

# Characterization of RuBP Carboxylase/Oxygenase of the *N. tabacum* Mutant Series “Consolation” with Homologous and Non-Homologous Antisera

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*Dedicated to Professor Wilhelm Menke at the occasion of his 80th birthday*

RuBP Carboxylase/Oxygenase, Antibodies, Antibody-Binding Capacity, Photorespiration, Enzyme Conformation

The molar ratio of RuBP carboxylase/oxygenase to the  $CF_1$  complex was determined in the wild type *N. tabacum* var. JWB and in various *N. tabacum* mutants, which differ with respect to their chloroplast structure and their photosynthetic and photorespiratory activity. It appears that the ratio RuBP carboxylase/oxygenase/ $CF_1$  is not constant in the different mutants, and apparently depends on the chloroplast structure and the photosynthetic capacity. In the green phenotypes of *N. tabacum* var. Consolation, var. NC 95 and var. Xanthi, whose lamellar system consists of a balanced ratio of grana and intergrana regions the ratio is 3–4 RuBP carboxylase/oxygenase molecules per  $CF_1$  complex. In chloroplasts of the yellow-green mutant Su/su, and the yellow mutant of *N. tabacum* var. Consolation, whose lamellar system consists of extended intergrana regions and low stacked grana the RuBP carboxylase/oxygenase/ $CF_1$  ratio is reduced to about one half. The aurea mutant *N. tabacum* Su/su var. aurea and the yellow-green leaf patches of variegated *tabacum* var. Xanthi are characterized by the fact that one molecule RuBP carboxylase/oxygenase correlates to two  $CF_1$  complexes. Also in the two *N. tabacum* mutants Su/su and Consolation green, that exhibit a 30% higher photorespiration these molar ratios depend on the chloroplast structure.

The determination of the maximal binding of antibodies out of homologous and non-homologous antisera onto RuBP carboxylase/oxygenase of the *N. tabacum* mutant Consolation showed, that the enzyme of the green mutant, which exhibits a higher oxygenase activity, has in comparison to the yellow-green and yellow phenotype of this mutant series and also in comparison to the wild type a 30 per cent higher antibody binding capacity. These differences in antibody binding are shown in both the region of enzyme antibody equivalence and that of antibody excess. With the methods of enzyme-antibody precipitation in agarose gels these differences of enzymes, exhibiting higher or lower oxygenase activities, cannot be detected. The native enzymes and the large subunits of the enzyme of the three Consolation mutants yield in these test reactions with the enzyme of the wild-type fusing precipitation bands.

Treatment with the chemical agent hydroxylamine, as well as heat treatment at 50 °C alters the enzyme conformation to such an extent that the antibody binding capacity is increased by 70%. The difference in the higher antibody binding capacity of the enzyme of the green mutant of *N. tabacum* var. Consolation is maintained also after these chemical modifications.

## Introduction

Activity of the bifunctional stroma enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase is, besides by external factors as light and temperature, determined by the partial pressure of  $CO_2$  and  $O_2$  [1–3]. High  $CO_2$ -partial pressure enhances carboxylation and with it photosynthetic  $CO_2$  fixation. High  $O_2$ -partial pressure on the other hand stimulates the oxygenase activity and diminishes due to photorespiration net synthesis of carbo-

hydrates. The work of many research groups has tried to find ways to influence the ratio of carboxylation/oxygenation in favour of  $CO_2$  fixation [4]. Thus, it was shown that hydroxylamine and its derivative *p*-nitrophenylamine inhibits the oxygenase activity of the higher plant enzyme [4–7]. In bacterial enzymes such as that of *Anabaena cylindrica* these chemical agents cause besides the inhibition of the oxygenase function a stimulation of the carboxylating activity [8]. On the other hand all temperature incubations of the enzyme at 50 °C also lead to stimulation of the carboxylation reaction [8–11]. The conformational change-induced inhibition of the oxygenase and the concomitant stimulation of the carboxylase function can be

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analyzed with immunological methods. Thus, Nespoulous *et al.* [7] were able to show, that the enzymes of the *N. tabacum* mutants Su/su and Su/su var. *aurea* as well as the enzyme of the tobacco wild type are modified by the chemical agent hydroxylamine and its derivative structurally to such an extent that the antibody binding capacity of the enzyme molecule decreases by 65 per cent. On the other hand precipitation reactions of the enzyme with antibodies in agarose gels cannot detect these molecular modifications.

In the present publication we study the effect of hydroxylamine on the tobacco mutant series "Consolation" of *N. tabacum*. The mutant series originates from a selected variegated plant, which upon selfing yielded green, yellow-green and yellow phenotypes, with chloroplasts that differ with respect to the structure of their lamellar system [12, 13]. The green phenotype is characterized in comparison to the yellow-green and normal wild type tobacco by a 30 per cent higher photorespiratory activity [14, 15]. With a freshly-prepared antiserum to the enzyme of "Consolation green" and one to "Consolation yellow" as well as with an antiserum to the wild type enzyme, we compare the structure of the enzyme with immunological methods. Moreover, we determine the molar quantity of RuBP carboxylase/oxygenase in this Consolation series as well as in a series of other mutants of *N. tabacum* and compare the amount of Rubisco with that of the CF<sub>1</sub> complex [16]. The determination is carried out in the rocket immunoelectrophoresis. In order to avoid enzyme losses in the chloroplast preparation, the enzymes are prepared directly from leaves.

## Materials and Methods

### Plant material

Plants of *N. tabacum* (Table I) were grown in a climatized growth chamber under conditions described earlier [7, 16].

### Chloroplast preparations

Chloroplasts of the tobacco mutants were obtained according to the procedure of Nespoulous *et al.* [7] and Beuttenmüller *et al.* [17] and suspended for the antibody-antigen reaction in agarose gels in a 0.06 M Na<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.4

or for immunoelectrophoretal analyses suspended in 0.03 M Tris barbiturate buffer, pH 8.6, containing 2% Triton X-100.

### Isolation of RuBP carboxylase/oxygenase

The enzyme and the large subunits were isolated of the *N. tabacum* mutants according to the procedures described by Nespoulous *et al.* [7] and Beuttenmüller *et al.* [17].

### Antisera

In order to obtain the antisera, 0.6 to 1.0 mg pure RuBP carboxylase/oxygenase were injected into rabbits according to an earlier described immunization method. Blood withdrawal and serum preparation have also been described earlier [18, 19]. The monospecificity of the antisera was proven by the double immunodiffusion test and by crossed immunoelectrophoresis (Fig. 1).

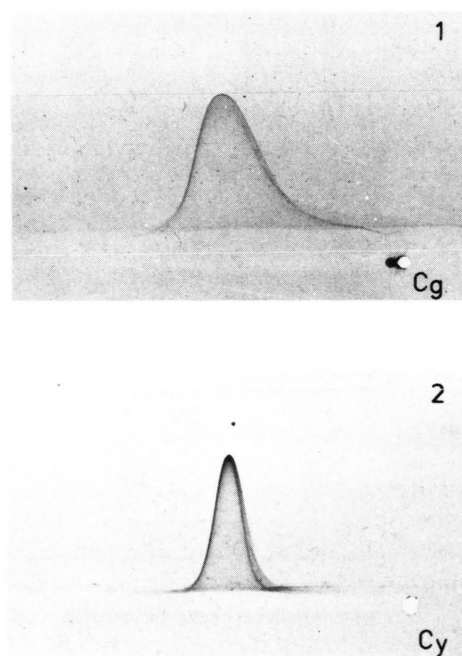


Fig. 1. Demonstration of monospecificity of the antisera to RuBP carboxylase/oxygenase of *N. t.* var. Consolation green and of *N. t.* var. Consolation yellow by means of crossed immunoelectrophoresis. Antigen: Chloroplast preparations of: Cg, *N. t.* var. Consolation green; Cy, *N. t.* var. Consolation yellow. Antiserum: 1, 0.45% antiserum to the enzyme of *N. t.* var. Consolation green in agarose gel; 2, 0.6% antiserum to the enzyme of *N. t.* var. Consolation yellow in agarose gel.

### Quantitative determination of RuBP carboxylase/oxygenase

Deveined leaves of *N. tabacum* were ground in a roller mill in 0.12 M Tris buffer, pH 7.6, containing 4 mM  $MgCl_2$ , 1 mM EDTA, 1% Triton X-100 and 0.1% polyvinylpyrrolidone. The brei was stirred for 1 h at 4 °C and passed through 8 layers of cheese-cloth. The filtrate was centrifuged at  $20,000 \times g$  for 30 min. The obtained supernatant was directly subjected to the rocket immunoelectrophoresis. The gel contained 1% agarose in 73 mM Tris-(hydroxymethyl)aminomethane, and 24 mM 5,5-diethylbarbiturate buffer, pH 8.6. The antiserum concentration was 1% (v/v) in the gel. Electrophoresis of the enzyme into the antibody containing gel was carried out for 18 h at 6 V/cm. Already after 16 h the length of the precipitation bands remained constant. The determination of the band length was carried out after staining with 0.1% amidoblack in 2% acetic acid. The calibration system was established under identical conditions with purified RuBP carboxylase/oxygenase according to Radunz and Schmid [20]. Protein determinations were carried out according to Lowry *et al.* [21]. Chlorophyll determination were done according to Schmid [22] in 90% methanol.

### Binding of antibodies onto the enzyme

The quantitative binding of antibodies onto the enzyme was carried out according to an earlier method of quantitative antigen-antibody precipitation described by Heidelberger and Kendall [23, 24] and by Radunz [25]. The used amount of antigen and antiserum, as well as the course of the antigen-antibody precipitations is depicted in Fig. 2.

### Binding of antibodies onto the enzyme after hydroxylamine and temperature treatment

The enzyme samples were suspended in 0.06 M phosphate buffer,  $Na_2HPO_4/KH_2PO_4$ , pH 7.4 and were prior to incubation with the antiserum.

- Heated for 10 min at 50 °C or
- supplemented with hydroxylamine to give a final concentration of 10 mM or
- heated for 10 min at 50 °C and subsequently supplemented with 10 mM hydroxylamine.

After treatment the preparations were cooled to room temperature and supplemented with the cor-

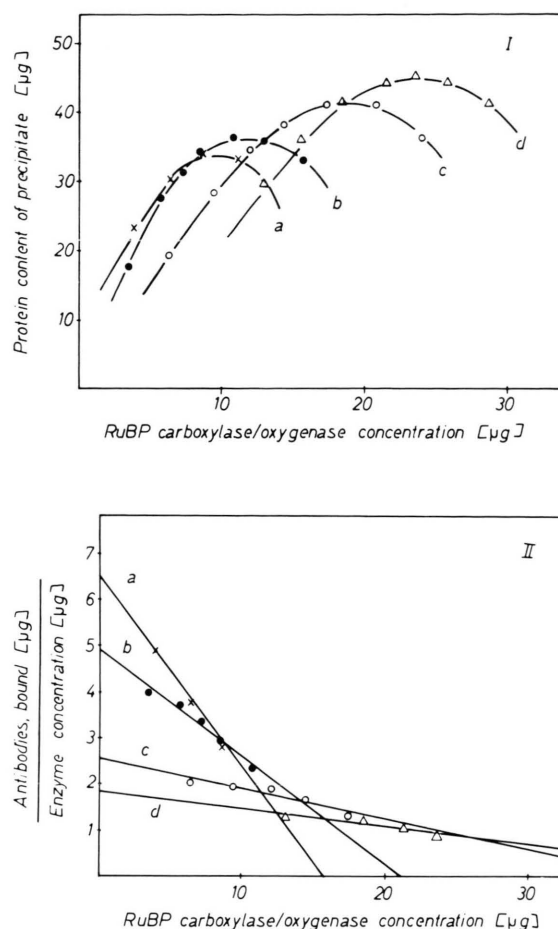


Fig. 2. Quantitative precipitations of RuBP carboxylase/oxygenase of *N. t.* var. Consolation green with the homologous antiserum after various treatments with hydroxylamine and temperature. I. The protein content of the precipitate was plotted as a function of the amount of enzyme added (4–28.8 μg). The amount of antiserum used was 30 μl. II. The ratio of bound antibodies and precipitated enzyme was plotted as a function of the amount of added enzyme. a. Native enzyme; b. with 10 mM hydroxylamine-treated enzyme; c. heat-treated enzyme (50 °C, 10 min); d. heat-treated enzyme subsequently treated with 10 mM hydroxylamine.

responding amounts of antiserum/control serum (Fig. 2).

### Results

The quantitative determination of RuBP carboxylase/oxygenase in the rocket immunoelectrophoresis with monospecific antisera showed that the absolute amount of the enzyme present in the

different mutants depends on the chloroplast structure and may differ considerably (Table I). In the green phenotypes of *N. tabacum* var. Consolation, var. NC 95 and var. Xanthi RuBP carboxylase/oxygenase makes up just as in the green wild type for 30 to 75% of the soluble leaf proteins. In the yellow and yellow-green phenotypes the enzyme represents only 10–50% of the soluble proteins. A clear dependency of the amount of RuBP carboxylase/oxygenase on the chloroplast structure is seen when the enzyme amount is referred to the amount of chlorophyll and when the molar ratio of the two components is determined. Whereas in the green phenotypes, the chloroplasts of which have a normal lamellar system with a normal ratio of grana to intergrana regions, 190 to 280 chlorophyll molecules per one RuBP carboxylase/oxygenase molecule are observed, the ratio in the yellow phenotype, the lamellar systems of which consists mainly of intergrana regions with occasional doublings, is found to be 25–35 chlorophyll molecules per 1 RuBP carboxylase/oxygenase molecule. Chloroplasts of the yellow-green phenotypes of both mutant series (*i.e.* Su/su and Consolation yellow-green), in which the ratio of grana and intergrana regions of the lamellar system is largely shifted in favor to the intergrana regions, are located in between the above described phenotypes. Here, 70–110 chlorophyll molecules are determined per 1 RuBP carboxylase/oxygenase molecule. The two tobacco mutants Su/su and Conso-

lation green, the enzymes of which exhibit a 30% higher oxygenase activity than all the other mutants tested, seemingly fit with their chlorophyll/enzyme ratio (but see Discussion) into the above described chloroplast structure row. A dependency or proportionality of a higher photorespiration on a higher amount of enzyme seems not to exist with these mutants at first glance.

In an earlier publication the content of coupling factor of photophosphorylation (CF<sub>1</sub> complex) in these mutants was determined [14]. The molar ratio of CF<sub>1</sub> to chlorophyll showed in these mutants also a dependency on the chloroplast structure. Therefore, we compare in Table II the molar ratio of RuBP carboxylase/oxygenase to CF<sub>1</sub> complex in these tobacco mutants. This molar ratio is also not constant in these mutants, and shows a dependency on the chloroplast structure. In the green phenotypes, in which the chloroplast structure is characterized by a normal ratio of grana to intergrana regions of the lamellar system, as already described above, just as in the wild type JWB, 1 ATPase molecule per 3–4 RuBP carboxylase/oxygenase molecules is determined. With the yellow and yellow-green phenotypes, in the lamellar system of which the intergrana regions prevail, this molar relationship is not so clearly recognized. Generally one can state that in these chloroplasts with more stroma lamellae and a considerably lower chlorophyll content the CF<sub>1</sub> content appears to be higher. This is expressed by a ratio of one

Table I. Comparison of the RuBP carboxylase/oxygenase content in leaves of the green, yellow-green and yellow phenotypes of different *Nicotiana tabacum* mutants, which are characterized by different chloroplast structures and different rates of photosynthesis and photorespiration. Chloroplast structure and photosynthetic efficiency of these mutants has been described earlier [12, 16, 28, 29].

	Chlorophyll in % of soluble leaf proteins	Chlorophyll ratio <i>a/b</i>	RuBP carboxylase/ oxygenase in % of soluble leaf protein	Molecular ratio RuBP carboxylase/oxygenase/ chlorophyll
<i>Nicotiana tabacum</i> var. John William's				
Broadleaf, green (wild type)	19.6	2.2/1	56.5	218/1
<i>N.t.</i> Su/su yellow-green	5.4	1.8/1	31.0	110/1
<i>N.t.</i> Su/su var. <i>aurea</i> , yellow	2.8	1.0/1	18.0	97/1
<i>N.t.</i> var. Consolation, green	23.2	2.5/1	51.5	275/1
yellow-green	4.9	4.4/1	45.8	66/1
yellow	2.8	5.8/1	49.1	35/1
<i>N.t.</i> var. NC 95, green	26.0	2.1/1	75.0	212/1
light-green leaf patches of variegated plant	6.0	1.8/1	36.4	102/1
<i>N.t.</i> var. Xanthi (D 523), green	9.3	2.6/1	30.4	185/1
yellow-green leaf	1.2	1.2/1	7.5	98/1
white-green patches	0.4	2.8/1	9.7	25/1



Table II. Molar ratio of RuBP carboxylase/oxygenase to coupling factor of photophosphorylation in chloroplasts of green, yellow-green and yellow phenotypes of different *Nicotiana tabacum* mutants.

<i>N. tabacum</i> types	Molar ratio of RuBP carboxylase/oxygenase CF <sub>I</sub>
<i>N.t.</i> var. JWB, green (wild type)	2.5/1
<i>N.t.</i> Su/su, yellow-green	1.7/1
<i>N.t.</i> Su/su var. <i>aurea</i>	0.6/1
<i>N.t.</i> var. Consolation, green	3.5/1
yellow-green	3.4/1
yellow	1.8/1
<i>N.t.</i> var. NC 95, variegated plant,	
green leaf areas	3.7/1
light-green areas	1.9/1
<i>N.t.</i> var. Xanthi, green	3.4/1
yellow-green	0.6/1

The values of RuBP carboxylase/oxygenase are taken from Table I and the values of CF<sub>I</sub> are taken from an earlier publication [16]. The molar ratio of both components was calculated *via* the ratio of the respective component to chlorophyll.

CF<sub>I</sub> complex per only 1–2 RuBP carboxylase/oxygenase molecules.

Comparative immunological studies of RuBP carboxylase/oxygenase of the green, yellow-green

and yellow phenotypes of the mutant series Consolation with homologous and non-homologous antisera in the double immunodiffusion test (Fig. 3) and in the earlier described tandem-crossed im-

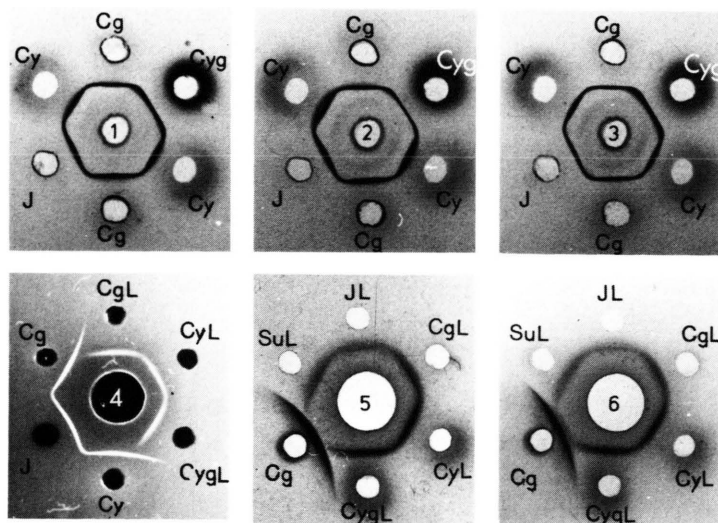


Fig. 3. Comparative analysis of native RuBP carboxylase/oxygenase and the large subunit of the mutant of *N.t.* var. Consolation with the enzyme of *N.t.* var. JWB in the double immunodiffusion. Antigen: Chloroplast preparation: J, of *N.t.* var. JWB; Cg, of *N.t.* var. Consolation, green; Cyg, of *N.t.* var. Consolation yellow-green; Cy, of *N.t.* var. Consolation yellow; CgL, CygL and CyL, large subunit of the green, the yellow-green and yellow mutant of *N.t.* var. Consolation; JL, SuL, large subunit of the wild type and of the mutant Su/su. Antiserum: 1, mixed antiserum to the enzyme of *N.t.* var. JWB and of the green mutant of *N.t.* var. Consolation; 2, mixed antiserum to the enzyme of the wild type and of the yellow mutant of *N.t.* var. Consolation; 3 and 4, mixed antiserum to the enzyme of the green and yellow phenotype of *N.t.* var. Consolation; 5, mixed antiserum to the native enzyme of the green and yellow phenotype of *N.t.* var. Consolation and to the large subunits of the enzyme of the wild type and the tobacco mutant Su/su; 6, mixed antiserum (3) and (4) and to the native enzyme of the wild type and of the tobacco mutant Su/su.

munoelectrophoresis have shown that all these enzymes are immunochemically identical to the wild type enzyme. Within the combined rocket line immunoelectrophoresis it is demonstrated in Fig. 4 that only fusing precipitation bands are observed between these enzymes, although the green Consolation mutant exhibits a 30% higher rate of photorespiration than the enzymes of the other mutants and of the wild type. Furthermore, the double diffusion test shows that the large subunits of the three mutant enzymes are serologically identical also to the subunit of the wild type enzyme.

For the comparative antibody-binding tests, antibodies out of the homologous as well as of the non-homologous monospecific antisera and also out of the antisera to the wild type enzyme were

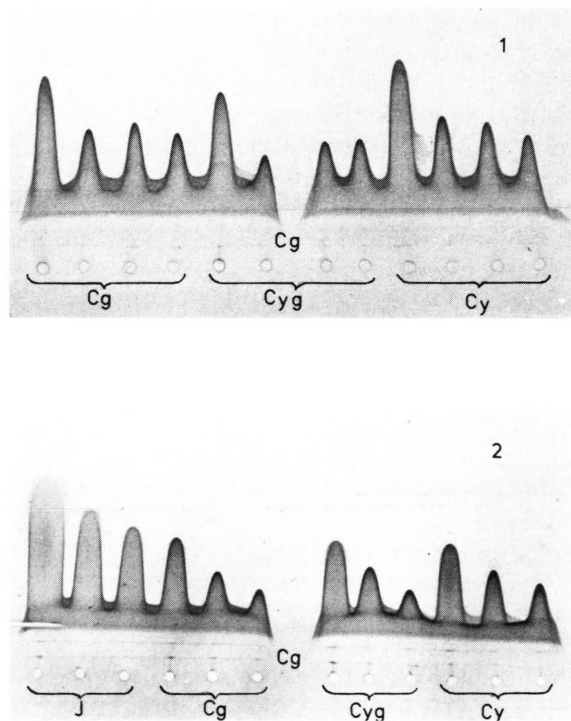


Fig. 4. Comparative analysis of RuBP carboxylase/oxygenase of *N.t.* var. JWB with the enzymes of the mutant series of *N.t.* var. Consolation in the line rocket immunoelectrophoresis. Antigen: Chloroplast preparations of: J, *N.t.* var. JWB; Cy, *N.t.* var. Consolation yellow; Cg, *N.t.* var. Consolation green; Cyg, *N.t.* var. Consolation yellow-green. Antiserum: 1, serum mixture of the antisera to RuBP carboxylase/oxygenase of *N.t.* var. Consolation green (1%) and of *N.t.* var. Consolation yellow (1%); 2, serum mixture of 1, and to RuBP carboxylase/oxygenase of *N.t.* var. JWB.

adsorbed onto RuBP carboxylase/oxygenase of the green, yellow-green and yellow phenotypes of the Consolation mutant series. The result is shown in Fig. 5 and demonstrates that the antibody-binding capacity of these enzymes is not the same. Whereas the enzymes of the yellow and yellow-green phenotypes, which exhibit an oxygenase activity comparable to that of the wild type, bind 9 antibody molecules per enzyme in the equivalence region, the enzyme of the green phenotype of the Consolation series (the one that exhibits a 30% higher oxygenase activity) binds 12 antibody molecules. This means that this mutant enzyme has a 30 per cent higher antibody-binding capacity. In the region of antibody excess, that is when antibodies are generally bound monovalently, the amount of adsorbed antibodies must be twice as high as in the region of equivalence. Also under these conditions, that is under a higher competition of the antibody-binding affinity, the higher antibody-

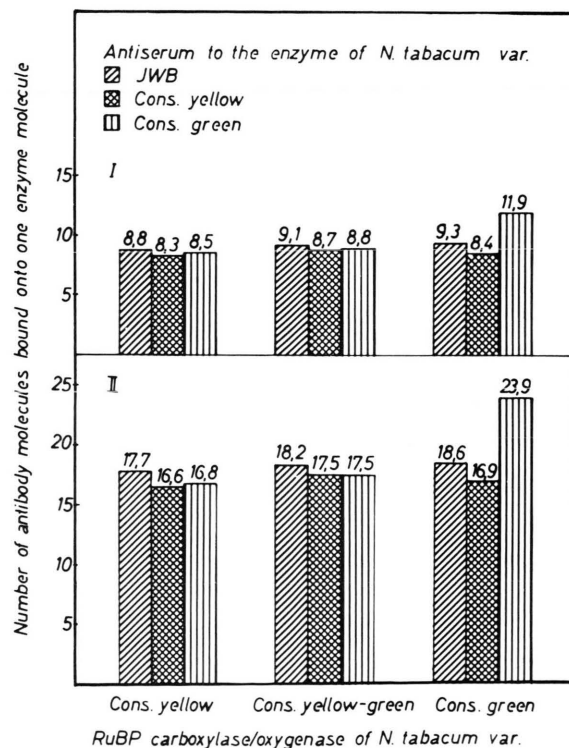


Fig. 5. Binding of antibodies onto untreated RuBP carboxylase/oxygenase of the mutant series of *N.t.* var. Consolation out of homologous and non-homologous antisera. I, in the region of equivalence; II, in the region of extreme antibody excess.

binding capacity of the enzyme with the higher oxygenase activity in the green phenotype of the Consolation mutant series is preserved.

Incubation of RuBP carboxylase/oxygenase at temperatures of 50 °C and treatment with the chemical agent hydroxylamine leads to a conformational change of the enzyme molecule. This conformational modification of the enzyme can be detected by a differing antibody binding. In the diagram of Fig. 6 the number of antibody molecules bound onto the enzymes of the green, yellow-green and yellow phenotypes of the mutant series *N. tabacum* var. Consolation out of an antiserum to the enzyme of the green mutant, which has the highest antibody-binding capacity, is compared. It is clearly seen that the number of reacting antigenic determinants decreases after the modification of the enzyme structure. Whereas treatment with 10 mM hydroxylamine leads to a 25% lower anti-

body binding, heat treatment leads to a 60% reduction of antibody binding. The most drastic effect on the enzyme structure is observed after heat treatment and subsequent hydroxylamine treatment. In comparison to the native enzyme 70% of the antigenic determinants disappear or become inaccessible to antibodies in the three enzymes. The higher binding capacity of the enzyme of the green mutant seems to be preserved even after these hydroxylamine treatments. The difference in antibody binding is, just as in the native enzyme, 30 per cent.

### Discussion

Comparative immunological studies on antibody binding have led to the conclusion, that the bifunctional enzyme RuBP carboxylase/oxygenase of the green phenotype of the *N. tabacum* mutant series Consolation must be structurally or conformationally different in comparison to that of all other mutants of this series and also to that of the wild type. This difference is maintained after hydroxylamine or heat treatment. In previous publications we were also able to show that the enzyme of the tobacco mutant Su/su, which also exhibits a higher oxygenase activity, is structurally different to the enzyme of the wild type and to the mutant *N. tabacum* Su/su var. *aurea*. Both enzymes, that of the new mutant which is a green phenotype derived from the earlier described mutant series Consolation [12] and the enzyme of the earlier described Su/su mutant [26] have a 30 per cent higher antibody-binding capacity. In the case of the *N. tabacum* mutant Su/su we were able to show that the antiserum to this enzyme causes with the homologous as well as with the non-homologous enzymes of the wild type and of spinach a considerably higher inhibitory effect of the oxygenase activity than the one which is inflicted by the homologous antisera. This inhibitory effect seems not to correlate with the number of antibodies bound. It should be emphasized, that these structural differences detected by a different antibody-binding capacity cannot be demonstrated by comparing enzyme-antibody reactions in agarose gels. In such comparing studies between the native enzyme as well as with the large subunits of the enzymes of *N. tabacum* Su/su and the wild type on the one hand and those of the enzyme of the green Conso-

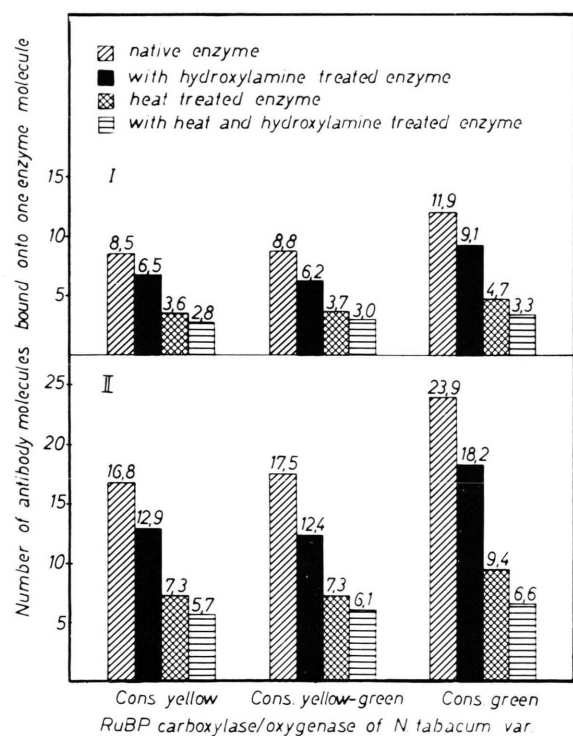


Fig. 6. Binding of antibodies onto RuBP carboxylase/oxygenase of the mutant series of *N. t.* var. Consolation out of the antiserum to RuBP carboxylase/oxygenase of *N. t.* var. Consolation green after various treatments. I, in the region of equivalence; II, in the region of extreme antibody excess.

lation mutant on the other hand only fusing precipitation bands are observed.

The quantitative determination of RuBP carboxylase/oxygenase in the rocket immunoelectrophoresis of leaves or leaf areas has shown that these two mutants with higher photorespiratory activity are in comparison to other mutants not distinguished by a higher enzyme content. The molar ratio of RuBP carboxylase/oxygenase to chlorophyll on the other hand seems to depend at first glance only on the chloroplast structure. In the chlorophyll deficient tobacco mutant *Su/su*, when referred to chlorophyll, twice the amount of enzyme molecules is present in comparison to the green mutant of the Consolation series. The comparison of this molar ratio depicted in Table I for the Consolation series, shows in comparison to the determination of the photosynthetic unit size of the same mutant series (Table VII in ref. [12]) that in comparison to electron transport chains present, the green phenotype (*i.e.* *N. tabacum* var. Consolation green) contains only 1/2 of the RuBP carboxylase/oxygenase molecules which the yellow-green phenotype has at its disposition, which explains that the light intensity curve of photosynthesis ( $O_2$

evolution or  $CO_2$  fixation) of the two plants plotted on a leaf area basis differs by the saturation level, the latter being twice as high for the yellow-green phenotype as that of the green one (see also Table III of ref. [26]), although this higher level is reached at only considerably higher light intensities.

If the molar ratio of RuBP carboxylase/oxygenase to the  $CF_1$  complex of photophosphorylation is compared, it is seen that this ratio between the energy consuming enzyme RuBP carboxylase/oxygenase and the "energy-equivalent producing" enzyme  $CF_1$  cannot only be put in relation to the chloroplast structure but also in relation to the photosynthetic capacity itself. Thus, only the yellow *i.e.* the "*aurea* phenotypes" of tobacco which have a higher photosynthetic efficiency, have twice the amount of  $CF_1$  ATPase in comparison to the RuBP carboxylase/oxygenase molecules present. Chloroplasts of these mutants have a lamellar system that consists of extended intergrana regions with low grana stacks or even only occasional doublings, the photosynthetic units of which generally contain less chlorophyll than those of green plants [12, 26, 27].

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