enzyme can be readily determined, and thus the relative photosynthetic rates be estimated.

I very much appreciate the opportunity to use biochemical and immunological technique in an analytical study. Clearly monoclonal antibody technology will have major impacts on plant physiology in the future years.

Figure 1: ***Mechanism of activation of RuBPC/O-ase.*** Acitvation of ribulose-1,5-bisphosphate carboxylase/oxygenase by carbon dioxide and magnesium ions. (Lorimer *et al.*,1976).


E + ACO2
slow
E.ACO2
+ M
fast
E.ACO2.M
(inactive)
(active)

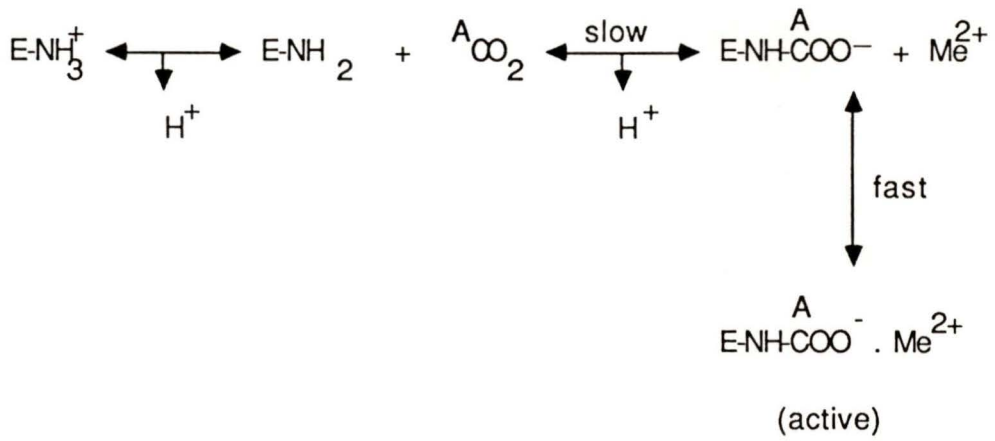
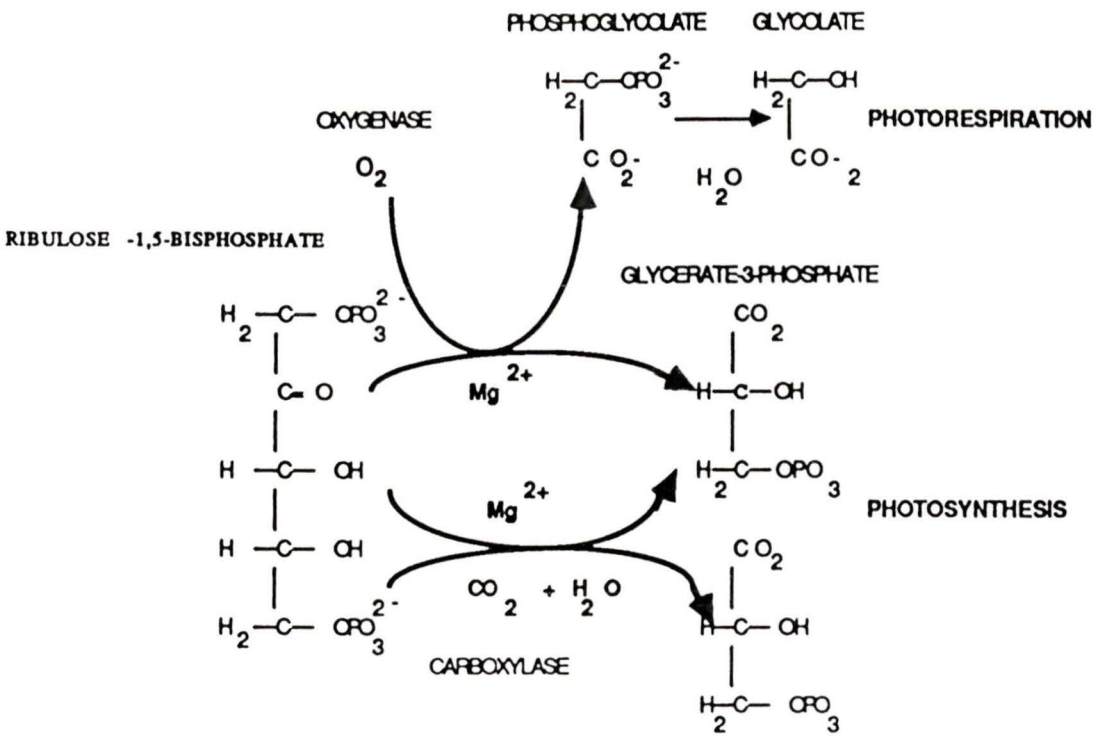
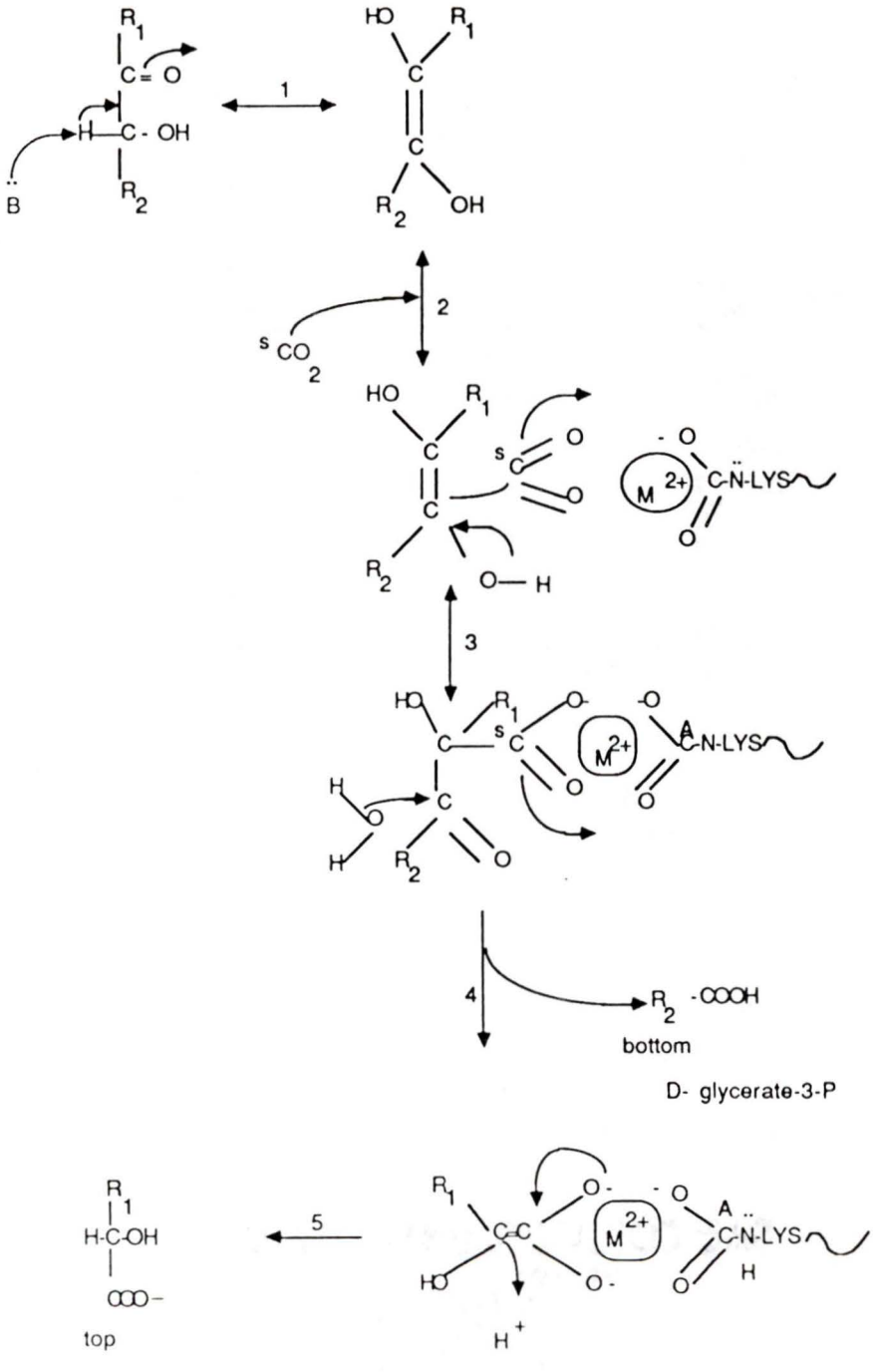

E-NH3+
E-NH2
H+
+ ACO2
slow
H+
E-NH-ACOO−
+ Me2+
fast
E-NH-ACOO-. Me2+
(active)

Figure 2: ***Reactions catalyzed by RuBPC/O-ase.*** Dual reaction catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase. (Jensen and Bahr, 1977).



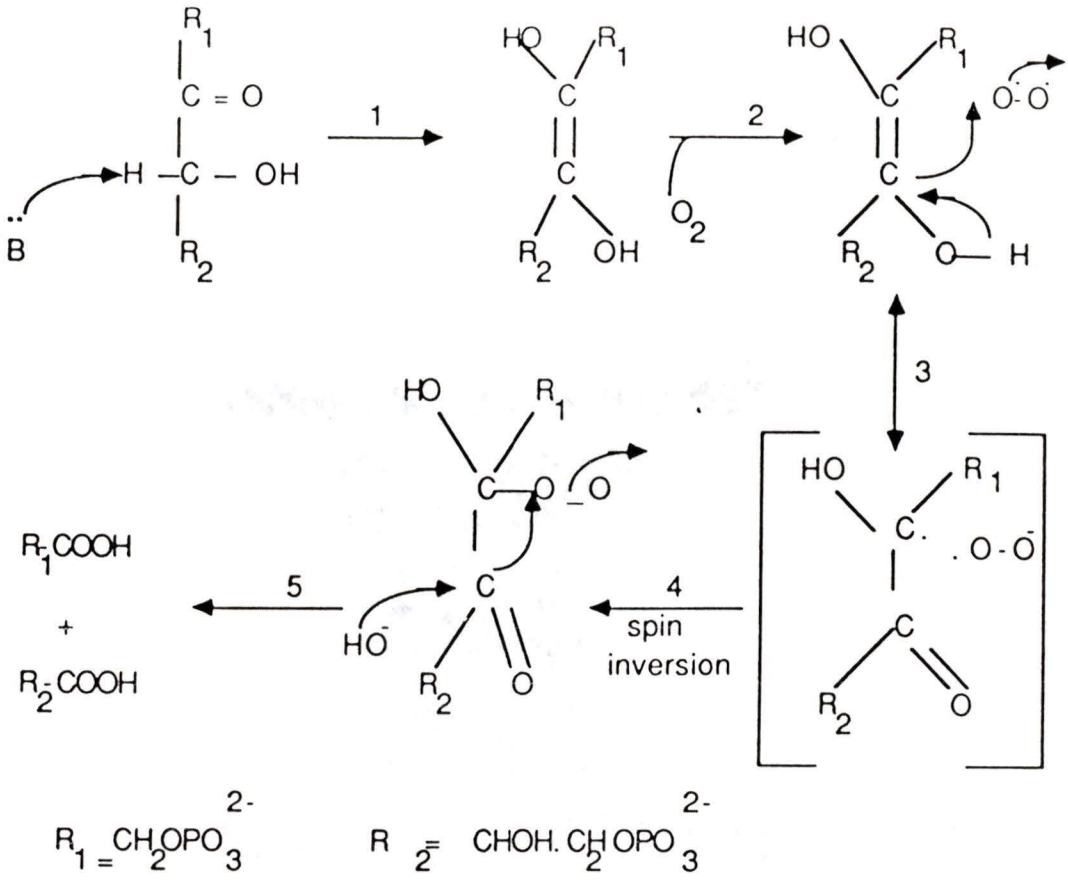
REACTIONS CATALYZED BY RIBULOSE-1,5BISPHOSPHATE CARBOXYLASE/ OXYGENASE

Figure 3: ***The Mechanism for carboxylation.*** A mechanism for the carboxylation of ribulose bisphosphate (Lorimer and Miziorko, 1980; Pierce *et al.*,1980).



$R_1 = CH_2OPO_3^{2-}$ $R_2 = CHOH.CH_2OPO_3^{2-}$

Figure 4: ***The mechanism for oxygenation.*** A mechanism for the oxygenation of ribulose bisphosphate (Kosman, 1978).

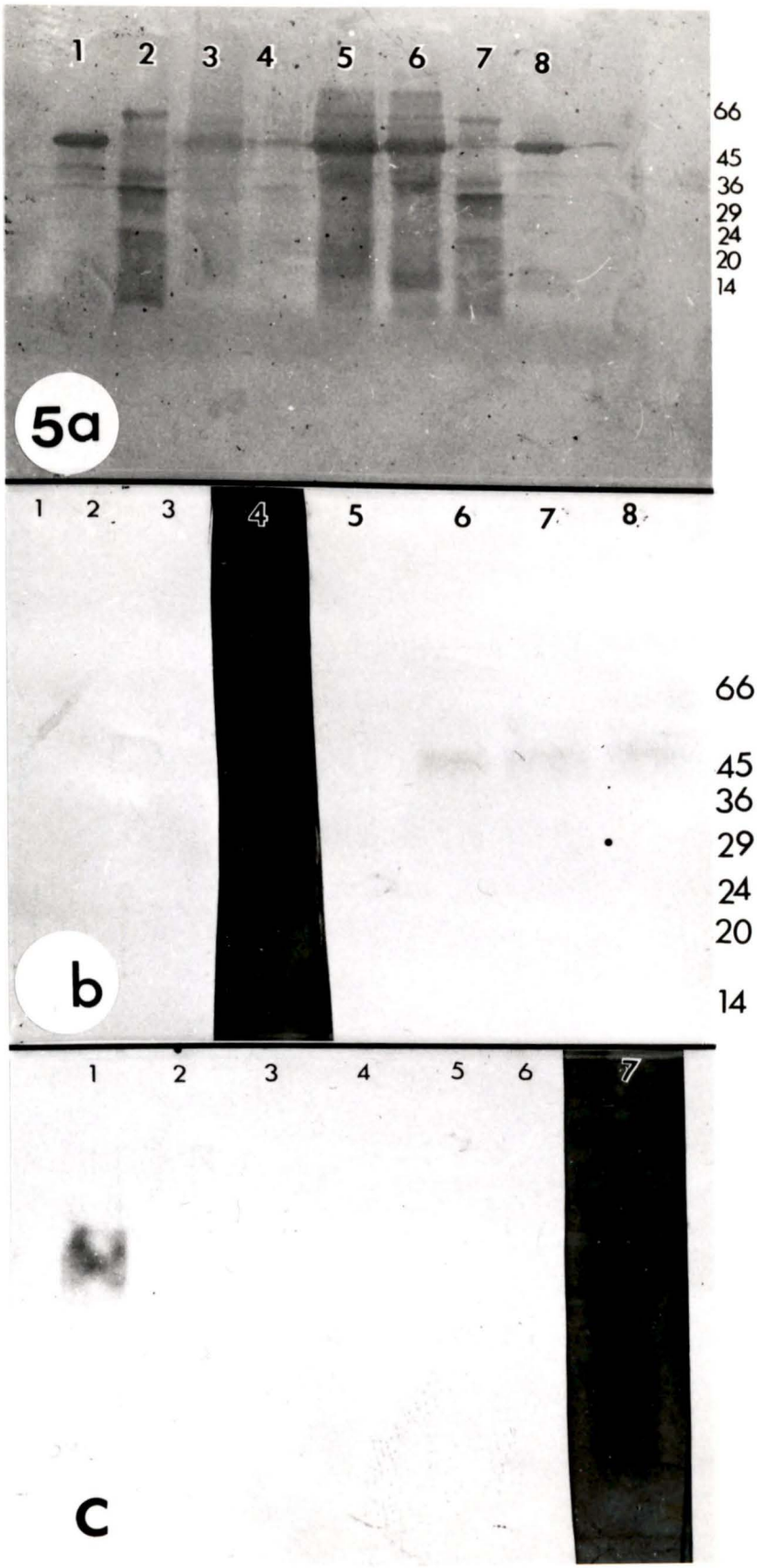


A PLAUSIBLE MECHANISM FOR THE OXYGENATIOIN OF RUBP

Figure 5: ***Western Blot.*** A.Protein bands visualized by staining with amido black. Lanes 1,3 and 4 are dilutions of spinach RuBPC/O-ase (Sigma). Lanes 2 and 7 are molecular weight markers, and lanes 5 and 6 are extracts from *Skeletonema costatum*

B. Immunostained to determine monospecificity Lanes 1 to 3 are non-relevant controls Lanes 1 and 2 were loaded with an extract from *Skeletonema costatum* and lane 3 was loaded with spinach RuBPC/O-ase. Lane 4 was loaded with marker proteins and stained with amido black. Lane 6 was spinach RuBPC/O-ase, and lanes 7 to 9 are dilutions of the *Skeletonema costatum* extract. Lanes 6 to 8 were stained with the anti-RuBPC/O-ase monoclonal antibodies.

C. Immunostained to determine cross-reactivity Lane 1, an extract from Skeletonema costatum, Lane 2, *Hedera helix*, Lane 3, *Lonisera ciliosa*. Lane 4, a diatom *Thalassiosira weissflogii* Lanes 5 and 6, Spinach RuBPC/O-ase. Lane 7, molecular weight markers.


1 2 3 4 5 6 7 8
66
45
36
29
24
20
14
5a
1 2 3 4 5 6 7 8
66
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36
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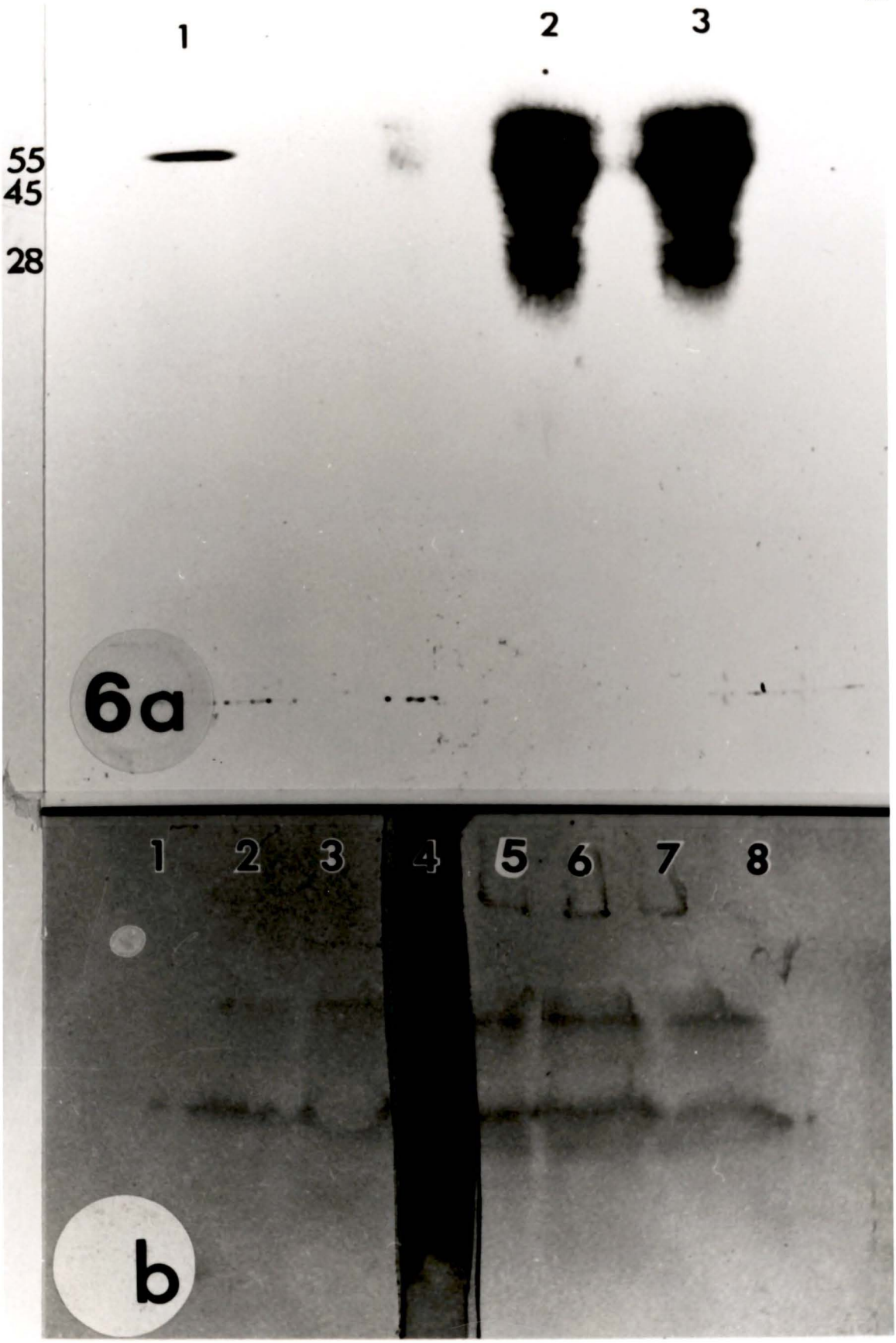
14
b
1 2 3 4 5 6 7
c

Figure 6: ***Assessment of affinity column.***

A. SDS-Polyacrylamide gel electrophoresis of SDS-boiled sepharose beads. Lane 1, spinach RuBPC/O-ase. Lanes 2 and 3 , SDS-boiled beads (supernatant).

B. Western blot and immunostaining of the SDS-boiled Sepharose beads. Lanes 1, 2 and 3 , non-relevant control. Lane 4, molecular weight markers stained with amido black. Lanes 5, 6 and 7, stained with anti-RuBPC/O-ase.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55
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6a
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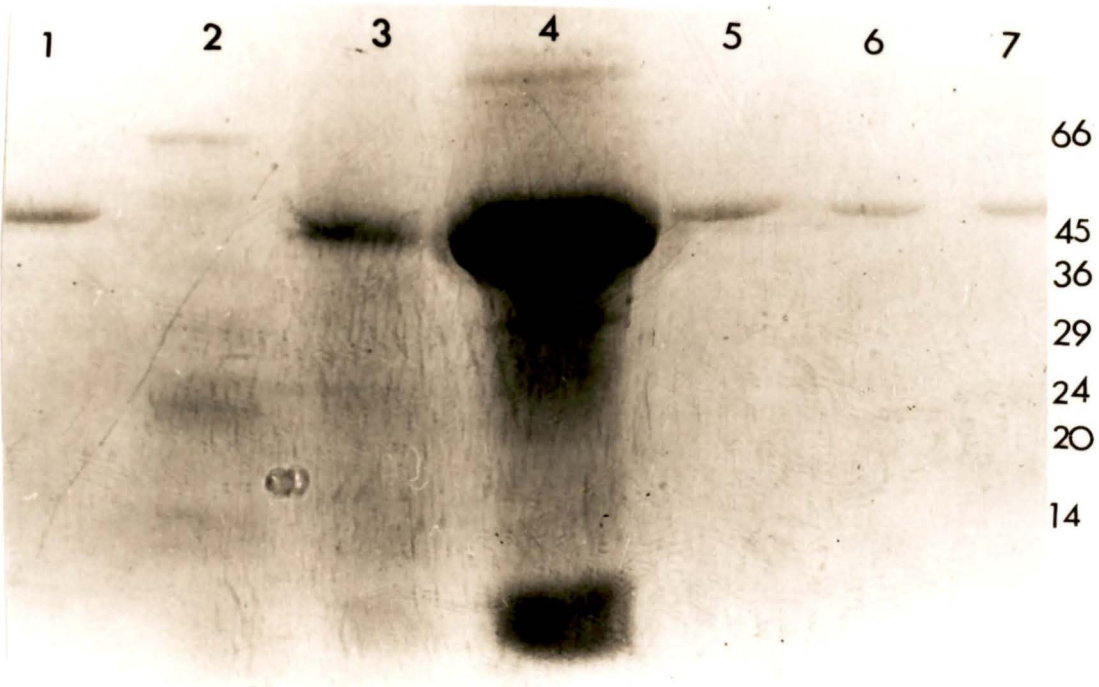
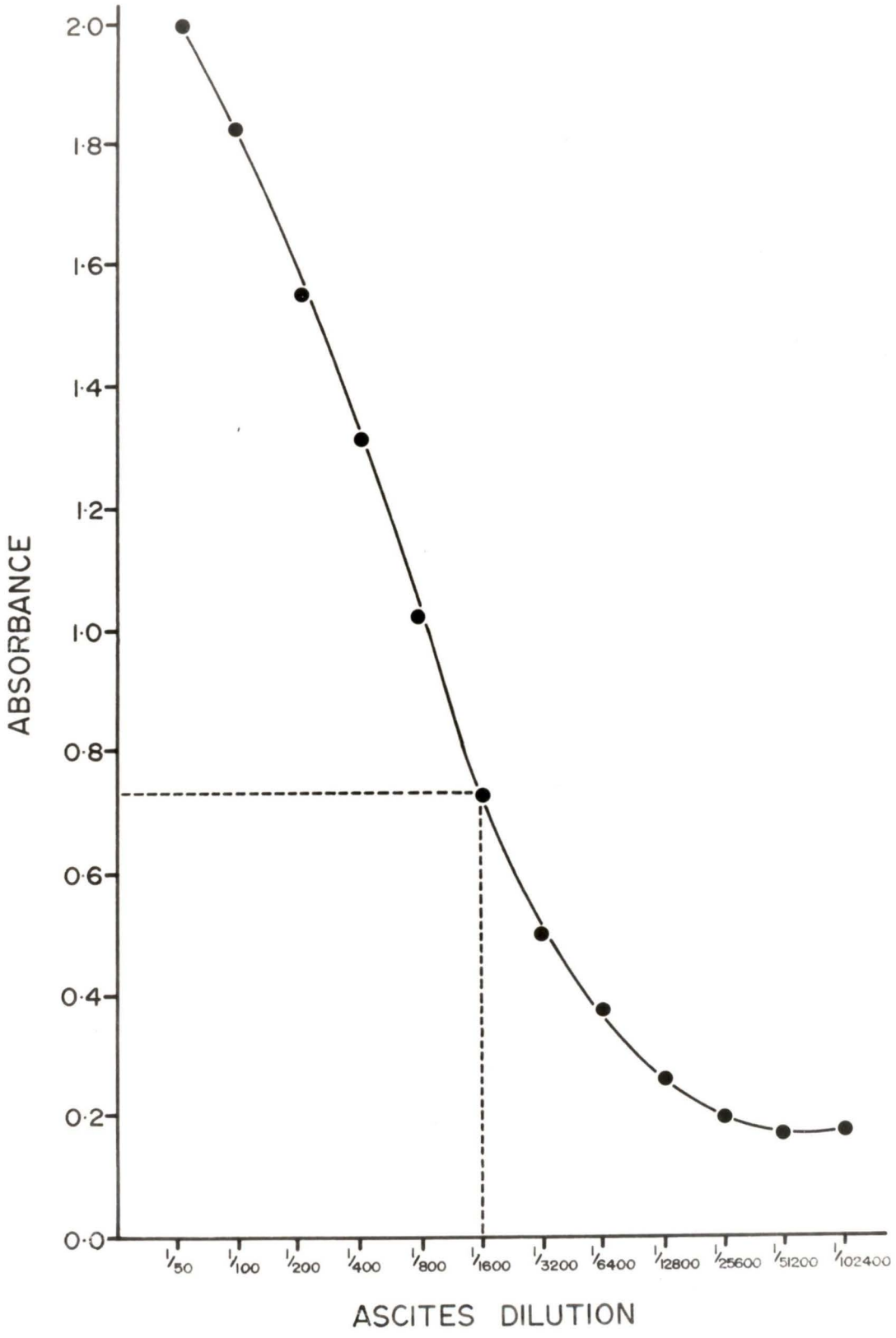
8
b

Figure 7: ***Electrophoretically pure RuBPC/O-ase.*** SDS-Polyacrylamide gel electrophoresis of ammonium sulphate precipitated and sucrose density ultracentrifuged RuBPC/O-ase. Lane 1, Spinach RuBPC/O-ase (Sigma). Lane 2, molecular weight markers. Lane 3, spinach RuBPC/O-ase contaminated by spillage from lane 4. Lane 4, ammonium precipitated *Skeletonema costatum* extract. Lanes 5, 6 and 7, serial dilutions of RuBPC/O-ase from *Skeletonema costatum* after ultracentrifugation.



7

Figure 8: ***Ascites titration.*** Curve relating absorbance to ascites dilution. Each point is an average of 3 replicas. Absorbance decreases with icreasing antibody dilution. 1/x, the antibody dilution at 50-80% reaction was chosen at 1/1600 for use in the Inhibition Assay


2·0
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1·0
0·8
0·6
0·4
0·2
0·0
ABSORBANCE
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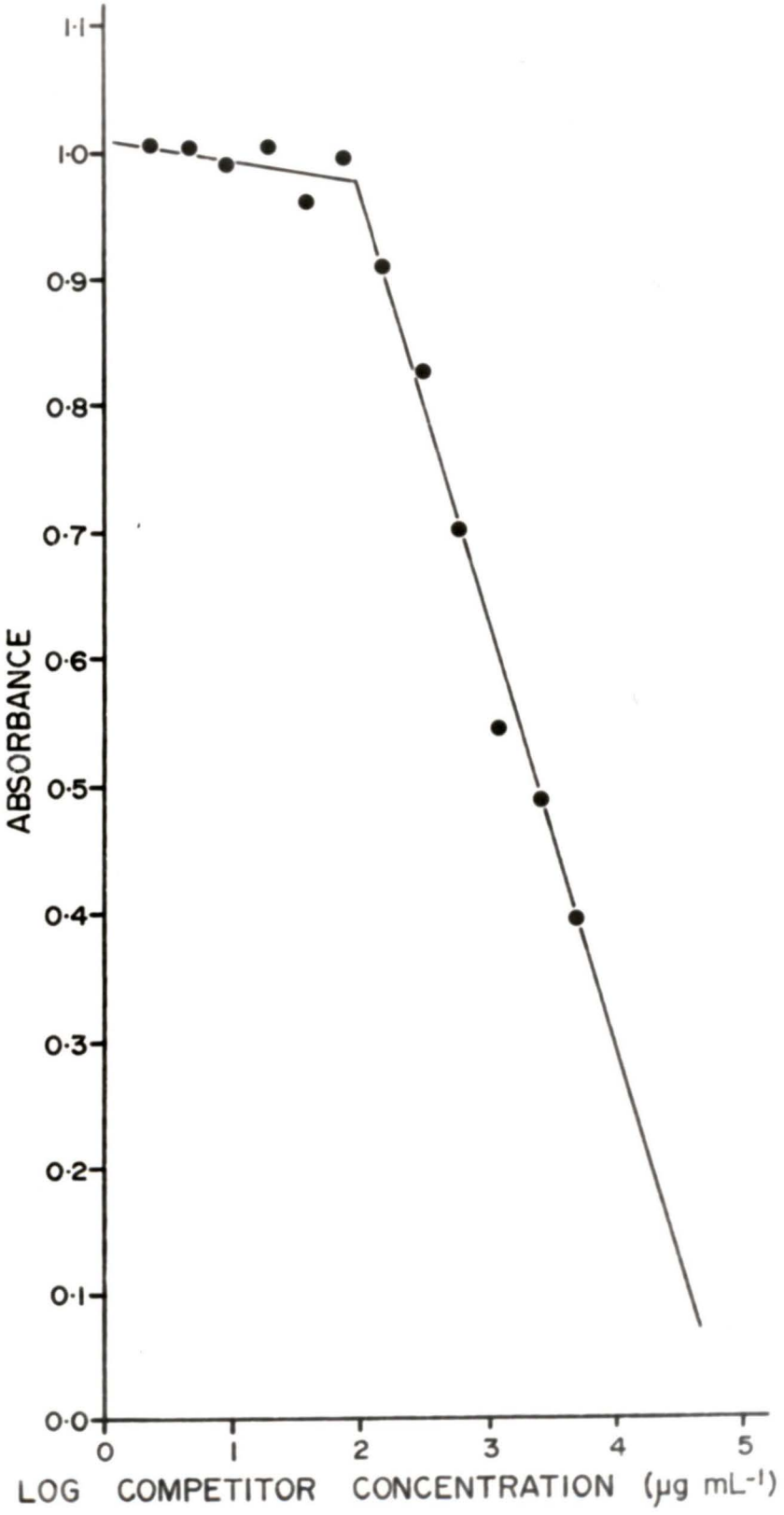
1/100
1/200
1/400
1/800
1/1600
1/3200
1/6400
1/12800
1/25600
1/51200
1/102400
ASCITES DILUTION

Figure 9: ***Standard curve.*** Curve relating absorbance to the logarithm of the concentration of competing antigen. Each point is an average of 3 replicates.

Coating antigen, 50 µl of a 50 µg/ml solution; first antibody, 100 µl of a 1/1600 dilution


1·1
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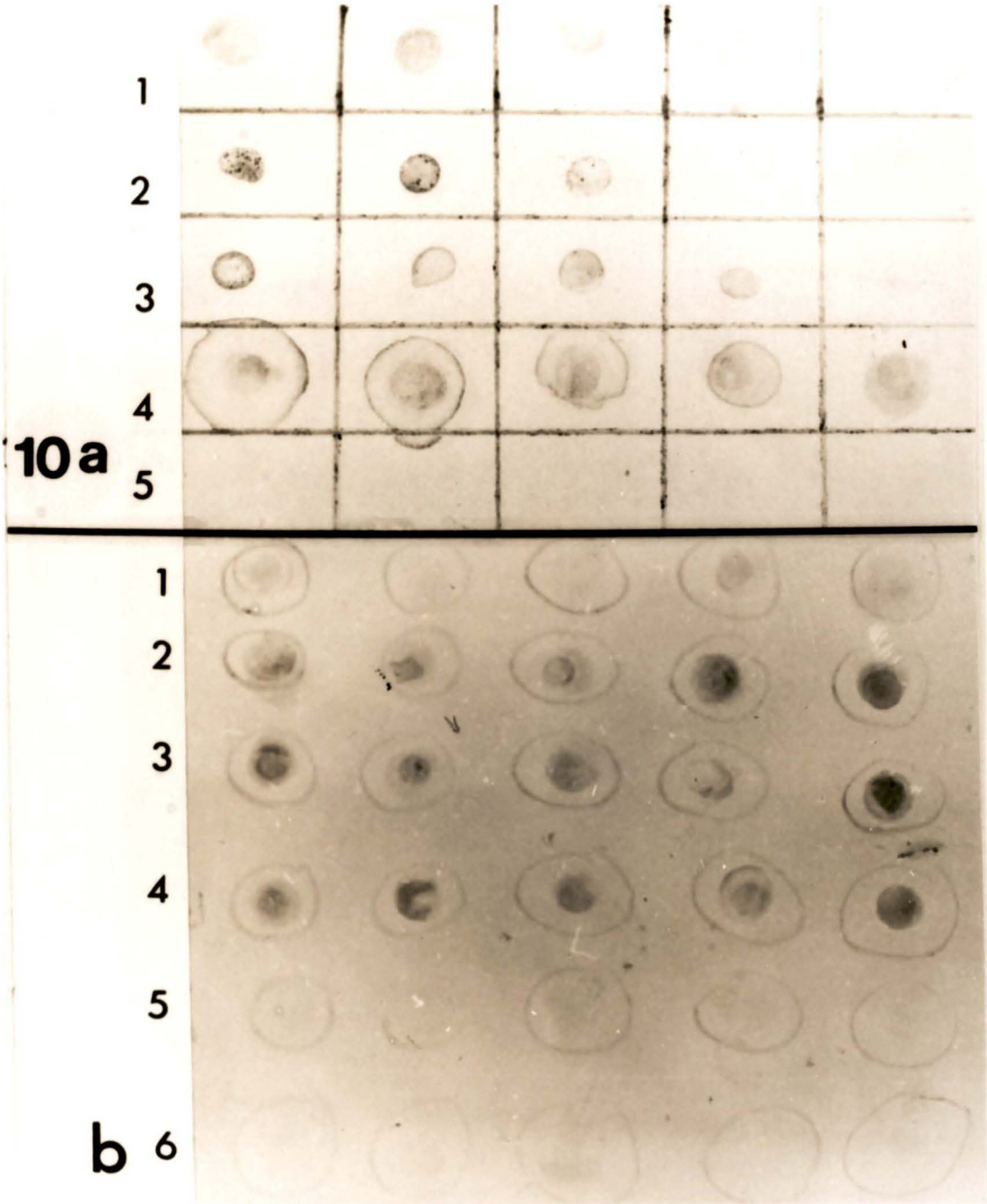
0·6
0·5
0·4
0·3
0·2
0·1
0·0
ABSORBANCE
0
1
2
3
4
5
LOG COMPETITOR CONCENTRATION (μg mL-1)

Figure 10: ***Dot Immunobinding Assay.***

A. Native proteins. Row 1, dilutions of spinach RuBPC/O-ase. Row 2, dilutions of *Hedera helix* Row 3, dilutions of *Lonicera ciliosa*. Row 4, dilutions of *Skeletonema costatum* extract. Row 5, dilutions of BSA.

B. Denatured proteins (SDS-boiled). Row 1, dilutions of spinach RuBPC/O-ase. Row 2, dilutions of *Hedera helix*. Row 3, dilutions of *Lonicera ciliosa*. Row 4, dilutions of *Skeletonema costatum* extract. Row 5, dilutions of BSA. Row 6, All antigens incubated with non-relavent antibody.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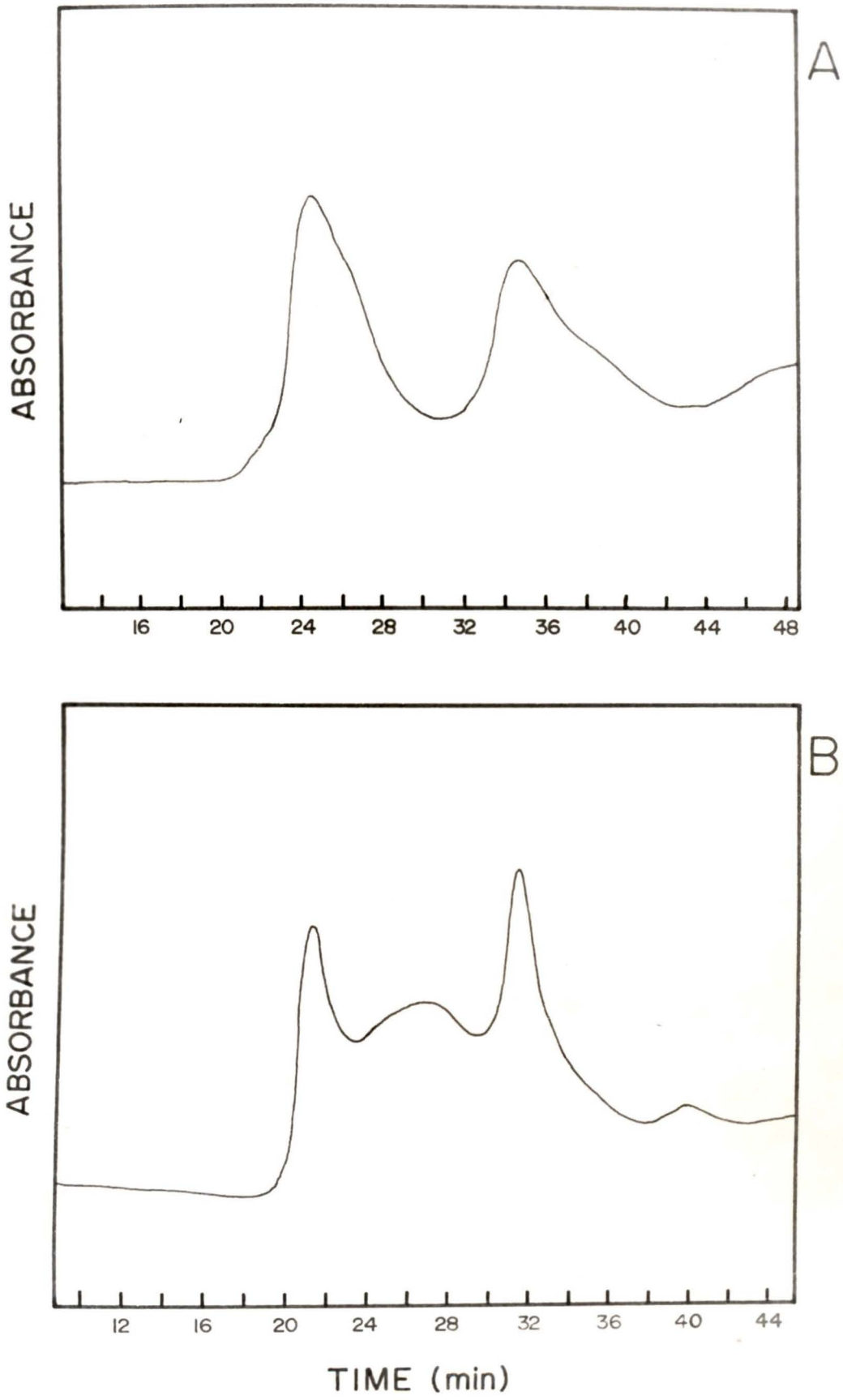
b 6

Figure 11: ***Gel permeation chromatograms.*** A. Chromatogram for spinach RuBPC/O-ase.

B. Chromatogram for ammonium sulphate precipitated RuBPC/O-ase from pea (*Pisum sativum*)

Chart speed, 0.3 cm/min.; Flow rate, 0.15ml/min.; 1.0 aufs.

The first peak represents the large subunit and the second peak, the small subunit.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48
B
ABSORBANCE
12
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24
28
32
36
40
44
TIME (min)

Figure 12: ***Electrophoretically pure RuBPC/O-ase after GPC (HPLC).*** Lanes 1 and 2, pea RuBPC/O-ase after gel permeation chromatography. This is the sample shown as the first peak on the chromatogram. Lane 5, spinach RuBPC/O-ase. Lane 6, marker proteins. Lane 7, Spinach RuBPC/O-ase. Proteins visualized by staining with Coomassie blue.

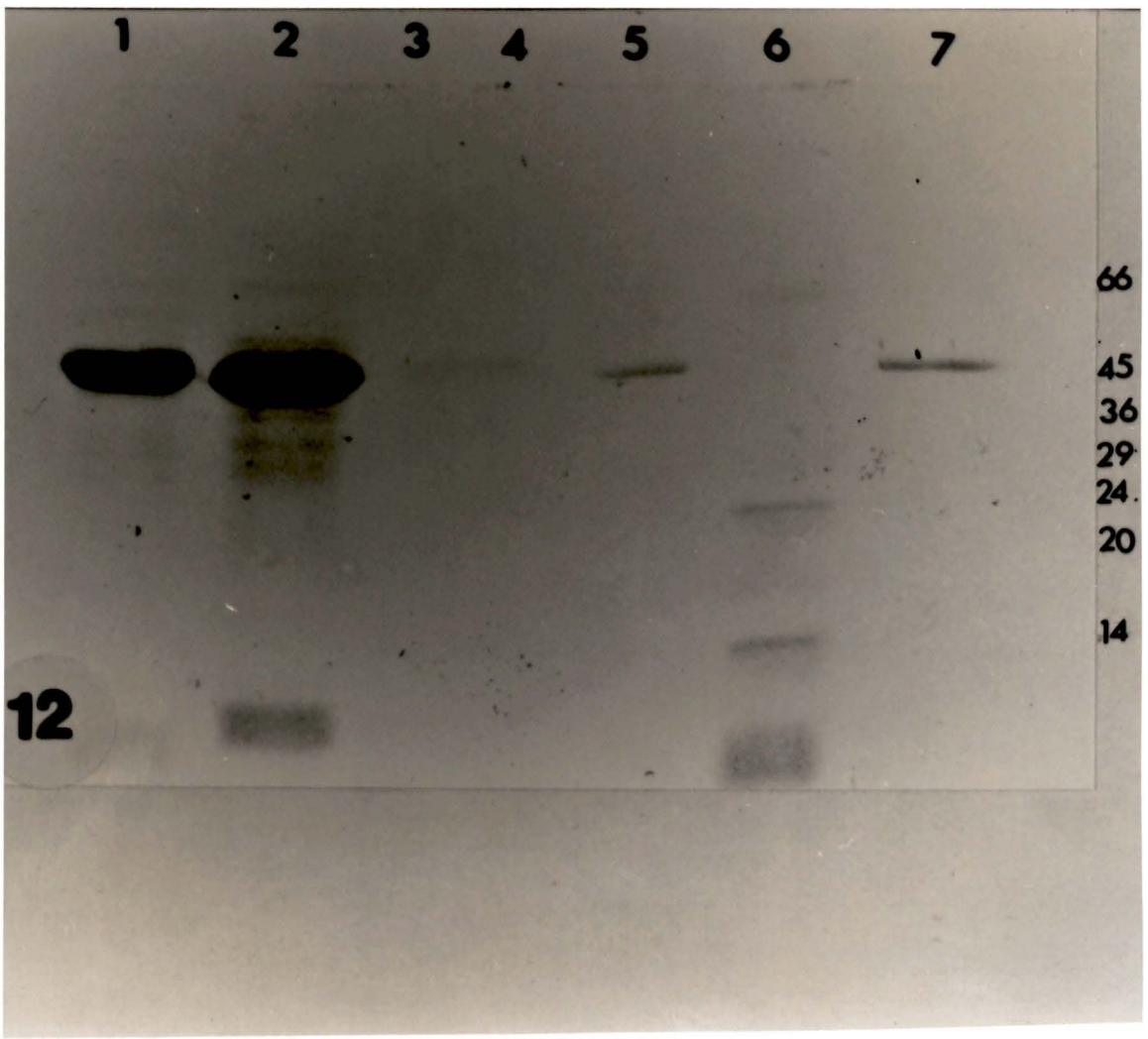
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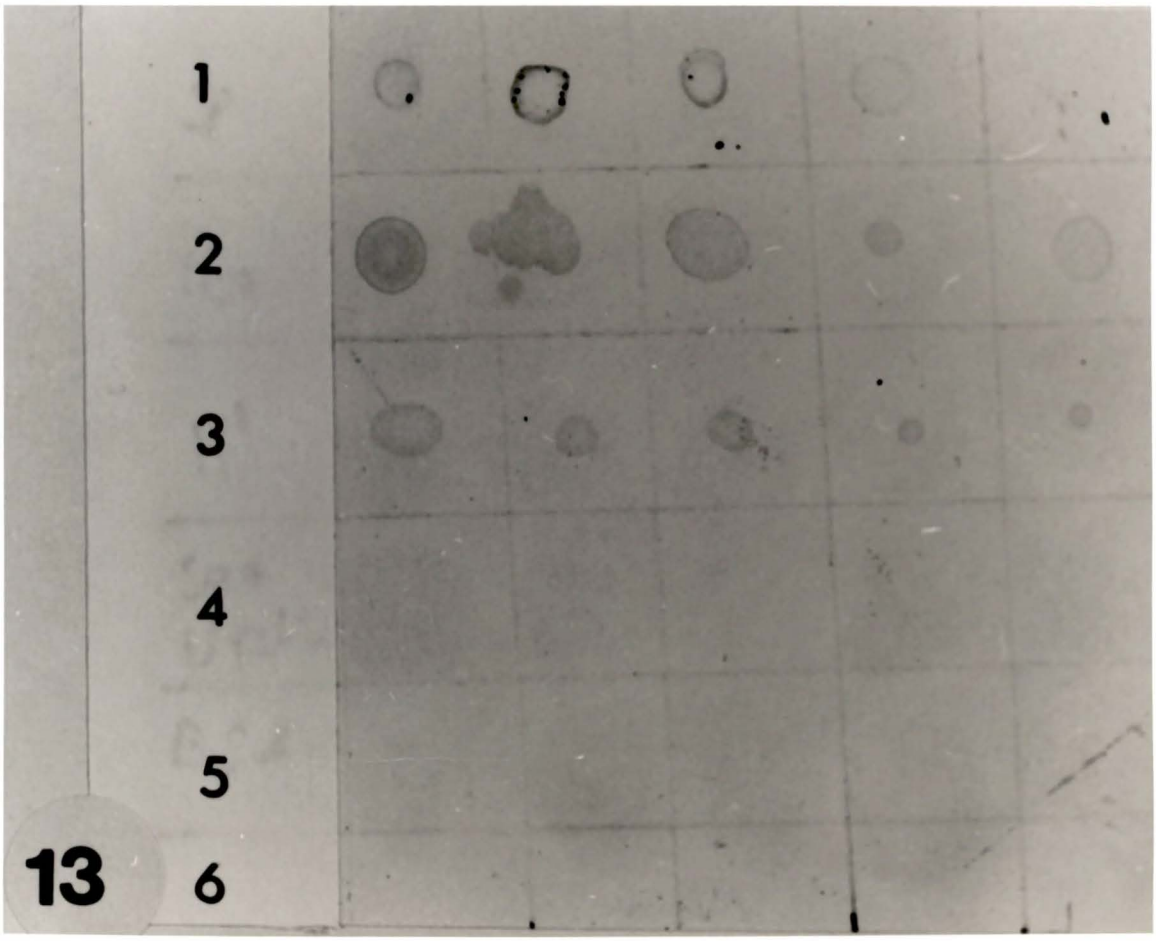
Figure 13: ***Confirmation of Cross-reactivity.*** Dot Immunobinding Assay of purified RuBPC/O-ases to confirm cross-reactivity. Row 1, dilutions of spinach RuBPC/O-ase (Sigma). Row 2, dilutions of the HPLC purified pea RuBPC/O-ase. Row 3, dilutions of an ammonium precipitated and sucrose density ultracentrifuge purified RuBPC/O-ase from higher *Hedera helix* Row 4, sea urchin soluble protein extract. Row 5,dilutions of bovine serum albumin. Row 6, all antigens incubated with non-relevant antibody prepared against sea urchin embryonic tissue.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APPENDIX A

PRODUCTION OF ASCITIC FLUID

Hybridomas will grow in the peritonial cavity of animals of the same strain as the tumour cell line donor and spleen cell line donor and secrete monoclonal antibody into the ascitic fluid formed within the cavity. By this procedure, large amounts of monoclonal antibodies can be produced (10 mg/ml of fluid) without the need for a large scale cell culture. Even though the monoclonal antibody was contaminated with immunoglobulins from the recipient animal, the monoclonal antibody can easily be partially purified, to satisfaction, by ammonium sulphate precipitation.

Materials and Equipment

Hybridomas (about 10 000 000 cells/animal)

Pristane (Aldrich)

Animals of the same strain as spleen cell donor and tumour cell line donor (Balb C mice).

Phosphate buffered saline (PBS)

Syringes (1 ml) with narrow gauge (23-27) hypodemic needles.

Autoclaved dissecting equipment

Conical centrifuged tubes

Pasteur pipets

Procedure

The animal is injected intraperitonially with 0.5 ml pristane (2,6,10,14-tetramethyl pentadecane) while hybridomas are being grown in culture. Seven days after pristane injection, the hybridoma culture is centrifuged (for 10 min at 300 g). The supernatant is discarded and the cell pellet resuspended in sterile PBS (10 000 000 cells/ml). 1 ml of this cell suspension is injected into each animal using narrow gauge needles. After 7 days the animals are inspected daily for swelling of the abdomen (indicator of fluid production) and the very swollen animals are dissected to locate the fluid tumours from which the ascitic fluid is sucked up into conical centrifuge tubes using pasteur pipets. Cells are removed by centrifugation at 500 g for 10 min. The fluid is stored frozen in aliquots.

APPENDIX B

ISOLATION OF MONOCLONAL ANTIBODIES FROM ASCITES FLUID

Materials

A saturated solution of ammonium sulphate is prepared by adding crystals to distilled water. Approximately 1000 g per liter is added and stirred at room temperature for 8 hours. When most of the salt is dissolved the solution is allowed to stand at 5°C overnight. The pH is adjusted to 7.0-7.1 with 30% NaOH.

Procedure

50% saturation is used to precipitate the monoclonal antibodies from the ascites fluid. 1 volume of saturated ammonium sulphate solution is added to 1 volume of ascites fluid. Mixing is achieved by swirling and precipitation allowed to occur for 1 hour. The whole procedure is carried out on ice. The precipitate is collected at 48 000 g for 30 min. After centrifugation, the supernatant is discarded and the precipitate is dissolved in a minimal amount of

distilled water. The solution is dyalyzed against several changes of the desired buffer (PBS, pH 7.4).

The sample is distributed into small freezing vials and kept at -70°C.

APPENDIX C

SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS

Reagents, Chemicals and Materials

30% acrylamide in distilled water

1% N, N'-methylene bisacrylamide

1.5 M Tris-HCl buffer, pH 8.7

0.5 M Tris-HCl buffer, pH 6.8

10% Ammonium persulphate (APS) prepared fresh

10% Sodium dodecyl sulphate (SDS)

N,N,N',N'-tetramethylethylene diamine (TEMED)

Water saturated butanol

Methanol

Acetic acid

Glycine

Bromophenol blue

Glycerol

2-mercaptoethanol

Coomassie blue

Eppendorf pipets

Aspirator

Vacuum grease

Gel apparatus

Shaking water bath

Gel drying apparatus

Power supplies

Recipes for discontinuous SDS separating gels

	Final concentration of acrylamide (%)						
Solution	5	7.5	10	12.5	15	17.5	20
30% acr	5.0	7.5	10	12.5	15	17.5	20
1% Bisacr	7.8	5.8	3.9	3.1	2.6	2.2	2.0
1.5 M Tris-HCl	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Water	9.3	8.8	8.2	6.5	4.5	2.4	0.1
10% APS	0.1	0.1	0.1	0.1	0.1	0.1	0.1
10% SDS	0.3	0.3	0.3	0.3	0.3	0.3	0.3
TEMED	0.01	0.01	0.01	0.01	0.01	0.01	0.01

The columns represent amounts (in ml) of the various solutions necessary to make a 30 ml gel solution. Before adding the SDS and TEMED, the solution is degassed.

The following recipe is used for the preparation of the stacking gel:

30% acrylamide	1.0 ml
1.0% bisacrylamide	1.0 ml
0.5 M Tris-HCl, pH 6.8	2.5 ml
Water	5.35 ml
10 % ammonium persulphate	0.05 ml
10% SDS	0.1 ml
TEMED	0.005 ml

SDS-Sample buffer

	1x	2x	3x
1 M Tris-HCl pH,8.6	6.25 ml	12.5 ml	18.75 ml
6 2-mercaptoethanol	5 ml	10 ml	15 ml
Glycerol	10 ml	20 ml	30 ml
SDS	2.3 g	4.6 g	6.9 g
0.1% Bromophenol blue	1 ml	2 ml	3 ml
Water	make up to 100 ml		

Electrode Buffers (upper and lower)

0.025 M Tris-base

0.192 M Glycine

0.1% SDS

pH, 8.3

Staining Solution

0.05% Coomassie blue in methanol: acetic acid:water (5:1:5) (v:v:v)

Destaining Solution

7% acetic acid in water (v:v)

Stored Gels

7% acetic acid in water (v:v)

Gel Polymerization

Separating gel: 2 hours at room temperature, or 1 hr at 37°C

Stacking gel:

30 min at room temperature

Electrophoresis

At constant current, 8 mA for 30 min, and then at 12 mA for 45 min.

Staining

At room temperature for 45-60 min.

Destaining

At room temperature, until background is clear.

APPENDIX D

ELECTROPHORETIC TRANSFER OF PROTEINS ONTO NITROCELLULOSE

Reagents, Chemicals and Materials

Reagents, chemicals, and materials for SDS-PAGE

Pure nitrocellulose membrane (BioRad)

Whatman 3 MM filter paper

Rabbit antimouse immunoglobulins (HPO-conjugated)

4-Chloro-1-naphthol

Transfer chamber

2 plexiglass plates,perforated

2 scouring pads (Scotch-Brite)

Hydrogen peroxide (30%)

Amido Black

Isopropanol

Transfer Buffer for SDS-Gels:

25 mM Tris-base	3.03 g/l
192 mM glycine	14.4 g/l
20% methanol	200 ml/l

Staining Solution for Nitrocellulose strips

0.1% amido black in acetic acid: isopropanol:water (1:1:8) (v:v:v).

Destaining Solution:

Acetic acid: isopropanol;water (1:1:8) (v:v:v)

Washing Buffer:

20 mM Tris-HCl	3.15 g/l
150 mM NaCl	8.75 g/l
0.1% BSA	1.0 g/l

Adjust pH to 8.2 with 1 N NaOH

Blocking Solution:

5% Fish Skin Gelatin (FSG) or 5% Milk powder in washing buffer

APPENDIX E

ENZYME LINKED IMMUNO SORBENT ASSAY (ELISA)

Reagents Chemicals and Materials

96-well polyvinyl (polysterene) plates (Costar)

Micropipets

Rabbit antimouse immunoglobulins (HPO-conjugated) (Sigma)

O-phenylenediamine

30% hydrogen peroxide

4 N sulphuric acid

Scanning spectrophotometer

Phosphate Buffered Saline (PBS) pH, 7.4

Solution A (1l)

NaCl	30 g
KCl	0.75 g
Na_2HPO_4	4.32 g
KH_2PO_4	0.75 g

Solution B (1l)

$CaCl_2.2H_2O$	1.0 g

Solution C (1l)

$MgCl_2.6H_2O$	1.0g

Final PBS pH, 7.4

Solution A	267 ml
Solution B	100 ml
Solution C	100 ml
Water	533 ml

Citric Acid Ammonia Buffer

3 ml glacial acetic acid is added to 522 ml distilled water. To 500 ml of this solution, 10.5 g citric acid is added. The pH is adjusted to 5.5 with strong ammonia solution and the solution is made up to 1l with distilled water.

Hydrogen Peroxide (3%)

7.5 ml distilled water is mixed with 0.5 ml of 30% hydrogen peroxide. This can be stored for 3 months at 4°C.

APPENDIX F

DOT IMMUNOBINDING ASSAY

Reagents, Chemicals and Materials

Pure nitrocellulose membrane (BioRad)

Soft pencil

Pipets

Milk powder

Bovine serum albumin

Tween-20

4-Chloro-1-naphthol

Methanol

Horseradish peroxide conjugated antimouse immunoglobulin (Sigma)

37°C incubator

APPENDIX G

SOLUBLE PROTEIN EXTRACTION BUFFER

(Paech and Dybing,1986).

100 mM Tris-HCl

100 mM NaCl

20 mM $MgCl_2$

10 mM $NaHCO_3$

1 mM EDTA

5 mM Dithiothreitol (DTT)

10 mM Isoascorbate

1 mM phenyl methyl sulfonylflouride (PMSF)

10 mM Iodoacetamide

20 mM 2-mercaptoethanol

APPENDIX H

TABLE OF ABBREVIATIONS

aufs	absorbance units full scale
BSA	bovine serum albumin
CPBP	carboxypentitol bisphosphate
C-2PCO	photorespiratory carbon oxidation cycle
C-3PCR	photosynthetic carbon reduction cycle
ELISA	enzyme linked immunosorbent assay
LS	large subunit
Me^{2+}	divalent metal ion
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PGA	phosphoglycerate
RuBP	ribulose bisphosphate
RuBPC/O-ase	ribulose bisphosphate carboxylase oxygenase
SDS	sodium dodecyl sulphate
SS	small subunit

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ANALYTICAL DETERMINATION OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE / OXYGENASE USING MONOCLONAL ANTIBODIES

Author 

Khisimuzi Mdluli

23rd October 1986

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