

DEHYDRATION OF MACROMOLECULES

I. EFFECT OF DEHYDRATION-REHYDRATION ON INDOLEACETIC ACID OXIDASE, RIBONUCLEASE, RIBULOSEDIPHOSPHATE CARBOXYLASE, AND KETOSE-1-PHOSPHATE ALDOLASE

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[Manuscript received 21 August 1972]

Abstract

A pressure-membrane technique has been developed to physically manipulate the water potential of *in vitro* enzyme preparations. Enzyme preparations were subjected to a range of water potentials using this technique. Indoleacetic acid oxidase and ribonuclease activities were unaffected by changes in water potential. Ribulose-diphosphate carboxylase was sensitive to water potential change and its activity was reduced to 20% of controls at a water potential of -14 bars. Loss of activity was attributed to fragmentation of the molecules to an apparent molecular weight of less than 1500. Ketose-1-phosphate aldolase lost all activity when subjected to slight water potentials (-1 bar) and fragmented in a similar fashion to ribulose-diphosphate carboxylase. Fragmentation in both cases was not due to protease activity. It is concluded that water has an important structural role in maintaining the molecular integrity of ribulosediphosphate carboxylase and ketose-1-phosphate aldolase, but not in indoleacetic acid oxidase and ribonuclease.

I. INTRODUCTION

The physiological effects of water deficits are well documented. However, the causes of these effects are not so clear. It is known that dehydration or water stress in plants results in changes in the activities of a number of enzymes. For example water stress decreased the activity of nitrate reductase in barley (Huffaker *et al.* 1970) and phenylalanine ammonia-lyase in maize (Bardzik *et al.* 1971) and the combined activities of ribosephosphate isomerase, ribulosephosphate kinase, and ribulose-diphosphate carboxylase in spinach chloroplasts (Plaut 1971). It also increased the activity of ribonuclease in tomato (Dove 1967) and indoleacetic acid (IAA) oxidase in pea and tomato (Darbyshire 1971*a*, 1971*b*). However, the chemical relationships between these changes in enzyme activity and the physical effect of water stress is not clear.

The two orthodox methods used to determine effects of plant water deficits on enzyme activities suffer from disadvantages. If a plant is stressed and an enzyme system of interest extracted, the enzyme environment is changed for the assay from one of low (perhaps -15 bars *in vivo*) to one of higher (-4 bars for phosphate

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buffer *in vitro*) water potential, with the result that it is not known how low water potentials influence the catalytic function of the enzyme. Alternatively, if an enzyme is extracted from plant tissue and the osmotic potential of the reaction environment adjusted by the addition of a suitable solute, it is not possible to distinguish between the direct effects of different concentrations of solute molecules on enzyme activity and the environment they create.

This paper describes how physical manipulation of an enzyme environment may be achieved, obviating the use of solutes. A number of enzymes have been quantitatively dehydrated to particular water potentials and then rehydrated. The various effects of this treatment are reported.

II. METHODS

(a) *Pressure Membrane Apparatus*

(i) *Manipulation of Water Potential*

The term "water potential", which has been defined by Slatyer and Taylor (1960) as the difference between the partial specific Gibbs free energy of water in a system compared with that of pure water, has been used to describe the energy relations of the system throughout this paper. Thus the water potential of an enzyme system will decrease with increasing solute concentration, increase with increasing temperature, and increase with increasing pressure.

A physical method using a pressure membrane apparatus (Darbyshire and Anlezark 1972) was used to dehydrate enzyme molecules. There is no record of this apparatus being hitherto employed as a tool for biological and biochemical research, although it has been used in wood technology (Barkas 1950) and extensively in soil-water research (e.g. Richards and Wadleigh 1952). The pressure membrane apparatus has a cylindrical brass body closed at one end. The closed end includes a solid filter support with a spider's web pattern cut into the surface and leading to a drain. A dialysis membrane underlain by a Millipore filter is held in position over the filter support by an acrylic sleeve containing a teflon insert which defines the filter area and acts as a basal seal. The filter surface has an effective diameter of 25 mm. After closing the apparatus with a screw top, air pressure from a cylinder is applied to the contents.

The membrane used in all experiments was Visking dialysis tubing (36/32). The membranes were soaked in changes of deionized water for 4 days. They were then placed in the apparatus and flushed with at least 3 ml of the buffer to be used with the particular enzyme preparation. In one series of experiments the membranes were prepared more thoroughly by washing in two changes of hot 50% ethanol, two changes of 10 mM NaHCO₃, one change of 1 mM EDTA, and two changes of deionized water. This rigorous washing did not influence the results obtained with ribulosediphosphate carboxylase and ketose-1-phosphate aldolase and hence results were not due to membrane artifacts. Membranes were underlain by a Millipore filter (0.01 μ m pore size) to ensure a smooth surface for the dialysis membrane.

After an aliquot of enzyme preparation is added to the chamber a pressure is applied. This results in an increase in the total water potential (see Fig. 1) and water and salts pass through the membrane, but the enzyme molecules are retained. Movement of water through the membrane continues until the solution external to the membrane at atmospheric pressure is in equilibrium with the internal phase (see Fig. 1). When the pressure is released the enzyme phase inside the chamber is subjected to a tension equivalent to the applied pressure. Thus by varying the applied pressure the enzyme environment can be manipulated to a range of water potentials. After rehydration with a suitable buffer the effect of the dehydration-rehydration treatment may be determined by assaying for enzyme activity.

(ii) *Operation of the Pressure Membrane Apparatus with Enzymes*

After 0.1 ml of the enzyme preparation was placed in the pressure membrane apparatus and a selected pressure applied, equilibration was allowed to occur for 90 min. Pressure was then

released and at the same time the desired water potential was generated (Fig. 1). The system was allowed to stand for 5 min before rehydrating with 2 ml of buffer. A 20-min rehydration period was followed before assaying for enzyme activity.

The water potentials of the buffers were determined using thermocouple psychrometers except in the case of the acetate buffer where the water potential was calculated. The water potential of the phosphate (IAA oxidase), acetate (ribonuclease), and Tris buffers (ribulose-diphosphate carboxylase and ketose-1-phosphate aldolase) were -4 , -4 , and -2 bars respectively. Corrections for these values were made when estimating the final water potential of the enzyme phase.

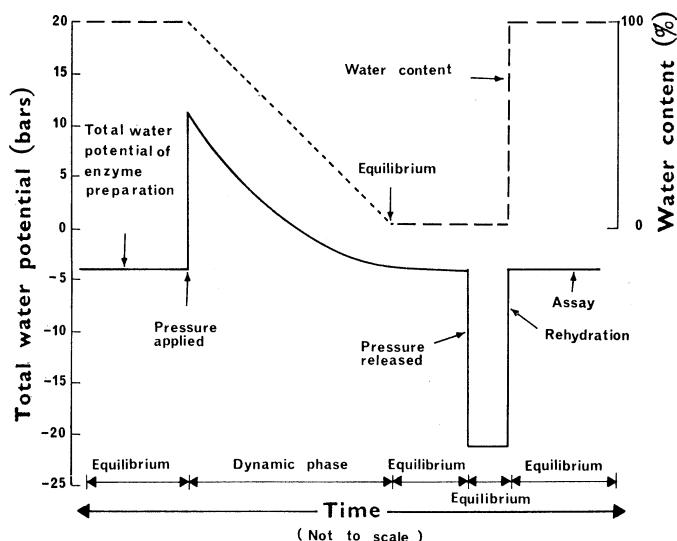


Fig. 1.—Theoretical changes in water potential and water content in the pressure-membrane apparatus. The water potential of the buffer for the enzyme preparation must be considered when determining total water potential generated after pressure release.

(iii) Hydrostatic Control

To determine the effects of pressure on an enzyme system, hydrostatic controls were routinely included by placing a neoprene rubber membrane under the Millipore filter to prevent equilibration. Pressure had no effect on IAA oxidase or ribonuclease activities where hydrostatic controls were the same as unpressured controls. Ribulosediphosphate carboxylase and ketose-1-phosphate aldolase activities were about 80 and 90% of unpressured controls. This did not influence the results obtained as all results were expressed in terms of activity with respect to the hydrostatic control.

(iv) Water Content and Ionic Control

The above technique is limited as the water content and ionic strength of the enzymes' surroundings are not characterized. When using enzyme preparations in buffer solutions, the major part of the aqueous phase passes through the membrane and upon equilibration the enzyme is supported on an apparently dry membrane. These objections have been overcome by using Sephadex resins. A resin is hydrated in buffer, an aliquot added to a chamber, and equilibration allowed to occur after a desired pressure is applied. The water content of the resin at a particular water potential may then be estimated from:

$$\text{Water content (\%)} = \left(\frac{\text{equilibrated weight} - \text{dry weight}}{\text{equilibrated weight}} \right) \times 100.$$

Six resins subjected to treatments resulting in a range of water potentials are shown in Figure 2. Each resin has a characteristic water content–water potential curve, and Sephadex G10 and G15 are particularly useful because for a wide range of water potential values their respective water

contents do not vary to any great extent. Other resins are useful in particular situations; for example, Sephadex G100 may be used to control water contents at a high level while water potentials are higher (Fig. 2). With the resins present the ionic concentrations are also better controlled as water and ions can now be held within the lattice of the resin beads. Another compound, Dextran T20 (Pharmacia Chemicals), which is soluble in water, has been used in some experiments. Its water content curve for a range of water potentials is also shown in Figure 2. As it has a molecular weight of approximately 20,000 it does not contribute significantly to the water potential of its solution. However, after equilibration of an aliquot of a solution of Dextran T20, its increased concentration due to water loss results in a characteristic water potential.

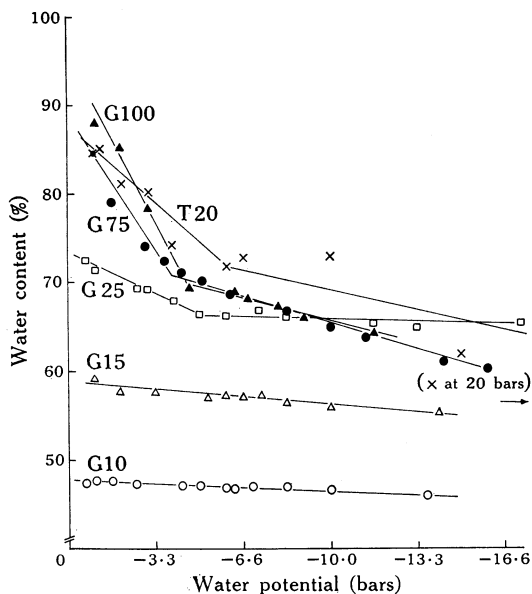


Fig. 2.—Relationships between the water potential and water content of a number of Sephadex resins and Dextran T20.

In experiments where Sephadex G15 was included in chambers, 1.5 ml of the resin was allowed to equilibrate for 24 hr before adding 0.1 ml of the enzyme preparation and following the above procedure. The rehydrated resin, enzyme, and buffer phase was removed from the apparatus and placed in a column. The enzyme was eluted from the resin by collecting a 3-ml fraction. Treated enzyme preparations were then assayed to determine the effect of dehydration upon molecular integrity, at particular water contents and water potentials.

Ribulosediphosphate carboxylase and ketose-1-phosphate aldolase were equilibrated in the presence of Sephadex G15 in all but preliminary experiments. IAA oxidase and ribonuclease preparations were equilibrated in both the presence and absence of G15 over a range of water potentials.

(v) Physiological Comparisons

The water relations of the Sephadex resins were expressed in terms of percentage water content. The water status of a plant is often described by the relative water content

$$\left(\frac{\text{fresh weight} - \text{dry weight}}{\text{turgid weight} - \text{dry weight}} \right) \times 100$$

but it has not been possible to express the water content of resins in this manner. In order to achieve a physiological comparison the water content, i.e. $[(\text{fresh weight} - \text{dry weight}) / \text{fresh weight}] \times 100$, of leaves from plants of three species has been determined. Leaves were equilibrated

in a Scholander pressure chamber (Scholander *et al.* 1964) at a pressure just exceeding their equivalent water potential pressure. The leaves were then weighed and dried. Their respective water contents (as % water content) were plotted against the associated water potential. The water content–water potential relationship for tomato, pepper, and citrus are shown in Figure 3. While there are inherent differences between species it is obvious that solute concentration rather than water content must be the controlling factor of leaf water potential. These data have been included to compare plant–water relationships with the Sephadex resin–water relationships.

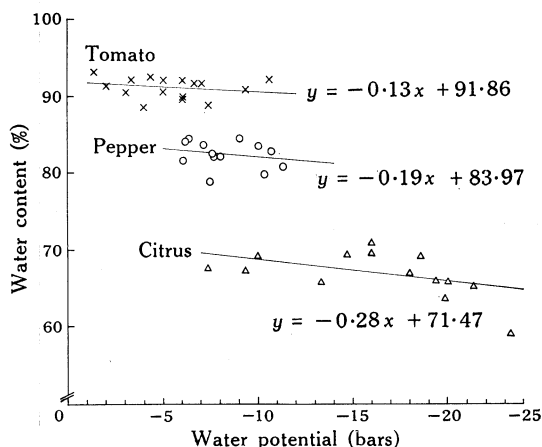


Fig. 3.—Relationship between water potential and water content of tomato, pepper, and citrus. The fact that water content does not appreciably change with decreasing water potential indicates control of plant water potential by changing solute.

(b) Enzyme Preparation and Assay

(i) Indoleacetic Acid Oxidase

(1) *Source*.—Seeds of *Pisum sativum* (cv. Greenfeast) were soaked in deionized water for 12 hr, planted in perlite, and grown in a controlled-environment growth cabinet (16 hr day at 23°C) for 10 days. 16 g of roots were harvested, homogenized in chilled acetone (−15°C), and the resulting powder was further washed three times with acetone. The powder was then dried in a desiccator for 1 hr, extracted into phosphate buffer (pH 6.4), and centrifuged at 18,000 g for 20 min at 4°C. The supernatant liquid was concentrated under vacuum to 10 ml. This was then fractionated on a column of SE Sephadex C50 (bed volume 100 ml), previously equilibrated with the buffer, and 5-ml fractions collected. All fractions displaying IAA oxidase activity were combined. The combined fractions were then concentrated to 10 ml using an Amicon model 12 ultrafiltration cell with a Diaflo membrane UM-20E. No IAA oxidase activity was detected in the filtrate. The concentrated fractions were used as the enzyme preparation for experiments.

(2) *Assay*.—IAA oxidase activity was determined colorimetrically. The reaction mixture consisted of 5 ml IAA (5.8 mM), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (100 μM), 2,4-dichlorophenol (40 μM) in buffer, and 1 ml of enzyme. The reaction occurred at 34°C for 1 hr, then 3 ml of Salkowski reagent (consisting of 20 ml of 0.5M FeCl_3 , 500 ml deionized water, and 500 ml of 72% HClO_4) was added. The colour was allowed to develop for 30 min and the absorbance read at 530 nm with a Bausch and Lomb Spectronic 20 colorimeter.

(ii) Ribonuclease (E.C. 2.7.7.16)

(1) *Source*.—A commercial preparation of the enzyme was used (Calbiochem 55674) at two concentrations, 2 and 4 $\mu\text{g}/\text{ml}$ in acetate buffer (pH 5.0).

(2) *Assay*.—1 ml of substrate (RNA, Sigma R6625) was added to 0.1 ml of the enzyme. The reaction was allowed to occur at 37°C for 4 min before being stopped by the addition of 1 ml of uranyl acetate (1% uranyl acetate in 25% HClO_4). The reaction mixture was then centrifuged at 22,000 g for 20 min. 0.1 ml of the supernatant was diluted with 3 ml of deionized water and

the absorbance of this read at 260 nm. Activity was expressed in terms of optical density and the effect of dehydration-rehydration treatment expressed as a percentage of the hydrostatic controls.

(iii) *Ribulosediphosphate Carboxylase* (E.C. 4.1.1.38)

(1) *Source*.—A commercial preparation from spinach (Sigma Chemical Company) was normally used, but in some preliminary experiments it was prepared from *Capsicum frutescens* L. by homogenizing leaves in 50 mM tricine-NaOH or Tris-H₂SO₄ (pH 8.0) + 10 mM MgCl₂ + 1 mM dithiothreitol in the presence of Polyclar AT (insoluble polyvinyl pyrrolidone). After filtering through muslin the filtrate was centrifuged at 100,000 g for 90 min at 4°C. The total soluble protein fraction was used for the low water potential treatments.

The Sigma preparation was dissolved in Tris-HCl or Tris-H₂SO₄ (pH 8.0) + 10 mM MgCl₂ + 1 mM dithiothreitol (1.7 mg protein per millilitre) and left overnight.

(2) *Assay*.—The activity of the carboxylase was assayed in a medium containing 30 μ moles Tris buffer pH 8.0, 10 μ moles MgCl₂, 1 μ mole dithiothreitol, and 10 μ moles NaH¹⁴CO₃ containing 10 μ Ci in 0.4 ml. An 0.5-ml aliquot of the enzyme solution was incubated in the reaction mixture for 5 min before the reaction was started by the addition of 0.1 ml ribulose diphosphate (0.5 μ mole). The temperature was 25°C and the reaction mixture was sampled at intervals by withdrawing 0.2 ml and adding it to a vial containing concentrated HCl. Usually the assay was carried out over 4 min. The acidified samples were dried under infrared lamps and counted in a scintillation spectrometer. Radioactivity was corrected for background and quenching and the enzyme activity was expressed as nanomoles CO₂ fixed per minute per 170 μ g of original enzyme. The activity of the treated enzyme was expressed as a percentage of the hydrostatic control.

(iv) *Ketose-1-phosphate Aldolase* (E.C. 4.1.2.7)

(1) *Source*.—A commercial preparation (Sigma Chemical Company A 6253) of the aldolase was used. 0.3 ml of this preparation was diluted to 5 ml with Tris-HCl buffer (pH 7.8) so that 0.1 ml of the diluted enzyme contained 0.21 units of activity. One unit has been defined in this case as the amount of enzyme required to convert 1.0 μ mole of fructose diphosphate to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate per minute at 25°C.

(2) *Assay*.—Enzyme activity was based on the method of Sibley and Lehninger (1949) as adapted by the Sigma Chemical Company (Sigma Technical Bulletin No. 750, March 1971).

(c) *Gel Electrophoresis*

Discontinuous electrophoresis in 7 or 15% polyacrylamide gels was carried out in the Tris-glycine system of Davis (1964). Separating gels were equilibrated before use at 3 mA per gel for 2 hr in the gel buffer system. Samples were applied above the stacking gel in Sephadex G15 or G200. After electrophoresis for 2 hr at 100 V and 2 mA per tube, gels were stained with amido black or coomassie blue.

III. RESULTS

(a) *Indoleacetic Acid Oxidase*

The effect of dehydrating IAA oxidase to a range of water potentials and then rehydrating with buffer is shown in Table 1. The activity of IAA oxidase is unaffected by dehydration from -5.6 to -17.3 bars both in the absence of Sephadex G15 and in its presence where the water content was from 57 to 54%. At no time in our experiments have we found IAA oxidase to be affected by the dehydration-rehydration treatment.

(b) *Ribonuclease*

It may be seen from Table 2 that ribonuclease, like IAA oxidase, is quite insensitive to dehydration treatments, and the result was not affected by enzyme

concentration. When either 2 or 4 $\mu\text{g/ml}$ ribonuclease is dehydrated, upon rehydration all activity is retained indicating that a possible protein-protein interaction did not occur. Water content control with Sephadex G15 did not have any influence. With both ribonuclease and IAA oxidase no interaction with the membrane, or loss of molecules through the membrane, could have occurred otherwise these results would not have been obtained.

TABLE 1
ACTIVITY OF IAA OXIDASE AFTER BEING DEHYDRATED TO VARIOUS
WATER POTENTIALS THEN REHYDRATED WITH PHOSPHATE BUFFER

Sephadex G15 absent		Sephadex G15 present		
Water potential (bars)	Activity (as % of hydrostatic control)	Water potential (bars)	Water content (%)	Activity (as % of hydrostatic control)
- 5.6	97.2	- 6.0	57.25	101.6
- 7.3	95.1	- 7.3	56.75	98.3
- 9.0	96.0	- 9.3	56.25	98.3
-10.6	97.58	-10.6	55.75	100.0
-14.0	99.0	-14.0	55.00	101.7
-17.3	97.0	-17.3	54.00	100.0

TABLE 2
ACTIVITY OF RIBONUCLEASE AFTER BEING DEHYDRATED TO VARIOUS WATER POTENTIALS IN THE
PRESENCE OR ABSENCE OF SEPHADEX G15, AND REHYDRATED WITH ACETATE BUFFER

Sephadex G15 absent				Sephadex G15 present		
Water potential (bars)	Activity (as % of hydrostatic control)*	Water potential (bars)	Activity (as % of hydrostatic control)†	Water potential (bars)	Water content (%)	Activity (as % of hydrostatic control)†
- 7.3	109.0	- 5.6	91.6	- 5.6	57.25	100.0
-10.6	90.9	- 7.3	100.0	- 7.3	56.75	87.3
-14.0	118.2	-10.6	83.3	- 9.3	56.25	90.1
-17.3	100.0	-14.0	85.4	-10.0	56.00	94.9
-20.6	86.4	-17.3	91.6	-14.0	55.00	88.2
		-20.6	104.0	-17.3	54.00	92.7

* Ribonuclease concentration 2 $\mu\text{g/ml}$.

† Ribonuclease concentration 4 $\mu\text{g/ml}$.

(c) *Ribulosediphosphate Carboxylase*

The activity of treated enzyme as a percentage of the hydrostatic control is plotted for both enzyme sources in Figure 4 as a function of the total water potential experienced by the preparation at pressure release. In these experiments the water content of the system was not defined but later, by the inclusion of Sephadex G15, treatments were made with water contents of 58–65% over the water potential range of -3 to -9 bars. This constant water content did not alter the response of the

enzyme to low water potentials. All the preceding treatments were made at 4°C when a cold inactivation of ribulosediphosphate carboxylase (Kawashima *et al.* 1971) would be expected. The Sephadex G15 observations were repeated at room temperature (23°C) and inactivation appeared to be marginally more severe when compared with the lower temperature.

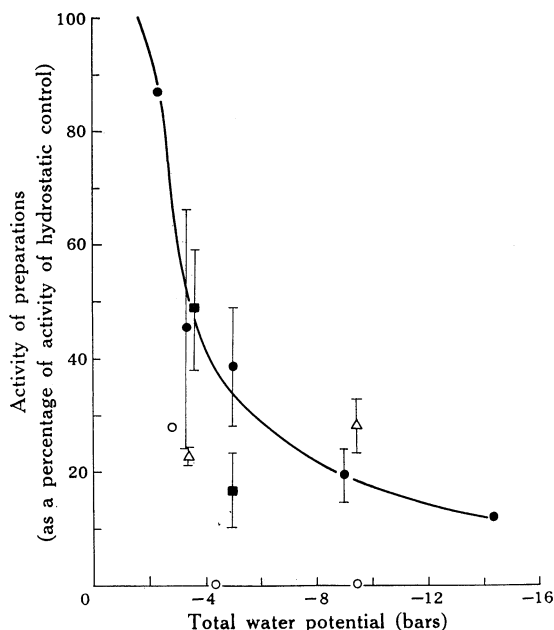


Fig. 4.—Effect of low water potential treatment on the activity of *in vitro* preparations of ribulosediphosphate carboxylase: ■ *Capsicum frutescens* L. preparation at 4°C. ● Sigma preparation at 4°C. △ Sigma preparation with water content controlled by the presence of Sephadex G15 at 4°C. ○ Sigma preparation at 23°C. Vertical bars represent 1 S.E. from five or six observations.

The effect of membrane interaction with ribulosediphosphate carboxylase was determined but no residual activity on the membrane could be detected. Loss of activity was associated with the fragmentation of the enzyme, as judged from Sephadex and polyacrylamide-gel studies. Fractionation of the products of the low water potential treatment on Sephadex G25 column showed that they were not excluded from the resin, but discrimination was obtained by using Sephadex G15 (Fig. 5).

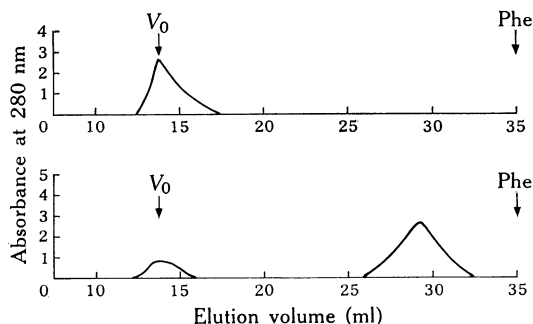


Fig. 5.—Elution profiles (relative absorbance units) at 280 nm of Sigma ribulosediphosphate carboxylase prepared in Tris-H₂SO₄. Upper: native enzyme. Lower: enzyme desiccated at -9.3 bars. V_0 is the void volume and Phe the elution volume of phenylalanine. Sephadex G15 was used in a column 0.9 by 50 cm.

Thus the apparent molecular weight of the fragments was less than 1500. There was no indication of the presence of separate subunits in the treated preparation. Any

residual ribulosediphosphate carboxylase activity was due to the presence of native enzyme. These findings are consistent with those from polyacrylamide-gel electrophoresis where only the residual native protein band could be located by amido black or coomassie blue staining on samples after treatment at low water potentials. Mobilities relative to the front for the native protein on 7% gels were 0.097, and on 15% gels 0.026. Quantitation on acrylamide gels of residual native ribulosediphosphate carboxylase (Racusen and Foote 1965) after desiccation at -9.3 bars indicated that only 10.01% (S.E. ± 1.43 ; $n = 10$) of native protein remained after treatment when compared to unpressured controls.

To determine if any enzyme or protein material remained on the membrane after low water potential treatments, two procedures were followed. Membranes were included in reaction mixtures but assays did not detect any carboxylase activity. Subsequently the carboxylase preparations were confined to a small section of the membrane while equilibration continued. After pressure release and rehydration the membrane was excised and placed on a polyacrylamide gel. After electrophoresis no protein bands could be visualized after staining with coomassie blue. These results indicated that no material remained on membranes after rehydration.

An obvious agent in the generation of these fragments from ribulosediphosphate carboxylase during exposure to low water potentials would be a protease, and a number of experiments have been carried out to test for the participation of a proteolytic enzyme in the system. The influence of 1 mM Fe^{3+} , 1 mM EDTA, and the presence or absence of 1 mM dithiothreitol incorporated in the Sephadex G15 resin in the pressure chamber during treatment was ascertained by comparing polyacrylamide-gel electrophoresis patterns with controls without the agents and with native untreated enzyme. None of the additions prevented the disappearance of the native band of ribulosediphosphate carboxylase caused by exposure to low water potentials. If a protease was responsible for the fragmentation of this enzyme then it did not belong to the groups of proteases that are inhibited by the above reagents. Other proteases such as papain (Whitaker and Perez-Villasenor 1968) and a neutral protease from oats (Gardner *et al.* 1971) are inhibited by phenyl methyl sulphonyl fluoride (PMSF). The inclusion of 5 mM PMSF with ribulosediphosphate carboxylase during treatment did not prevent the fragmentation of the latter as determined from the 280 nm absorbance pattern of the eluate from a Sephadex G15 column; the low molecular weight fragments were present in treatments with and without PMSF.

Other evidence is also against protease participation in the fragmentation phenomenon. Sugiyama *et al.* (1968) found that preincubation of ribulosediphosphate carboxylase with ribulose diphosphate prevented proteolytic attack on the enzyme by a protease, Nagarse. The inclusion of 1 mM ribulose diphosphate in our system did not prevent the loss in carboxylative activity after treatment at low water potentials. A common substrate for the assay of protease activity is Azocoll (Calbiochem), but when it was included in the Sephadex G15 resin in the pressure chamber along with ribulosediphosphate carboxylase there was no release of dye from the Azocoll during or after low water potential treatment. For these reasons we think that fragmentation of ribulosediphosphate carboxylase at low water potentials is not the result of protease contamination of the system.

(d) Ketose-1-phosphate Aldolase

The results from dehydrating the aldolase were, as with ribulosediphosphate carboxylase, completely dissimilar to those with IAA oxidase and ribonuclease. We found that any dehydrating treatment, no matter how mild (-1 bar), resulted in a complete loss of activity. The presence of Sephadex G15 did not change this result. Teipel and Koshland (1971) found that inactivation of ketose-1-phosphate aldolase by 6M guanidine hydrochloride could be almost completely reversed within 10 min of removing the agent. Even after 20 min of rehydration no reassociation or activation of the molecules could be detected. This drastic effect of dehydration was examined more closely using Sephadex and polyacrylamide-gel characterization of native and dehydrated enzyme.

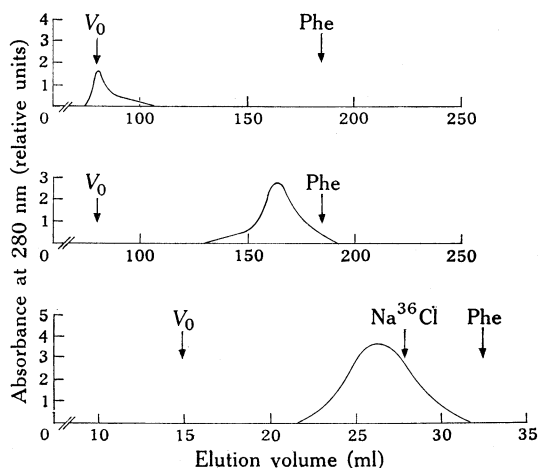


Fig. 6.—Elution profiles at 280 nm of ketose-1-phosphate aldolase including (upper) native enzyme through Sephadex G25, (middle and lower) dehydrated enzyme (-8.6 bars) through Sephadex G25 and G15. V_0 is the void volume of column. Phe and Na^{36}Cl are the elution volumes of phenylalanine and Na^{36}Cl .

Polyacrylamide-gel examination of the aldolase indicated that the native molecule was relatively immobile in a 15% gel but could be easily detected with coomassie blue staining. The dehydrated-rehydrated enzyme could not be detected on the gel. Sephadex G25 studies indicated that the native aldolase was eluted with the void volume. The treated enzyme was eluted much later at a volume similar to the elution volume of phenylalanine (Fig. 6). A further separation on Sephadex G15 (Fig. 6) indicated that the products of dehydration have a molecular weight of less than 1500. Thus the effects of dehydration of the aldolase were very similar to those found with ribulosediphosphate carboxylase. Dehydration of aldolase (mol. wt. 130,000) resulted in fragmentation to particles of a very small size. Incorporation of PMSF with treatments did not influence this result.

As Tris buffers were used with both ribulosediphosphate carboxylase and ketose-1-phosphate aldolase the effect of this buffer was tested on ribonuclease. A ribonuclease solution of concentration 4 $\mu\text{g}/\text{ml}$ was prepared in 50 mM Tris buffer adjusted to pH 6.5 with HCl. Aliquots were dehydrated to a range of water potentials from -3.3 to -17.0 bars. Upon rehydration and assay 100% recovery of activity was obtained when compared to the hydrostatic controls.

IV. DISCUSSION

The hydrostatic pressures required for the successful operation of the pressure membrane apparatus are small enough not to introduce any complications. However, hydrostatic controls are routinely used to avoid objection. Murakami (1970) and Landau (1970) indicate that at low hydrostatic pressures (less than 20 bars) conformational changes in molecules are unlikely to occur. Morita and Zobell (1956) demonstrated that the activity of succinic dehydrogenase was not affected up to pressures of 200–600 bars, when inhibition occurred. It is not envisaged that pressures in excess of 20 bars would be used to examine dehydration effects on macromolecules if normal physiological levels are being considered. This would then include a range of water potentials experienced by most crop plants.

The differential sensitivity to desiccation between IAA oxidase and ribonuclease on one hand and ribulosediphosphate carboxylase and ketose-1-phosphate aldolase on the other, is reflected in their molecular complexity. IAA oxidase and ribonuclease are comparatively small molecules (mol. wt. 40,000 and 13,700 respectively) and composed of a single polypeptide chain. However, the aldolase has a molecular weight of 130,000 and is composed of three subunits, while the carboxylase has a molecular weight of 550,000 and is composed of two dissimilar subunits, eight larger having a molecular weight of 50,000 and six smaller having a molecular weight of 24,000. Both the aldolase and the carboxylase dissociated under desiccating conditions to particles of a size smaller than molecular weight 1500. The molecular weight or nature of these particles have not been thoroughly examined. However, we have established that the fragments from both molecules are considerably smaller than the respective molecular subunits.

The possibility that proteases caused the fragmentation of the aldolase and the carboxylase when these enzymes experienced dehydration was investigated. Protease inhibitors did not prevent fragmentation of the carboxylase and if fragmentation is due to a protease it would need to have very specific environmental requirements. The results have led us to exclude the possibility of a protease being involved in the fragmentation of the aldolase and the carboxylase.

It is now established that water molecules are an integral part of protein structure. It is generally accepted that water molecules surrounding proteins are in an ice-like state (Klotz 1958; Berendsen 1962). Hydrophobic bonds and hydrophobic hydration are important in protein integrity and dependent upon structured water (Tait and Franks 1971). The most plausible explanation we are able to offer for the desiccation fragmentation of the carboxylase and the aldolase involves water associated with the protein molecules and we suggest that removal of water from these molecules has resulted in fragmentation. Until characterization of fragments is achieved we cannot suggest sites within protein molecules where such breakages may be anticipated, but it is conceivable, for example, that β bends (Lewis *et al.* 1971) may be susceptible rupture sites. It may also be proposed that water may play a more decisive role in the structure of ribulosediphosphate carboxylase and ketose-1-phosphate aldolase than in ribonuclease and IAA oxidase. This would mean that lack of response of IAA oxidase and ribonuclease to desiccation could be explained in terms of either the absence or minor role of water in the structure of these molecules.

An attempt was made to estimate the importance of water in these various enzymes. Bigelow (1967) suggested a method for determining the hydrophobic character of proteins by calculating an "average hydrophobicity" index. Goldsack (1970) applied this index to a number of proteins. The index is calculated from the residue composition of the protein, after each amino acid is assigned a factor based on its solubility in aqueous and non-aqueous solvents. We calculated the hydrophobicity index for ribulosediphosphate carboxylase and its subunits, ketose-1-phosphate aldolase, cytochrome *c*, ribonuclease, and vicilin (a storage protein) but no meaningful pattern could be interpreted to describe why the carboxylase and the aldolase were so sensitive to desiccation compared to ribonuclease.

Ribulosediphosphate carboxylase has been found to be protected from iodoacetamide inhibition by fructose 1-phosphate, ribulose diphosphate, and fructose 1,6-diphosphate (Akoyunoglou *et al.* 1967) and ribulose diphosphate has been found to protect the enzyme from *p*-chloromercuribenzoic acid inhibition (Sugiyama and Akazawa 1967). Results reported here indicated that ribulose diphosphate does not confer any protection on ribulosediphosphate carboxylase when the enzyme is desiccated. Sugar alcohols and other carbohydrates protect proteins against denaturation (Hardt *et al.* 1946) and inositol has been shown to effectively increase the resistance of bacteria to desiccation (Webb *et al.* 1964). Osmond and Greenway (1972) have shown that a mannitol concentration equivalent to -12 bars in the reaction mixture does not significantly alter the activity of ribulosediphosphate carboxylase isolated from bean. However, Cl^- at a concentration generating a similar water potential reduced activity to 20% of controls. The effects of sugar alcohols mentioned above suggest that mannitol may protect the carboxylase from adverse low water potential environments that the solute generates. Chloride ion on the other hand may have two deleterious effects on the carboxylase by displacing bonded water (McPhee 1959) and generating an environment of low water potential. The above considerations lead us to believe that while the carboxylase is extremely sensitive to *in vitro* desiccation and Cl^- exposure, the chloroplast must offer considerable *in vivo* protection or buffering against adverse environments. Plaut (1971) has shown that although CO_2 fixation by isolated chloroplasts is reduced with increasing sorbitol concentrations above 0.3M this reduction could not be accounted for by the slightly reduced activity of the carboxylase. Thus this enzyme within the intact chloroplast is in a closely regulated environment and it is conceivable that both protection by compounds within the chloroplast and membrane protection from adverse external environments could maintain enzyme integrity *in vivo*.

It should be emphasized that in our *in vitro* experiments, even at high water potentials, the highest water content attained was 90% (using G100 and not reported herein). It will be necessary to increase this level and examine the various parameters required to protect enzymes such as ribulosediphosphate carboxylase and ketose-1-phosphate aldolase from desiccation fragmentation.

It has been established, by the results presented in this paper, that the pressure membrane apparatus offers a valuable means to examine *in vitro* water relations of enzymes. The method is being extended to examine the effects of reaction environments of low water potential on enzymes such as IAA oxidase and ribonuclease.

V. ACKNOWLEDGMENTS

We wish to acknowledge the assistance of Dr. A. V. Blackmore, Division of Soils, CSIRO, and Dr. F. M. Melhuish, Division of Irrigation Research, CSIRO, during the early stages of the development of this work. We thank Dr. W. G. Crewther, Division of Protein Chemistry, CSIRO, and Dr. F. MacRitchie, CSIRO Wheat Research Unit, for criticism of the manuscript. Finally we thank our many colleagues from the Division of Irrigation Research for reading the manuscript at various stages of its preparation.

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