Immunochemical Comparative Study on RuBP Carboxylase/Oxygenase of Different Tobacco Mutants Exhibiting Different Levels of Photorespiration

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Nicotiana tabacum var. John William's Broadleaf, Antiserum, Chloroplast, Double Immuno Diffusion Test

By means of the immunochemical methods of double immuno diffusion and tandem crossed immuno electrophoresis we have compared the bifunctional enzyme RuBP carboxylase/oxygenase from tobacco mutants which differ with respect to their rates of photosynthesis and photorespiration. The comparative studies were carried out with a monospecific antiserum to the enzyme of the wild type *Nicotiana tabacum* var. John William's Broadleaf. RuBP carboxylase/oxygenase from the green, yellow-green and yellow phenotype of the mutants namely *N. tabacum* Su/su, *N. tabacum* Su/su var. Aurea, *N. tabacum* var. Consolation, *N. tabacum* var. NC 95 and *N. tabacum* Xanthi (D 523) are immunochemically identical to the enzyme of the wild type *N. tabacum* var. John William's Broadleaf. Furthermore, immunochemical identity of the RuBP carboxylase/oxygenase exists between *Nicotiana tabacum* and other representatives of the Solanaceae such as *Solanum tuberosum, S. lycopersicum* and *Datura suaveolens*. In contrast to this only partial identity to the enzymes of the C_3-plants *Antirrhinum majus, Spinacia oleracea, Sinapsis alba, Petroselinum crispum, Allium porrum, Hordeum vulgare, Avena sativa* to the enzymes of the green alga *Chlorella vulgaris* and to the blue-green alga *Oscillatoria chalybea* as well as to the enzyme of the C_4-plant *Zea mays* is observed.

Introduction

In a preceding paper [1] we have studied by means of quantitative rocket immuno electrophoresis in several tobacco mutants, whether there exists a relationship between photosynthetic or photorespiratory activity [2–6] and the amount of the bifunctional enzyme RuBP carboxylase/oxygenase. It was seen that although the amount of enzyme present in the tobacco mutants was low when compared to spinach *(Spinacia oleracea),* the different mutants which exhibit different rates of photosynthesis and photorespiration clearly contained different amounts of enzyme. Whereas the content of RuBP carboxylase/oxygenase in the green phenotypes of *N. tabacum* var. Consolation, *N. tabacum* var. NC 95 and *N. tabacum* var. Xanthi (D 523) makes up for approximately 10% of the stroma proteins, just as in the wild type *N. tabacum* var. John William's Broadleaf, the yellow-green and yellow phenotypes of the mutant *N. tabacum* Su/su, *N. tabacum* Su/su var. Aurea, *N. tabacum* var. Consolation yellow-green, yellow leaf patches of the variegated mutant from NC 95, and *N. tabacum* var. Xanthi may contain the threefold amount. In contrast to this in spinach chloroplasts the enzyme content may make up for up to 45% of the stroma proteins. Correlation between high photorespiration and a higher enzyme content was not established. However, it seems as if in some tobacco mutants a correlation exists between a high amount of enzyme and high photosynthetic rates. These results are obtained, if the amount of enzyme is referred to the total chloroplast proteins. However, it should be borne in mind that the amount of RuBP carboxylase/oxygenase itself varies considerably in dependence on the culture conditions of the plants, such as light, temperature, fertilizer and age. Thus, we observed that chloroplasts from leaves of the upper plant region of a NC 95 mutant contained approximately 30% more enzyme than chloroplasts from leaves of the lower plant region. Therefore we will report in a separate publication on the ratio of enzyme content to amount of chlorophyll present (Radunz and Schmid, in preparation).

In the present paper we study by means of double immuno diffusion and by tandem crossed immuno electrophoresis the question of immunochemical identity of RuBP carboxylase/oxygenase in different

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tobacco mutants which, as shown earlier, exhibit different rates of photorespiration. Furthermore, we compare with immunochemical methods the bifunctional enzyme of *N. tabacum* with that of other Solanaceae and with that of representatives of the families of the Scrophulariceae, Chenopodiaceae, Cruciferae, Papilionaceae, Umbelliferae, Liliaceae, Gramineae as well as with the enzymes of green and blue-green algae. The test reagent is a monospecific antiserum to RuBP carboxylase/oxygenase from the wild type tobacco *N. tabacum* var. John William's Broadleaf [1, 7–9].

Materials and Methods

Plant material

Plants of *Nicotiana tabacum* (listed in Table I) were cultured in climatized growth chambers under identical conditions. The light/dark cycle was 18/6 h. Temperature in the light was 27 °C and in the dark 22 °C. Relative humidity was kept at 60%. Silvania tubes with a light intensity of 12,000 lux were used as light source. The other C_3- and C_4-plants listed in the table were cultivated in a greeenhouse under natural sun light conditions.

Antiserum

The antiserum was obtained by immunization of rabbits with pure RuBP carboxylase/oxygenase from *N. tabacum* var. John William's Broadleaf and from *Spinacia oleracea* [7]. The monospecificity of the antiserum was established by means of the double immuno diffusion test [7] and by rocket immuno electrophoresis [1, 8, 9].

Chloroplast preparations

Chloroplasts were obtained by fractionating centrifugation in the following isolation buffer: 0.1 M Tris (N-(Tris)hydroxymethyl-aminomethan) containing 0.6 M sucrose, 4 mM $MgCl_2$ and 1 mM EDTA, pH 7.8. For the immunological study the chloroplast were suspended in 24 mM barbiturate buffer, pH 8.6 containing 2% Triton X-100, sonicated 3 times for 10 sec and centrifuged subsequently for 30 min at $20{,}000 \times g$. Protein determinations were carried out with Folin reagent according to Lowry *et al.* [10]. Chlorophyll was determined photometrically according to Schmid [11] in methanol/water 9/1.

Immunological tests

Agarose gels for the double immuno diffusion test were carried out in 0.06 M Na_2KPO_3 buffer, pH 7.4 according to Sörensen and the gels for the tandem crossed immuno electrophoresis in 24 mM barbiturate buffer, pH 8.6. Agarose concentration was for both methods 1%. The double diffusion test required 24 to 48 h. Conditions for the tandem crossed immuno electrophoresis were the following: Electrophoresis in the first direction (Fig. 2, 4 and 5,I) required 90 min at 160 V/cm and electrophoresis of the antigen proteine into the antibody containing gel (Fig. 2, 4 and 5,II) required 16–18 h at 6 V/cm. Subsequently the gel plates were washed in 1.7% NaCl solution, dried and stained with Amidoblack (0.2% in 2% acetic acid). The antibody concentrations used for the immuno electrophoresis are given in Fig. 2, 4 and 5.

Results

The bifunctional enzyme, RuBP carboxylase/oxygenase of different tobacco mutants and that of the wild type *N. tabacum* var. John William's Broadleaf were compared by means of double immuno diffusion tests and tandem crossed immuno electrophoresis. Fig. 1 and 2 clearly show that in the immuno diffusion test as well as in the immuno electrophoresis only fusing precipitation bands are observed between the chloroplasts tested. In no case, spurs or crossing bands are observed. The results of the two immunological reactions lead to the conclusion that the enzyme in all green phenotypes as well as in all yellow-green and yellow phenotypes such as in the mutants of *N. tabacum* Su/su., *N. tabacum* var. Aurea, *N. tabacum* var. Consolation, *N. tabacum* var. NC 95 and *N. tabacum* var. Xanthi is immunochemically identical to that of the wild type. This might imply that the molecular structure of the enzyme in mutants exhibiting low and high rates of photorespiration is identical.

In a further experiment RuBP carboxylase/oxygenase of *Nicotiana tabacum* was compared by means of the same antiserum on the one hand to the enzyme of other Solanaceae such as *Solanum tuberosum, S. lycopersicum* and *Datura suaveolens* and on the other hand to the enzyme of representatives of other plant families such as Scrophulariceae *(Antirrhinum majus)* or Chenopodiaceae (*Spinacia oleracea* and

Beta altissima), the Papilionaceae *(Phaseolus vulgaris)* or Cruciferae (*Brassica oleracea* and *Sinapsis alba*), the Liliaceae *(Allium porrum)*, the Gramineae (*Avena sativa, Hordeum vulgare* and *Zea mays*) as well as the enzyme of the green alga *Chlorella vulgaris* and the blue-green alga *Oscillatoria chalybea*. The investigations show that only between the enzyme of *N. tabacum* and other representatives of the Solanaceae fusing precipitation bands are observed (Fig. 1 and 2). Between the *Nicotiana tabacum* enzyme and that of all other plant preparations listed in Table I precipitation bands with one sided spures are observed (Fig. 3, 4 and 5). This means that RuBP carboxylase/oxygenase of tobacco and of the other listed plants are immunochemically not fully identical and are only partially identical. These results are summarized in Table I.

In the case of an enzyme comparison between *Nicotiana tabacum* and *Spinacia oleracea* we were able to show that use of a mixed antiserum in the tandem crossed immuno electrophoresis, containing antibodies to the tobacco and the spinach enzyme, also yield one sided spures just as in the case where only tobacco enzyme was tested. This means that even when using a mixed antiserum partial identity between the enzymes can be demonstrated (Fig. 4, AS + SpAS). A difference between the tobacco and blue-green alga as well as the green algal enzyme is inferred from the observation that the electrophoretic mobility of the tobacco enzyme is substantially higher (Fig. 5). The result means that the tobacco enzyme protein contains more negative charges in its surface then the blue-green algal or the green algal protein.

The major part of the bifunctional enzyme is localized in the chloroplast stroma. However, as shown by studies of Kannangara *et al.* [12], Strotmann *et al.* [13], Henriques and Park [14] and Staehelin [15] some molecules are adsorbed onto the surface of the thylakoid membrane, which is directed towards the stroma. These enzyme molecules are so tightly associated with the thylakoid membrane that repeated washings of the lamellar system of *Antirrhinum* chloroplasts with 0.8% NaCl do not remove the protein. Our own investigation on the maximal binding of antibodies onto the thylakoid membrane surface

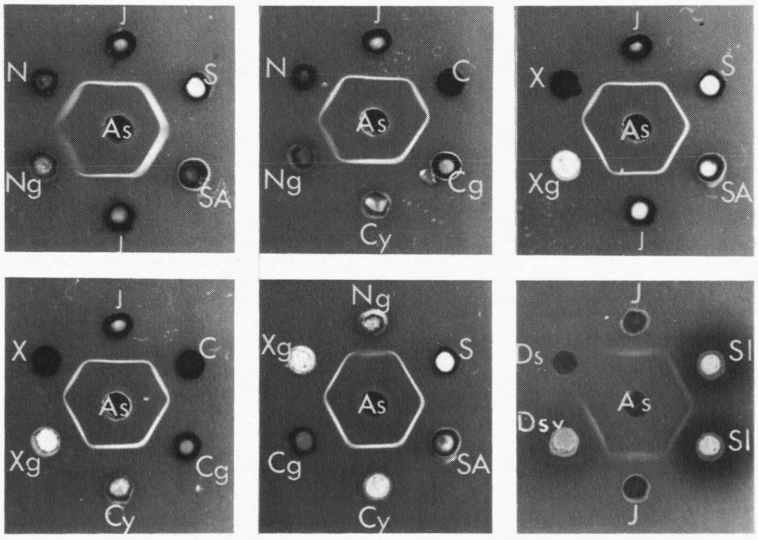

Fig. 1. Comparison of RuBP carboxylase/oxygenase of *Nicotiana tabacum* var. JWB with that of *Nicotiana tabacum* mutants by means of the double immuno diffusion test in agarose gel.
Antiserum: As, Antiserum to RuBP carboxylase/oxygenase of *Nicotiana tabacum* var. JWB.
Antigen: Chloroplast preparations of: J, *Nicotiana tabacum* var. JWB, green; S, *N. tabacum* Su/su, yellow-green; SA, *N. tabacum* Su/su var. Aurea, yellow; C, *N. tabacum* var. Consolation, green; Cg, *N. tabacum* var. Consolation, yellow-green; Cy, *N. tabacum* var. Consolation, yellow; N, *N. tabacum* var. NC 95, green; Ng, *N. tabacum* var. NC 95, yellow-green; X, *N. tabacum* var. Xanthi, green; Xg, *N. tabacum* var. Xanthi, yellow-green; Sl, *Solanum lycopersicum;* Dsv, *Datura suaveolens;* Ds, *Datura sanguinea*.

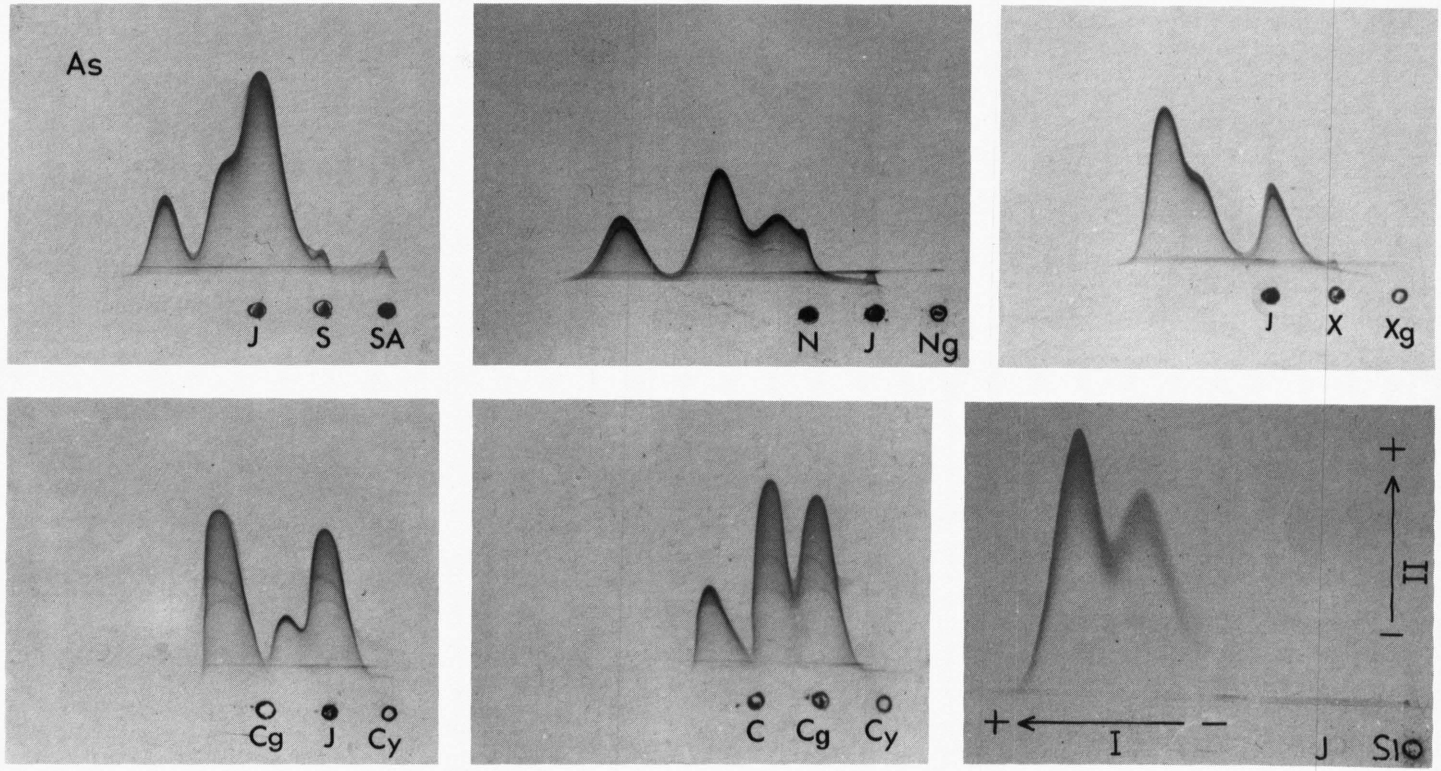

Fig. 2. Demonstration of immunochemical identity of RuBP carboxylase/oxygenase between *Nicotiana tabacum* var. JWB, *Nicotiana tabacum* mutants and the Solanaceae *Solanum lycopersicum* by means of tandem crossed immuno electrophoresis in agarose gel.
Antiserum: As, 1% antiserum to RuBP carboxylase/oxygenase of *Nicotiana tabacum* var. JWB in agarose gel.
Antigen: Chloroplast preparations of: J, *Nicotiana tabacum* var. JWB, green; S, *N. tabacum* Su/su, yellow-green; SA, *N. tabacum* Su/su var. Aurea, yellow; C, *N. tabacum* var. Consolation, green; Cg, *N. tabacum* var. Consolation, yellow-green; Cy, *N. tabacum*, var. Consolation, yellow; N, *N. tabacum* var. NC 95, green; Ng, *N. tabacum* var. NC 95, yellow-green; X, *N. tabacum* var. Xanthi, green; Xg, *N. tabacum* var. Xanthi, yellow-green, Sl, *Solanum lycopersicum*.

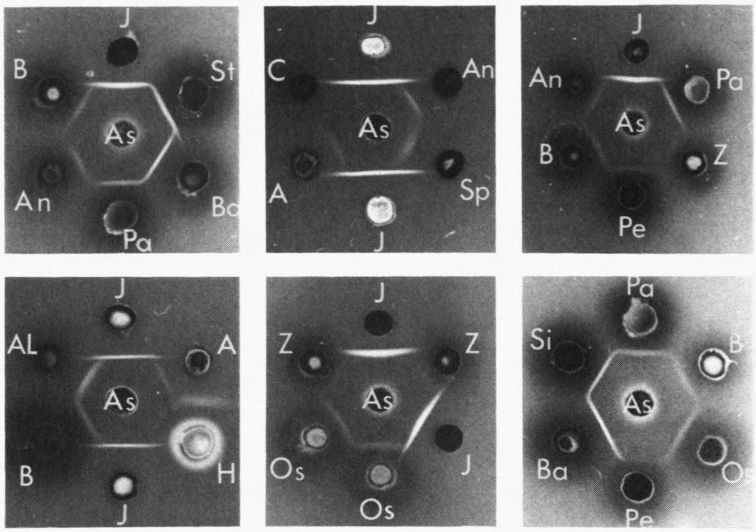

Fig. 3. Comparison of RuBP carboxylase/oxygenase of *Nicotiana tabacum* var. JWB with the enzyme of other C_3-plants and with that of the C_4-plant *Zea mays* by means of the double immuno diffusion test in agarose gel.
Antiserum: As, antiserum to RuBP carboxylase/oxygenase of *Nicotiana tabacum* var. JWB.
Antigen: Chloroplast preparations of: J. *Nicotiana tabacum* var. JWB; St, *Solanum tuberosum;* An, *Antirrhinum majus;* Sp, *Spinacia oleracea;* B, *Brassica oleracea;* Pa, *Phaseolus vulgaris;* Ba, *Beta altissima;* Pe, *Petroselinum crispum;* Si, *Sinapis alba;* Al, *Allium porrum;* A, *Avena sativa;* H, *Hordeum vulgare;* Z, *Zea mays;* C, *Chlorella vulgaris;* Os, *Oscillatoria chalybea*.

Table I. Immunological comparison of the RuBP carboxylase/oxygenase of *Nicotiana tabacum* var. JWB with other C_3- and C_4-plants.

Plants	Family	Immunochemical identity of the enzyme from *Nicotiana tabacum* var. JWB with	
		identical	partially identical
Nicotiana tabacum Su/su, yellow-green	Solanaceae	+	
N.t. Su/su var. Aurea, yellow		+	
N.t. var. Consolation, green		+	
N.t. var. Consolation, yellow-green		+	
N.t. var. Consolation, yellow		+	
N.t. var. NC 95, green		+	
N.t. var. NC 95, yellow-green		+	
N.t. var. Xanthi*, green		+	
N.t. var. Xanthi, yellow-green		+	
Solanum tuberosum		+	
Solanum lycopersicum		+	
Datura suaveolens		+	
Antirrhinum majus	Scrophulariceae		+
Spinacia oleracea	Chenopodiaceae		+
Beta altissima			+
Phaseolus vulgaris	Papilionaceae		+
Brassica oleracea	Cruciferae		+
Sinapis alba			+
Petroselinum crispum	Umbelliferae		+
Allium porrum	Liliaceae		+
Avena sativa	Gramineae		+
Hordeum vulgare			+
Zea mays			+
Chlorella vulgaris			+
Oscillatoria chalybea			+

Results were obtained according to the precipitation behaviour of RuBP carboxylase/oxygenase in the double immuno diffusion test and in the tandem crossed immuno electrophoresis with the antiserum to RuBP carboxylase/oxygenase from the wild type *N. tabacum* var. JWB.
* The geno- and phenotype of this mutant were characterized by Burk *et al.* [17].

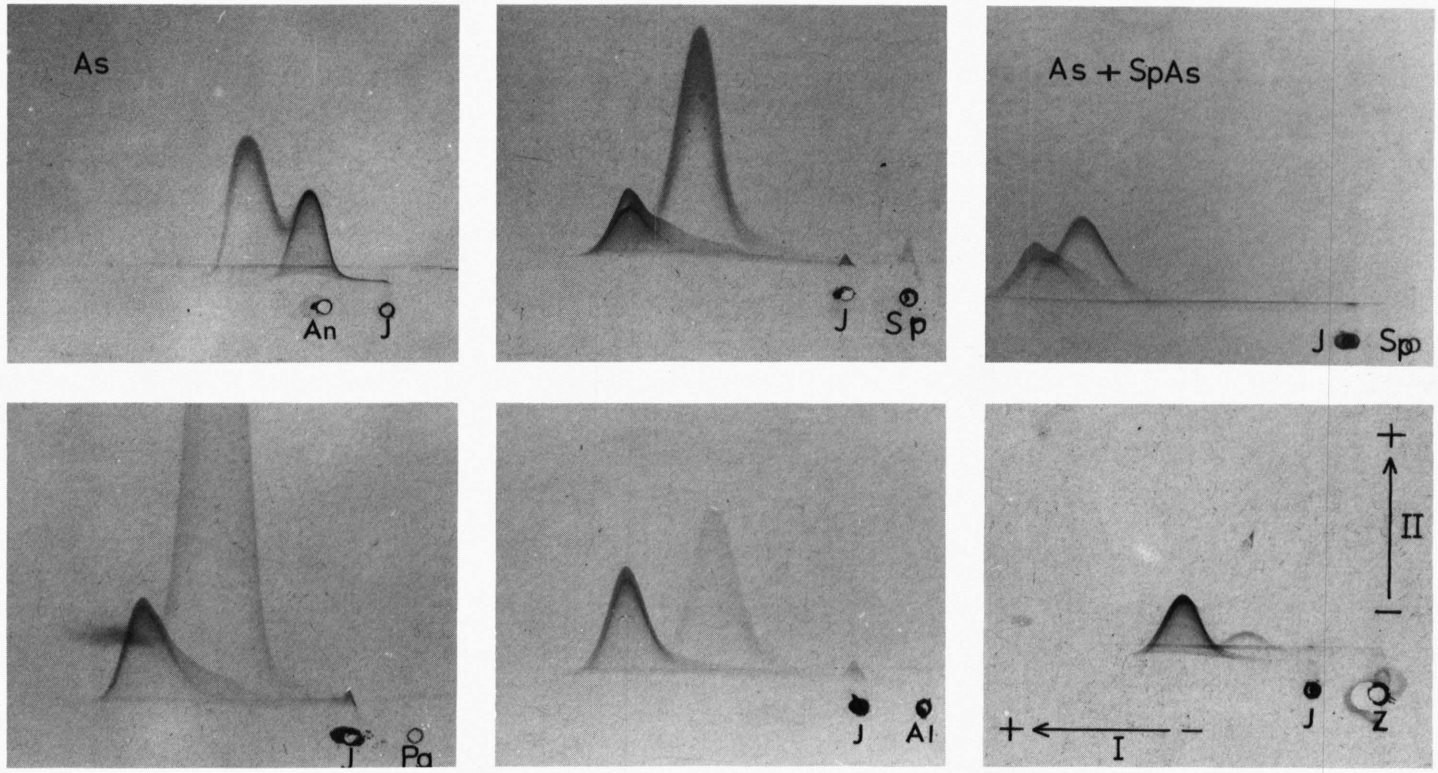

Fig. 4. Demonstration of immunochemical partial identity of RuBP carboxylase/oxygenase of *Nicotiana tabacum* var. JWB with that of some C_3-plants and with the C_4-plant *Zea mays* by means of tandem crossed immuno electrophoresis in agarose gel.
Antiserum: As, 1% antiserum to RuBP carboxylase/oxygenase of *Nicotiana tabacum* var. JWB in agarose gel; AS + SpAS, mixed antiserum to the RuBP carboxylase/oxygenase of *Nicotiana tabacum* var. JWB and *Spinacia oleracea.*
Antigen: Chloroplast preparations of: J, *Nicotiana tabacum,* var. JWB: An, *Antirrhinum majus;* Sp, *Spinacia oleracea;* Pa, *Phaseolus vulgaris;* Al, *Allium porrum;* Z, *Zea mays.*

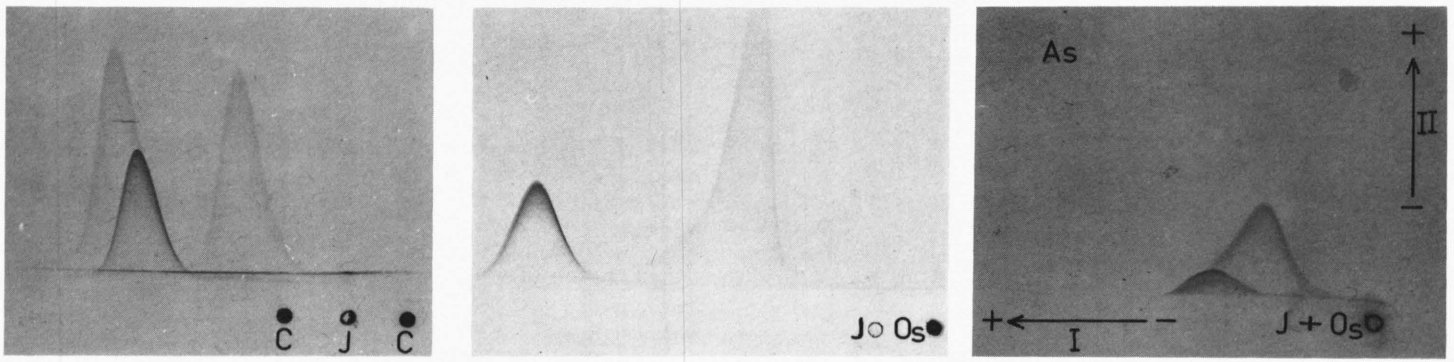

Fig. 5. Demonstration of immunochemical partial identity between RuBP carboxylase/oxygenase of *Nicotiana tabacum* var. JWB and the enzyme of green and blue-green algae by means of the tandem crossed immuno electrophoresis.
Antiserum: As, 1% Antiserum to RuBP carboxylase/oxygenase of *Nicotiana tabacum* var. JWB in agarose gel.
Antigen: J. Chloroplast preparation of *Nicotiana tabacum* var. JWB; C. Preparation of the green alga *Chlorella vulgaris;* Os, Preparation of the blue-green alga *Oscillatoria chalybea;* J + Os, mixed preparation of *N. tabacum* chloroplasts and *Oscillatoria chalybea* in one hole.

has shown that 13% of the total antibody amount which can be bound at the surface are antibodies to RuBP carboxylase/oxygenase [7, 16]. This does not mean, however, that the thylakoid membrane surface is composed by 13% of RuBP carboxylase/oxygenase. This surface must be smaller, since binding of antibodies onto membrane-bound antigenes leads at the margin of these components to overlappings due to the size of antibody molecules. From the double immuno diffusion tests and the tandem-crossed immuno electrophoresis we can conclude, that soluble and bound RuBP carboxylase/oxygenase is immunochemically identical. The amount of this bound enzyme makes up for only 6% of the enzyme localized in the stroma.

Discussion

Comparative immunological studies have led to the result, that RuBP carboxylase/oxygenase of all tested tobacco mutants and the wild type *N. tabacum* var. John William's Broadleaf are immunochemically identical, despite the fact that these plants exhibit distinctly different rates of photosynthesis and photorespiration. This might imply that this bifunctional enzyme has not undergone any molecular modification in the different phenotypes. However, it should be pointed out, that this result has been obtained with one single antiserum, which is the antiserum to the enzyme from the wild type. Strictly this means that the mutant enzyme has the same antigenic determinants which the wild type enzyme has. But since we use the antiserum to wild type enzyme towards the mutant enzymes we cannot exclude that additional antigenic determinants are present which only a homologous antiserum would detect. This is to say, that no mixed antiserum was used, which also contained antibodies to the RuBP carboxylase/oxygenase of the respective mutant to be compared. The further comparative studies prove, that molecular differences of the enzyme of *N. tabacum* var. John William's Broadleaf and other C_3-plants irrespective whether these are representatives of monocotyledons or dicotyledons, green algae or blue-green algae, can be demonstrated with a single monospecific antiserum, provided these differences refer to differences of the antigenic determinants. Also, the enzyme comparison between *N. tabacum* var. JWB and *Spinacia oleracea* obtained by means of a mixed antiserum confirms the results obtained by one single antiserum, namely that to the enzyme of *Nicotiana tabacum* var. JWB. In both cases partial identity of the enzymes from tobacco and spinach was demonstrated. Thus, one might be tempted to conclude that RuBP carboxylase/oxygenase from *N. tabacum* var. John William's Broadleaf and from the tobacco mutants tested and other Solanaceae are immunochemically identical. On the other hand it would mean that between the tobacco enzyme and the enzyme of the plants listed in Table I or green and blue-green algae, only partial identity would exist.

The quantitative serological determination of the enzyme content in tobacco mutants and the wild type [1] together with the present study in which immunochemical identity between the tobacco enzyme of Su/su and the wild type is demonstrated fully fits into what has been reported earlier [18]. In earlier publications we have shown that the photosynthetic light intensity curve of leaves of *N. tabacum* Su/su showed light saturation at higher light intensities than the green control and when compared on a leaf area basis at a higher saturation level than the green control. This was interpreted at the time as being due to the presence of higher concentrations of dark enzyme [19]. Very recently Canaani *et al.* [20] have analyzed the yellow-green tobacco mutant *N. tabacum* Su/su and the wild type by the photoacoustic method. The authors show that saturation curves yield rates for the mutant which are more than twice higher *per* leaf area and about five times higher *per* chlorophyll as compared to the wild type. The present study which demonstrate immunochemical identity between the Su/su enzyme and that of the wild type suggests that Su/su obtains its high photosynthetic rates by using higher amounts of an enzyme as shown earlier. However, there must be a difference between the two enzymes, since Okabe and Schmid [21] have reported that the Su/su enzyme exhibits higher affinities towards O_2 and lower ones towards CO_2 when compared to the wild type. This difference, however, is serologically not detectable with the method used in this paper. Very recent studies show that despite the serological identity of the JWB- and Su/su-enzyme both enzymes seem to be maintained under activated conditions in different conformational states (Nespoulous, Fabisch, Radunz and Schmid, in preparation).

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