SEPARATION AND CHARACTERIZATION OF SUBUNITS OF RIBULOSE DIPHOSPHATE CARBOXYLASE

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Abstract

Ribulose-1,5-diphosphate carboxylase (fraction I protein) from spinach beet has been separated into its subunits after maleylation of the *S*-carboxymethyl derivative and gel filtration using only aqueous buffers.

The separated subunits have been further characterized with respect to molecular weight by gradient gel electrophoresis and by acrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. The molecular weight of subunit A was 55,400 in agreement with previous estimates using a calibrated Sephadex column. For subunit B a molecular weight of 12,100 was found, lower than the previous estimate of 16,000.

Peptide maps of tryptic digests of subunit A gave a number of peptides more consistent with a molecular weight of 27,000. *N*-Terminal group studies have given no stoicheiometrical yield of amino acid residues for either subunit.

I. Introduction

In a previous paper (Moon and Thompson 1969) evidence was presented to support the existence of two dissimilar subunits in ribulose-1,5-diphosphate carboxylase (fraction I protein) from spinach beet. The subunits differed in molecular weight and amino acid composition (for a recent review see Kawashima and Wildman 1970*a*). For separation of the subunits, gel-filtration of the *S*-carboxymethylated protein in 8M urea solutions or of the aminoethylated derivative in the presence of sodium dodecyl sulphate (Rutner and Lane 1967) has been used.

In the present studies the *S*-carboxymethylated fraction I protein was converted to the maleyl derivative (Butler *et al.* 1969) and the negatively charged substituent groups facilitated separation of the subunits by gel-filtration in the absence of dissociating agents.

The separated subunits have been studied further with regard to molecular weight, end groups, and peptide maps.

II. Materials and Methods

Fraction I protein was purified from spinach beet (*Beta vulgaris*) and *S*-carboxymethylated as previously described (Moon and Thompson 1969).

Maleic anhydride (Sigma Chemical Co.) was resublimed and stored in the cold.

Sodium lauryl sulphate was a specially pure grade supplied by British Drug Houses.

Gradipore gels were supplied by Townson & Mercer Ltd., Sydney, for use in a Gradipore^R electrophoresis apparatus.

Proteolytic enzymes used in these studies were TPCK-trypsin (Worthington) and Pronase (Kaken Chemical Co., Japan).

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(*a*) *Preparation of Maleyl-S-carboxymethyl Fraction I Protein*

The *S*-carboxymethylated protein was dialysed against 0·4M sodium borate–0·001M EDTA at pH 8·5. Maleylation of the protein was then carried out by a method similar to that of Butler *et al.* (1969). To approximately 100 mg of *S*-carboxymethylated protein solution (20 ml) at room temperature 300 mg of resublimed maleic anhydride in dry acetone (0·5 ml) was added dropwise over a period of 15–20 min. The pH of the solution was kept between 8·5–9·0 by the addition of 1M NaOH. After maleylation of the protein the solution was freeze-dried and then either dialysed against 0·01M Tris–0·001M EDTA (pH 8·3) to prepare the sample for Sephadex chromatography, or against dilute ammonia and freeze-dried. A column of Sephadex G100 (2·5 by 88 cm) was used to fractionate the dissociated products.

(*b*) *Removal of Maleyl Groups*

When removal of the maleyl groups from the amino groups of the protein was required the protein was suspended in 0·2M acetic acid, adjusted to pH 3·5 with ammonia, and heated at 60°C for 8 hr (Butler *et al.* 1969). The reaction was stopped by freeze-drying.

(*c*) *Polyacrylamide Disk Electrophoresis in the Presence of Sodium Dodecyl Sulphate (SDS)*

Acrylamide gels (10% w/v) containing 0·1% SDS were used. The method of preparing the gels was essentially the same as Dunker and Rueckert (1969) except "split" gels were not used. It was not possible to ensure that the wedge used in forming a split gel was absolutely leak-proof.

Protein samples were prepared for electrophoresis by first dissolving them at a concentration of about 2 mg/ml in 1% 2-mercaptoethanol (v/v)–4M urea and about 1% SDS, and then leaving to stand at room temperature for approximately 1 hr. Electrophoresis was carried out at approximately 6–8 mA per tube for 4 hr.

After electrophoresis the gels were stained with Coomassie brilliant blue GS (I.C.I. Ltd.), Colour Index 42660, in a methanol–acetic acid mixture and subsequently destained by extensive washing in a mixture of methanol–acetic acid–water (5 : 7·5 : 87·5 v/v, Weber and Osborn 1969).

(*d*) *Slab Acrylamide Gel Electrophoresis*

Slab acrylamide gel electrophoresis (Margolis and Kenrick 1968) was performed in a Gradipore^{R} electrophoresis apparatus using commercially available gels having a continuous acrylamide concentration gradient. The gels had a concave gel gradient of 4–26%. The buffer system used was 0·05M Tris HCl–0·01M 2-mercaptoethanol (pH 8·5) and gels were equilibrated with this buffer by a preliminary run of 2 hr at 50V/20 mA. After loading the proteins electrophoresis conditions were 75V/25 mA at the beginning of the run; no attempt was made to keep either voltage or current constant over the duration of the run. The current usually dropped to 15 mA after 10 hr. The buffer was cooled by circulating it through a heat-exchange plastic bag immersed in ice.

At the completion of the run the gels were sliced horizontally into two pieces and the cut surface immediately stained with a saturated solution of Coomassie brilliant blue GS (I.C.I. Ltd.) in 15% trichloroacetic acid (Massaro and Markert 1968). The protein on the cut surface was in immediate contact with the dye and stained rapidly. The gels were destained by numerous washings with 7% acetic acid.

(*e*) N-*Terminal Amino Acid Analysis*

N-Terminal analysis was carried out by the manual procedure of Blomback *et al.* (1966) with the modifications described by Moss and Thompson (1969).

(*f*) *Tryptic Digestion and Peptide Mapping*

This was carried out as described previously (Moss and Thompson 1969).

(*g*) *Examination for Blocked* N-*terminal Groups*

The larger subunit A was dissolved in 0·2M $KHCO_3$ buffer, pH 8·2. Pronase was added at an enzyme–protein ratio 1 : 20 (w/w) and digestion carried out at 37°C for 3–4 hr. The digest was then treated in one of two ways.

A column 2 by 30 cm of Dowex-50 (X2,H^+ form, 200–400 mesh) was poured and washed well with water at 4°C. The digest was run on to the column and washed through with water at 4°C (Ikenaka *et al.* 1966). Peptides with the amino end blocked (provided they have no positively charged side-chains) will not bind on to the column and will be present in the water eluant.

The second way in which the digest was treated was that of Wilkinson, Press, and Porter (1966). The digest was treated with 1-fluoro-2,4-dinitrobenzene and stirred vigorously for another 3 hr to block all free amino groups. The reaction mixture was acidified to pH 2 by the addition of 26M formic acid. Most of the DNP-peptides and excess fluorodinitrobenzene were removed by several extractions with ethyl acetate and ether. The remaining solution was concentrated and then passed through a small column (8·0 by 0·5 cm) of talc (Sanger 1949) in 1N acetic acid. The DNP-peptides, but not other peptides, were retarded. The eluant was concentrated and passed through a Dowex-50 column as before.

Aliquots of the water wash from the Dowex-50 columns were dried and hydrolysed in 6N HCl for 24 hr at 110°C before drying and amino acid analysis. As well, paper electrophoresis at 4000V under Varsol at pH 3·5 for 45 min was performed. The peptides were detected by the chlorine–tolidine–KI technique (Reindel and Hoppe 1954) after lightly spraying the paper with 0·05M sodium tetraborate solution (O'Donnell and Thompson 1964).

III. Results

(*a*) *Separation of Maleylated Subunits*

The separation of maleylated carboxymethylated fraction I protein subunits A and B by gel filtration on Sephadex G100 at pH 8·3 is shown in Figure 1. Peak A was eluted in the void volume of the column well separated from peak B. On a dry weight basis the yields of the two subunits were 65–70% of subunit A and 30–35% of subunit B.

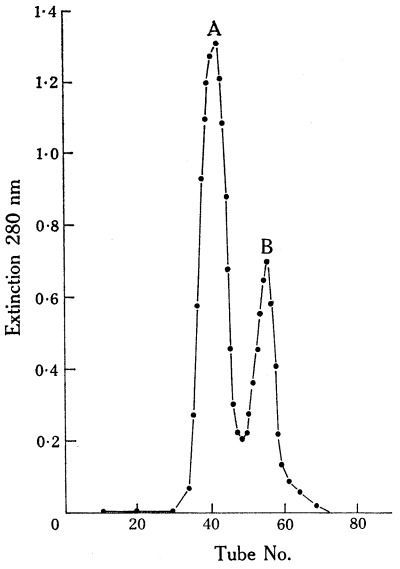


Fig. 1.—Gel filtration in 0·01M Tris–0·001M EDTA, pH 8·3, on a Sephadex G100 column (88 cm by 2·5 cm diam.) of a sample of *S*-carboxymethylated and maleylated fraction I protein. Fraction size 4·5 ml.

The amino acid composition of the two fractions was comparable with that of the subunits previously isolated using urea buffers (Moon and Thompson 1969).

Sugiyama and Akazawa (1970) have recently reported a similar method of fractionation but they carboxymethylate their subunits after separation as maleylated derivatives.

(*b*) *Molecular Weight Studies of the Subunits*

Electrophoretic examination of these two components in a Gradipore^{R} gel further revealed molecular weight dissimilarities. Gradipore^{R} gel electrophoresis will separate proteins on the basis of molecular weight and the effect of charge differences will be minimized. Figure 2 shows the results obtained after electrophoresis for 7 hr. The proteins have not moved to their equilibrium positions in this period but clearly show good separation of the subunits from each other and from the native protein.

Subunit A migrated to a position between human carbonmonoxyhaemoglobin and human serum albumin while subunit B migrated to a position ahead of ovalbumin, but behind low molecular weight peptides (obtained when maleylated kangaroo α-globin was digested with trypsin). The denatured proteins, i.e. subunits A and B, are not as compact on the gels as were the native protein and standards, possibly due to the fact that these denatured proteins are uncoiled and thread their way through the gel pores.

Molecular weight determinations of the polypeptide chains in oligomeric proteins using acrylamide electrophoresis in the presence of sodium dodecyl sulphate have been well characterized (Dunker and Rueckert 1969; Weber and Osborn 1969) and results obtained by this method correlate with results obtained using other physico-chemical means.

The isolated subunits from carboxymethylated maleylated fraction I protein were run on 10% polyacrylamide gels containing sodium dodecyl sulphate to determine the molecular weight of the subunits. A graph of the relative mobility (trypsin = 1·0) of each protein against the logarithm of their respective molecular weights yielded a straight line which allowed the molecular weights of the two maleylated subunits A and B to be determined as 58,000 and 13,000 respectively.

The introduction of the maleyl group increases the molecular weight of the protein by about 100 for each free amino group with which it reacts. The lysine content of the subunit A as determined from an amino acid analysis was 26, thus the molecular weight of the unmaleylated subunit A was estimated to be 55,400, which compares favourably with the value of 54,000 determined using the calibrated Sephadex G200 column (Moon and Thompson 1969).

The molecular weight of subunit B after correcting for maleyl groups is 12,100 (assuming 9 lysine residues for a molecular weight of 12,100). This is considerably lower than our previous value of 16,000 using a calibrated Sephadex column.

The present values for the molecular weights of subunits A and B are in excellent agreement with those recently published by Rutner (1970) using the same method. Sugiyama and Akazawa (1970), using the subunits from spinach leaf, however, obtained values of 60,000 for the large subunit by both ultracentrifugation and polyacrylamide–SDS gel electrophoresis, whereas the small subunit gave 20,800 by analytical ultracentrifugation and 14,000 by polyacrylamide–SDS gel electrophoresis. Discrepancies of this order in our own results and from different laboratories are difficult to explain but it is not clear if the latter authors corrected their results for the weight of maleyl groups.

(*c*) *Peptide Mapping of the Isolated Subunits*

The isolated subunits were treated at pH 3·5 to remove maleyl groups and thereby regenerate the free lysine ε-amino groups. The subunits were then digested

with trypsin and fingerprinted, the results of which are presented in Figures 3 and 4. These peptide maps clearly show that these two subunits differ in structure from each other as would be predicted from their amino acid composition.

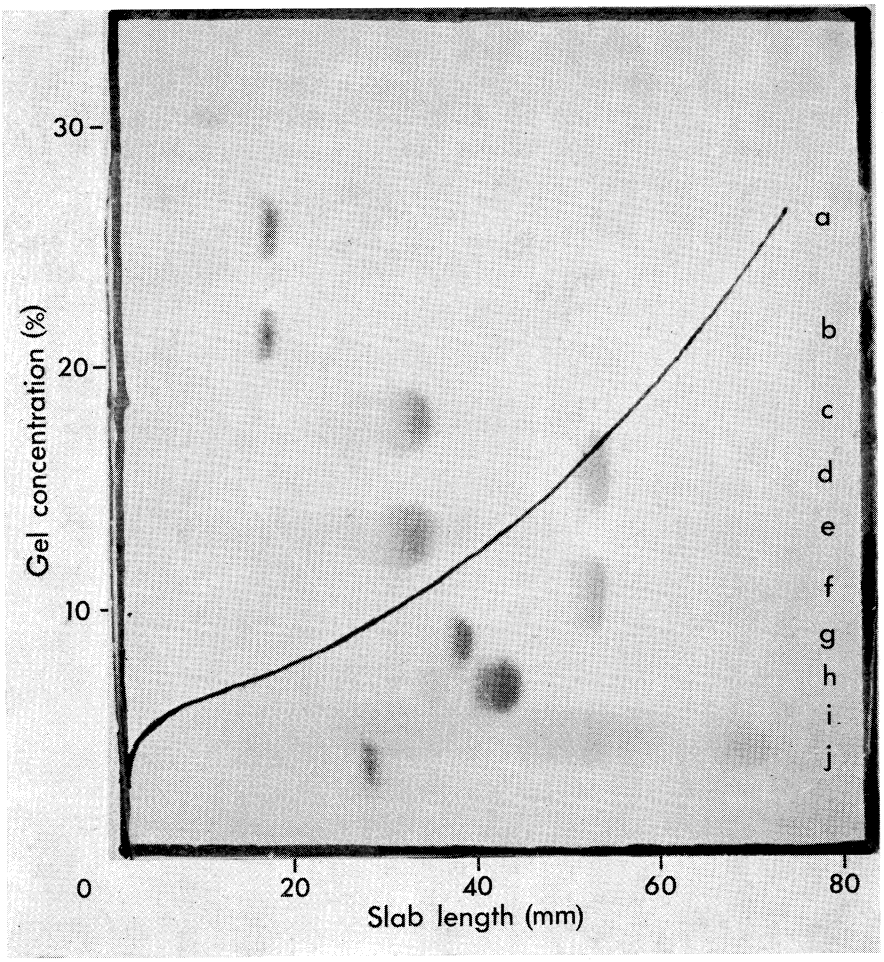


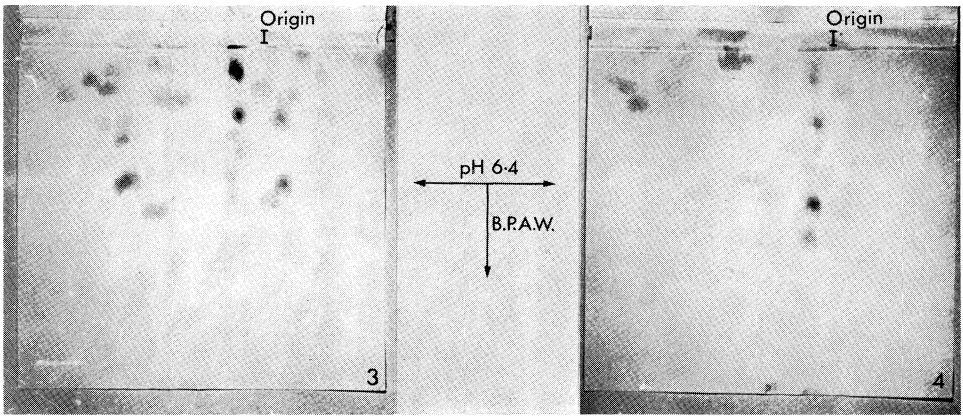
Fig. 2.—Electrophoretic pattern after 7 hr at pH 8·5 in a Gradipore^{R} polyacrylamide slab. Migration of the proteins is from left to right. The solid line indicates the acrylamide concentration at a particular point in the gel. (*a*), (*b*) native fraction I protein; (*c*), (*e*) maleylated subunit A; (*d*), (*f*) maleylated subunit B; (*g*) human serum albumin; (*h*) ovalbumin; (*i*) tryptic digest of maleylated kangaroo α-globin; (*j*) human carbonmonoxyhaemoglobin.

If the isolated subunit A, of molecular weight approximately 54,000, consisted of a single chain or two non-identical chains, peptide mapping of the carboxymethylated subunit A, completely hydrolysed by trypsin at all lysine and arginine bonds, should reveal approximately 55–60 ninhydrin-positive spots. In fact, the peptide map contained only about one-half this number. After digestion with trypsin and freeze-drying not all the digest was soluble in the pH 6·4 electrophoresis buffer. It was important to determine whether this insoluble fraction contained several inaccessible

trypsin-sensitive bonds, thus rendering an estimate of the molecular weight of the subunit speciously low.

Relative total amino acid contents of 11·8% and 88·2% were obtained for the insoluble residue and soluble peptides, respectively, by acid hydrolysis and assay on the amino acid analyser.

The amino acid composition of the insoluble and soluble peptide fractions was determined and related to that of the undigested *S*-carboxymethylated subunit A. The results are shown in Table 1. The insoluble fraction contained a small amount of lysine and arginine. On the assumption that this fraction contained one lysine residue and the soluble peptides contained the remaining lysine residues present in the intact molecule, the sum of the residues in both fractions agreed well with that of the undigested subunit. The values of 52 residues for the insoluble fraction and 444 residues for the soluble peptides, 10·5% and 89·5% of the total respectively, match the results of the acid hydrolysis quantitative analysis closely.



Figs. 3 and 4.—Peptide maps obtained by ionophoresis at pH 6·4 and chromatography in butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12 v/v) of tryptic digests of *S*-carboxymethylated subunit A and *S*-carboxymethylated subunit B obtained by demaleylation of the separated maleylated derivatives.

Thus the potential maximum number of tryptic peptides contained in the insoluble residue is only three and consequently has no significant effect on a molecular weight estimate by peptide mapping. It would appear that the minimum molecular weight of the subunit A, based on peptide maps, is 27,000, one-half of the molecular weight of the isolated subunit, and that the identical chains are held together by some bond, probably covalent, other than a disulphide bond.

The peptide map of subunit B revealed 13–14 ninhydrin-positive spots in close agreement to that expected from the total lysine and arginine residues of the protein.

(*d*) N-*Terminal Analyses*

No significant yield of phenylthiohydantoins or regenerated amino acids was obtained using the Edman method. This absence of a free *N*-terminal amino acid could not be due to failure to remove the maleyl group from the *N*-terminal amino acid for similar results have been obtained with the subunit A isolated on Sephadex G200 columns in the presence of 8M urea.

TABLE 1

AMINO ACID COMPOSITION OF CARBOXYMETHYLATED SUBUNIT A AND ITS SOLUBLE AND INSOLUBLE TRYPTIC PEPTIDE FRACTION

Amino Acid	Tryptic Peptide Fraction Insoluble at pH 6·4			Tryptic Peptide Fraction Soluble at pH 6·4			Sum of Residues in both Fractions	Carboxymethylated Subunit A: No. of Residues
	Amount (μmole)	Calc. No. of Residues	Nearest Integer	Amount (μmole)	Calc. No. of Residues	Nearest Integer		
Lysine	0·0207	1·0	1	0·450	25·0	25	26	26
Histidine	0·0456	2·2	2	0·2070	11·5	12	14	14
Arginine	0·0276	1·3	1	0·510	28·1	28	29	28
Aspartic acid	0·0852	4·1	4	0·7530	41·7	42	46	44
Threonine*	0·0712	3·4	3	0·4760	26·5	27	30	32
Serine*	0·0558	2·7	3	0·2540	15·7	16	19	20
Glutamic acid	0·0776	3·8	4	0·8420	46·8	47	51	52
Proline	0·0713	3·4	3	0·3740	20·8	21	24	24
Glycine	0·1566	7·6	8	0·7520	41·8	42	50	50
Alanine	0·0924	4·5	5	0·8000	44·4	44	49	48
Valine	0·1017	4·9	5	0·5940	33·0	33	38	35
Methionine	0·0110	0·53	1	0·0860	4·8	5	6	8
Isoleucine	0·0567	2·8	3	0·3440	19·1	19	22	20
Leucine	0·0884	4·3	4	0·7250	40·3	40	44	43
Tryosine	0·0400	1·9	2	0·3420	19·0	19	21	17
Phenylalanine	0·0556	2·7	3	0·3210	17·8	18	21	22
SCM-cysteine	Trace			0·1150	6·4	6	6	8
Total			52			444	496	491

* Uncorrected for decomposition.

This inability to detect an *N*-terminal amino acid with the Edman reagent could mean that the amino end of the protein is masked by some blocking group such as a formyl or acetyl group or the *N*-terminal residue is pyroglutamic acid.

The peptides unadsorbed on sulphonated polystyrene from a Pronase digest of subunit A contained several amino acids with glutamic acid predominating (column 1, Table 2). The presence of glutamic acid in the hydrolysate of the water wash from the

TABLE 2

AMINO ACID COMPOSITION OF HYDROLYSED ACIDIC PEPTIDES ISOLATED BY CHROMATOGRAPHY ON DOWEX-50 FROM SUBUNIT A DIGESTED WITH PRONASE

Maleylated and *S*-carboxymethylated subunit A used for experiments 1 and 2; *S*-carboxymethylated subunit A used for experiment 3. All values expressed as moles of amino acid per mole of subunit A

Amino Acid	Expt. 1*	Expt. 2†	Expt. 3‡
Aspartic acid	0·45	Trace	0·54
Threonine§	0·87	0·69	0·64
Serine§	0·56	0·60	0·64
Glutamic acid	1·20	1·20	1·76
Proline	0·61	0·50	0·70
Glycine	0·50	0·73	0·54
Alanine	0·44	0·44	0·39
Valine	0·30	0·43	—
Isoleucine	0·10	—	—
Leucine	0·21	—	—
Phenylalanine	0·12	—	—

* Subunit A Pronase digest not treated with fluorodinitrobenzene.

† Subunit A Pronase digest reacted with fluorodinitrobenzene.

‡ For this experiment the time of digestion was 12 hr and carboxymethylated subunit A prepared on a Sephadex G200 column in the presence of 8M urea was used. The values represent the mean of two experiments. Fluorodinitrobenzene was reacted with the digestion mixture.

§ Uncorrected for decomposition.

sulphonated polystyrene column suggested that perhaps the absence of an *N*-terminal amino acid in subunit A was due to a terminal pyroglutamic acid residue. However, there is the possibility that the pyroglutamic acid is an artefact formed by the cyclization of a glutaminyl residue under the acidic conditions on the ion-exchange column. Thus a further Pronase digest of subunit A was performed followed by the immediate addition of fluorodinitrobenzene to the reaction mixture to block all free amino groups (Press, Piggot, and Porter 1966). It has been established that glutamine does not form any significant amounts of pyroglutamic acid under these conditions (Press, Piggot, and Porter 1966; O'Donnell 1968). After removal of DNP-peptides, the extract was passed through a sulphonated polystyrene column as before and an aliquot hydrolysed and its amino acid composition determined. The result (column 2) was similar to that in which no fluorodinitrobenzene was added. The predominant amino acid was glutamic acid accompanied by significant amounts of six other amino

acids. The maleylation of the protein served as a precaution against there being a lysine residue close to the *N*-terminal. A peptide containing a free lysine residue would bind on to the column of sulphonated polystyrene and be undetected. As column 3 shows, however, it was possible to isolate a similar acidic peptide fraction from subunit A in which the lysine residues had not been modified. The similar amino acid content for the non-maleylated subunit A non-adsorbed fraction also serves as a check that there is no free α-amino group not detected by the phenylisothiocyanate method. By masking the α-amino group the *N*-terminal peptide can be isolated as an acidic peptide not adsorbed on sulphonated polystyrene.

It was clear from Table 2 that the eluate from the Dowex-50 column was a mixture of peptides.

After electrophoresis of the eluate from the Dowex-50 column, four bands which were ninhydrin negative (indicating no free amino group) but which gave a positive reaction with the chlorine–tolidine–iodide reagent were detected on the paper. One of these bands having an R_F value of 0·41 (taking the mobility of pyroglutamic acid as 1·00) was the major band and had the following amino acid composition: Glu 2·00 (2); Ser 0·94 (1); Thr 0·94 (1); Pro 0·91 (1). Low recovery of this peptide prevented any further characterization but it could represent an *N*-terminal pyroglutamyl peptide.

Using similar techniques to that employed for subunit A no conclusive evidence was obtained to allow a definite amino acid to be ascribed to the amino end of subunit B.

IV. Discussion

Despite the obvious importance of fraction I protein in the photosynthetic carbon fixation cycle of higher plants and the extensive studies that have been made on it since its first detection, there are still numerous problems associated with our understanding of the structure and the mechanism of action of this protein. Perhaps the most concentrated study has been made on characterizing the binding site for ribulose 1,5-diphosphate. Since the original observation by Mayaudon, Benson, and Calvin (1957) that *p*-chloromercuribenzoic acid and iodoacetamide inhibited the activity of ribulose-1,5-diphosphate carboxylase a number of studies using sulphydryl reagents have been reported (see Kawashima and Wildman 1970*a* for a summary). We have also shown (unpublished observations) that sodium tetrathionate can be used as an inhibitor of ribulose-1,5-diphosphate carboxylase and that the inhibition can be reversed by cysteine. However, there is still no general agreement as to whether ribulose-1,5-diphosphate binds specifically to the sulphydryl group of a cysteinyl residue of the protein. A knowledge of the subunit structure of fraction I protein should assist greatly in further investigations into this aspect of the enzyme.

This present study has confirmed previous estimations of the molecular weight of the larger subunit. While there is now more general agreement regarding the molecular weight of the smaller subunit between our present results and those of Rutner (1970) the results of Sugiyama and Akazawa (1970), previously referred to, give different values.

Analysis by weight of the subunits suggests that there is approximately twice the weight of the larger subunit (68%) compared with that of the smaller subunit

(32%). Allowing for the different molecular weights of 54,000–55,500 for the larger subunits and 12,100–16,000 for the smaller subunit the relative number of molecules of each subunit could be approximately one is to two respectively. More exact estimates must await the determination of the amino acid sequence of the individual chains.

The exact number of peptide chains is not known and in this respect the lack of clearly defined *N*-terminal residues is a disappointment. Results of tryptic peptide mapping of the larger subunit suggests that its minimum molecular weight is considerably less than its isolatable molecular weight, which is in agreement with the report by Kawashima and Wildman (1970*a*, 1970*b*). In contrast, however, Sugiyama and Akazawa (1970) found that carboxypeptidase A hydrolysis of the larger subunit from spinach released 1 mole of valine per mole of subunit assuming a molecular weight of 60,000. The amino end group analysis as reported in this study does not clarify the position but the isolation and characterization from the larger subunit of peptides containing, for example, unique cysteine residues should assist in determining its minimum molecular weight.

The uncertainty surrounding estimates of the molecular weights of both the native enzyme and the individual subunits obviously hampers accurate calculations as to the number of each subunit in the native protein but tentative models have been proposed (Rutner 1970; Kawashima and Wildman 1970*a*, 1970*b*). The arrangement of these subunits to yield the characteristic regular-shaped molecules of fraction I protein seen in the electron microscope is clearly an interesting and complex problem.

V. Acknowledgments

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