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ACTION OF SULPHITE ON THE SUBSTRATE KINETICS OF CHLOROPLASTIC NADP-DEPENDENT GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

IRMGARD ZIEGLER, AJA MAREWA and ELSE SCHOEPE
Institut für Biochemie der Ges.f.Strahlen- und Umweltforschung and Botanisches Institut der Technischen Universität, München, W. Germany

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Key Word Index—*Spinacia oleracea*; spinach; Chenopodiaceae NADP-dependent glyceraldehyde-3-phosphate dehydrogenase; chloroplasts; sulphite action; regulation.

Abstract—The kinetics of NADP-GPD from spinach chloroplasts are biphasic vs NADPH and PGA. Thus, two maximum velocities exist with an intermediary plateau and two K_m values. Activation by NADPH + DTT increases V_{max} of both sections, but does not change the substrate affinities. Sulphite reduces the maximum activities of both sections vs NADPH, however, it causes normal substrate kinetics vs PGA; even V_{max} is reduced. Sulphite, present only during the activation process, suppresses the enzyme form with the higher V_{max}. The kinetics vs NADH are also biphasic; the activity is strongly reduced by preincubation of the chloroplasts with NADH + DTT or at NADH concentrations > 0.4 mM. Using NADH as cofactor, inverted peaks in the kinetics vs PGA occur; sulphite is active in a similar way as when NADPH is used as cofactor. The biphasic kinetics are discussed with respect to additional potential for regulation of enzyme activity according to illumination and NADPH concentrations respectively.

INTRODUCTION

NADP–GPD (EC 1.2.1.13) and NADP–MDH (1.1.1.82) are chloroplastic enzymes which undergo reversible activation by light [1,2]. In peas, after *in vivo* activation by light, NADP–GPD shows enhanced V_{max} vs both NADPH and PGA, whereas affinity is not changed [3]. *In vitro*, full activation of NADP–GPD in spinach chloroplasts can be achieved by incubation of sonicated chloroplasts with NADPH + DTT [4]. The nucleotide seems to exert a strong influence even on gross enzyme characteristics like MW: when present during gel chromatography, it causes dissociation into protomers, which show increased affinity for NADP [5]. NADP-MDH of Zea mays is activated by DTT alone [2], but the presence of NADPH during gel filtration is necessary to stabilize a MW form of about 180 000 daltons. Otherwise, a high MW form (see [2]) is split into a number of smaller ones with very low activity [6].

Preceding investigations on NADP–MDH in Zea mays showed unusual biphasic kinetics, which, under the action of sulphite, are further divided into several peaks; this indicates a conformational change in the enzyme [7,8]. Up to now, the number of enzymes found with biphasic kinetics has considerably increased (for references see 9). Such kinetics seem to be characteristic of regulated enzymes. On account of the similarity in molecular behaviour of chloroplastic NADP–MDH and NADP–GPD, it was of interest to investigate the kinetics of this enzyme and the effect of sulphite on these kinetics, especially as biphasic kinetics have been found for NAD–GPD from honey-bee muscle [10], yeast [11] and *Bacillus subtilis* [12]. In yeast GPD, cAMP causes inhibition along with subdivision of the biphasic substrate kinetics into several peaks; this was interpreted with a conformational change in the subunits [11].

Abbreviations: NADP (or NAD)-GPD = NADP (or NAD) dependent glyceraldehyde-3-phosphate dehydrogenase; NADP (or NAD)-MDH = NADP (or NAD) dependent malate dehydrogenase; DTT = dithiothreitol; PGA = 3-phosphoglycerate; chlorophyll = chl.

RESULTS

Activation rate after preincubation with NADPH + DTT

The activation rate of NADP–GPD is dependent on the concentration of the NADPH + DTT present during preincubation, when the enzyme activity is measured under substrate saturating conditions [4]. The maximum of activation is reached with about 1.5 mM NADPH [4]. As the substrate kinetics involved were seen to be biphasic (Fig. 1) the question arises whether V_{max} of both sections is about equally increased, or whether only one of both is enhanced. Table 1 and 2 show that $V_{max\,I}$ and $V_{max\,II}$ respectively are both increased by about the same factor. Moreover, it is seen that the affinity towards the substrate is not markedly changed by the activation process.

In the following, the section with $V_{max\,I}$ and $V_{max\,II}$ respectively should be termed "$V_{max\,I}$ form" and "$V_{max\,II}$ form" without anticipating an interpretation of the molecular mechanism.

Action of sulphite on substrate kinetics

In enzymes which undergo activation both *in vivo* and *in vitro*, sulphite may interfere not only with its activity, but also with the activation process. Therefore, sulphite had to be present both during preincubation, and during measurement of the activated enzyme.

Table 1. Kinetic constants of NADP–GPD vs PGA. For concentrations of the other components see Experimental

	Non-activated enzyme	Partially activated enzyme (preincubation with 0.54 mM NADPH + 10 mM DTT)	Fully activated enzyme (preincubation with 2.7 mM NADPH + 50 mM DTT)
$V_{max\,I}$ (μmol NADPH/mg chlor.h)	8.9	20.3	36.0
$V_{max\,II}$ (μmol NADPH/mg chl.h)	33.3	111.0	166.0
$K_{m\,I}$ (mM PGA)	0.016	0.018	0.020
$K_{m\,II}$ (mM PGA)	0.14	0.20	0.20

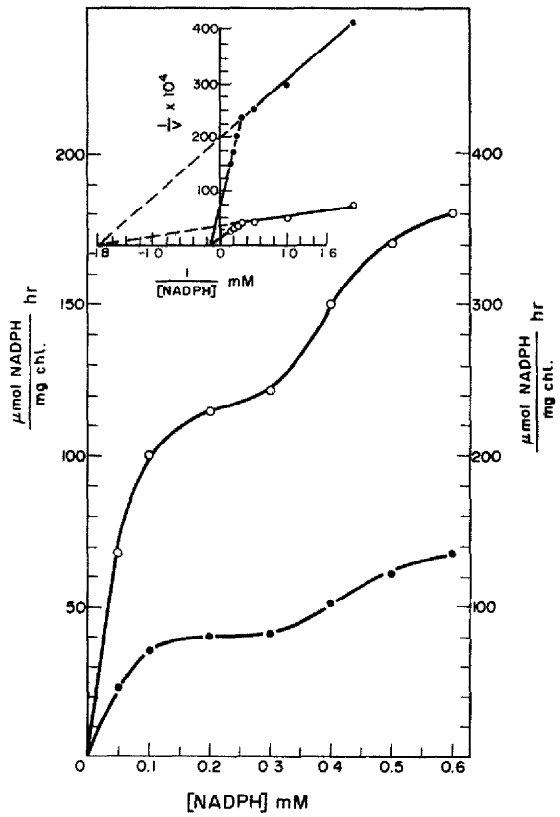

Fig. 1. Kinetics of NADP–GPD vs NADPH: ●—● without preincubation scale left side; ○—○ after preincubation with NADPH + DTT scale right side. Insert: Lineweaver–Burk plot.

Action of sulphite on enzyme activity

With respect to NADPH (Fig. 2) the biphasic kinetics are not abolished by sulphite, and the affinity of both V_{max} forms is not changed. Only the maximum activity of both forms is strongly reduced. Thereby, as calculated from the Lineweaver–Burk plot (see insert in Fig. 2), both V_{max} forms are equally affected (Fig. 3). It further shows that half maximum inhibition occurs at about 1.5 mM, but that the inhibition curve levels off, at higher sulphite concentrations and about 20% of the normal activity remains at even higher concentration levels.

With respect to varying PGA concentrations, the kinetics are also biphasic (Fig. 4). The addition of sulphite produces normal ones. The substrate affinity of the inhibited enzyme corresponds to that of the $V_{max\,II}$ form; even its maximum activity is strongly reduced.

Thus, sulphite is a non-competitive inhibitor with respect to both NADPH and PGA; simultaneously, with respect to PGA, it abolishes the enzyme form with $V_{max\,I}$ and fixes the form with $V_{max\,II}$ throughout the entire substrate concentration range.

Action of sulphite on the activation process

Addition of sulphite to the medium used for preincubation, even up to 5 mM, causes a sulphite concentration of up to 0.04 mM in the assay medium. This concentration has no effect on the GPD activity, and thus the sulphite action restricted to the activation process can be studied.

The kinetics with respect to NADPH (Fig. 5) show that the activity of the enzyme form with $V_{max\,I}$ is not or only slightly affected. In contrast, $V_{max\,II}$ is completely suppressed and does not exceed $V_{max\,I}$. The intermediary plateau has changed to an "inverted peak" described as a "trough region" in the case of DT-diaphorase [9].

The action of sulphite on the kinetics vs PGA completely parallels that vs NADPH, resulting in strong inhibition of $V_{max\,II}$ and thus showing an inverted peak.

Action of sulphite on the substrate kinetics of NADP–GPD with NADH used as cofactor

There is good evidence that, in principle, NADP–GPD reacts with both NADPH and NADH, but that its activity vs each cofactor is dependent on the MW

Table 2. Kinetic constants of NADP–GPD vs NADPH. For concentrations of the other components see Experimental

	Non-activated enzyme	Fully activated enzyme (Preincubation with 2.7 mM NADPH + 50 mM DTT)
$V_{max\,I}$ (μmol NADPH/mg chl/hr)	50.0	280.0
$V_{max\,II}$ (μmol NADPH/mg chl/hr)	166.0	625.0
$K_{m\,I}$ (mM NADPH)	0.058	0.060
$K_{m\,II}$ (mM NADPH)	0.80	0.60

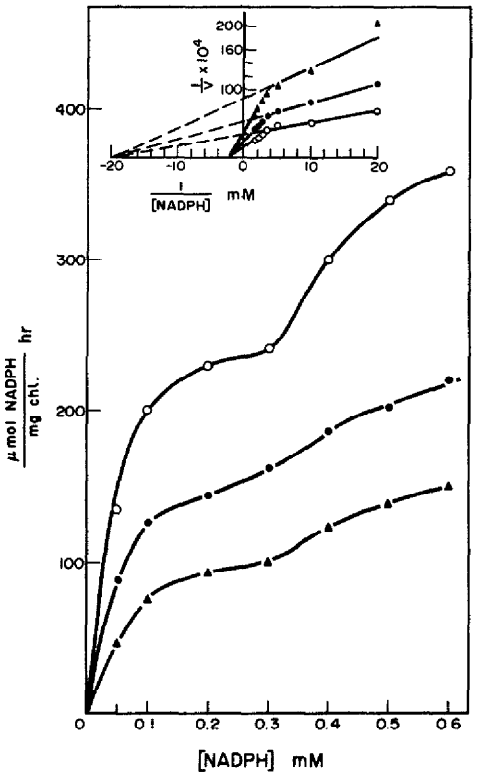

Fig. 2. The action of sulphite on the kinetics of NADP-GPD vs NADPH. Sulphite was added to the assay medium after activation by NADPH + DTT. ○—○ without sulphite; ●—● 1 mM sulphite; ▲—▲ 2 mM sulphite. Insert: Lineweaver–Burk plot.

form [5,13], which, in turn, changes according to its pretreatment.

It is evident that vs NADH the enzyme shows similar biphasic kinetics to those existing vs NADPH (Fig. 6; Table 3). Preincubation with NADPH + DTT does not change the affinity for NADH but considerably increases V_{max}. Moreover, the kinetics reveal complete inhibition at a NADH concentration of 0.6 mM. Preincubation with NADH + DTT strongly reduces the enzyme's affinity for NADH; also here, NADH concentrations

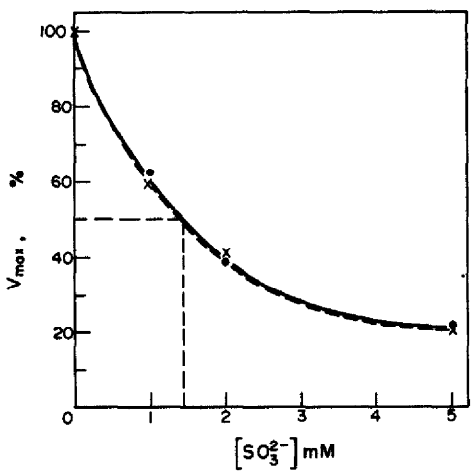

Fig. 3. Percentage inhibition of $V_{max\,I}$ and $V_{max\,II}$ by sulphite. Data from Fig. 2. ×—× $V_{max\,I}$; ○—○ $V_{max\,II}$.

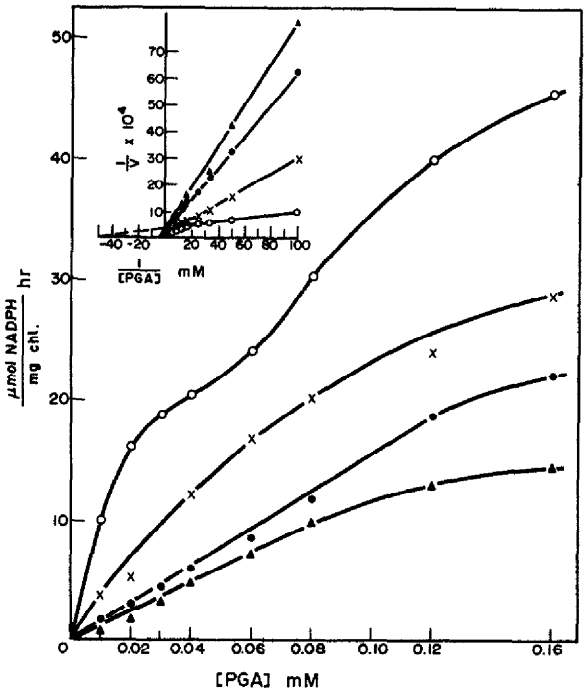

Fig. 4. The action of sulphite on the kinetics of NADP-GPD vs PGA. For further indications see Fig. 2. ○—○ without sulphite; ×—× 0.5 mM sulphite; ●—● 1.0 mM sulphite; ▲—▲ 2.0 mM sulphite.

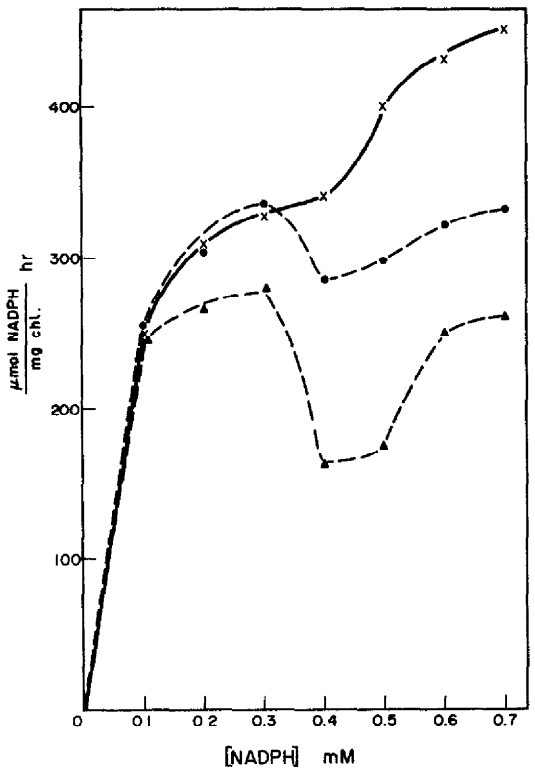

Fig. 5. The action of sulphite, present during enzyme preincubation, on the kinetics vs NADPH. ×—× without sulphite; ●—● 1.0 mM sulphite; ▲—▲ 2.0 mM sulphite.

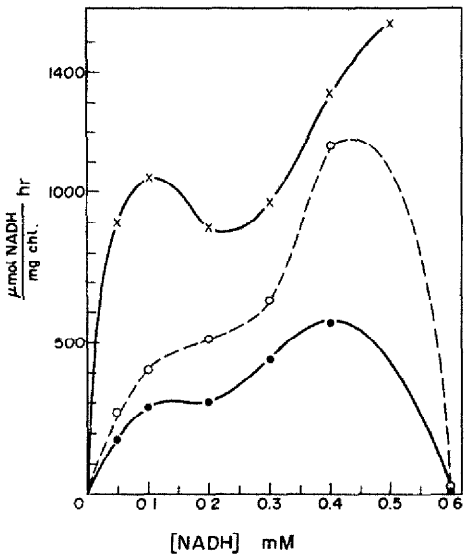

Fig. 6. Kinetics of NADP–GPD vs NADH under various preincubation conditions. O—O no preincubation; ×—× preincubation with DTT + NADPH; ●—● preincubation with DTT + NADH.

> 0.4 mM are completely inhibiting. Consequently, the theoretical maximum velocities are never reached.

For the experiments with sulphite, no preincubation, but only sonicated chloroplasts were used. Sulphite, present in the assay medium, strongly inhibits the enzyme form with $V_{max\,II}$ (Fig. 7), whereas the form with $V_{max\,I}$ is only slightly affected. Thus, the inhibition pattern parallels that obtained with NADPH as cofactor.

The kinetics vs PGA are no longer biphasic, as they were with NADPH used as cofactor. Rather, they show inverted peaks (Fig. 8). The activity is low. Sulphite (1 mM) has only a slight effect on the activity, but changes the kinetics back to a biphasic curve. Its K_m values are identical with those obtained when NADPH is used as cofactor, but both maximum velocities are strongly reduced.

DISCUSSION

It is generally accepted that the chloroplasts contain one GPD, active with both NADPH and NADH (see [5]). The advantage of using isolated chloroplasts instead of partially purified enzyme preparations was recently demonstrated by Bahr and Jensen [14]: ribulosediphosphate carboxylase preparations, even in crystallized form, show unphysiologically high K_m values, whereas the enzyme from freshly lysed chloroplasts fixes with high affinity towards HCO_3^-, showing transitions in enzyme forms during incubation.

Biphasic kinetics, recently found with numerous enzymes (see Introduction), were explained by negative cooperativity [15]; in such cases, occupation of the substrate binding site in one subunit hinders substrate binding to neighbouring subunits until the first ones are filled up. Trough regions in DT-diaphorase were explained on the same basis, but they require a four site enzyme, which exhibits positive and negative cooperativity with respect to the catalytic activity of the sites involved. As an alternative, two dimeric enzyme species, each with negative cooperativity, may exist [9].

Although on the basis of the experiments described above no decisive comments on the molecular mechanism of those kinetics can be made, the findings with ribulosediphosphate carboxylase [14] offer an explanation which may apply to chloroplastic, regulated enzymes: a "low K_m form" (with low activity) and a "high K_m form" (with high activity) exist not only in ribulosediphosphate carboxylase, but also in NADP–GPD. Depending on the concentration of the cofactor and/or the substrate, they change into one another. This makes

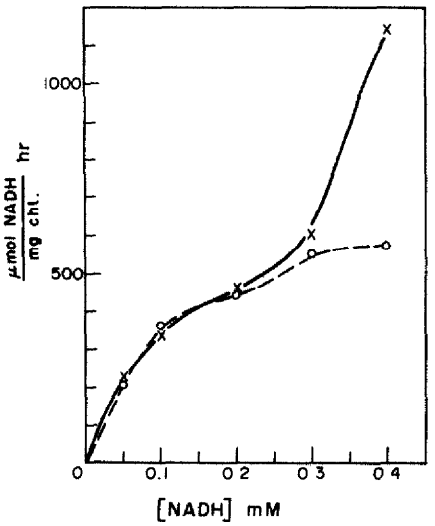

Fig. 7. The action of sulphite on the kinetics of NADP–GPD vs NADH. Sulphite was added to the assay medium. ×—× without sulphite; O----O 1.0 mM sulphite.

Table 3. K_m and V_{max} values of NADP–GPD versus NADH after different preincubation conditions (without consideration of substrate inhibition)

	No preincubation	Preincubation with NADPH + DTT	Preincubation with NADH + DTT
$V_{max\,I}$	769 μmol NADH/mg chl/hr	2220 μmolNADH/mg chl/hr	769 μmol NADH/mg chl/hr
$V_{max\,II}$	1430 μmol NADH/mg chl/hr	2220 μmol NADH/mg chl/hr	3330 μmol NADH/mg chl/hr
$K_{m\,I}$ (mM NADH)	0.09 mM	0.09 mM	0.16 mM
$K_{m\,II}$ (mM NADH)	0.33 mM	0.33 mM	2.0 mM

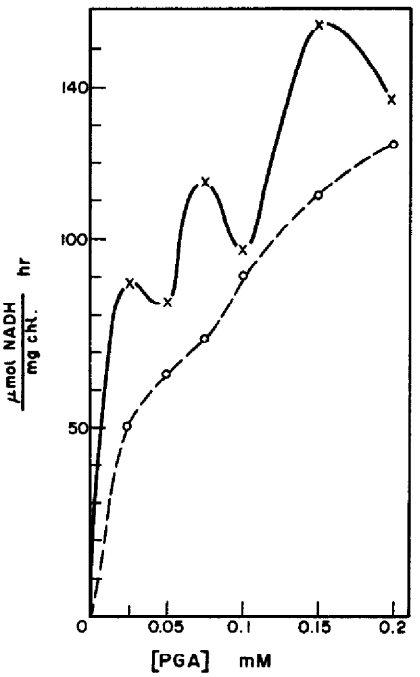

Fig. 8. The action of sulphite on the kinetics of NADP–GPD vs PGA with NADH used as cofactor. ×—× without sulphite; O——O 1 mM sulphite. In the presence of sulphite: $V_{max\,I}$ 33.3 μmol NADH/mg chl/hr. $V_{max\,II}$ 200 μmol NADH/mg chl/hr; K_{mI} 0.020 mM NADH; K_{mII} 0.10 mM NADH.

for an additional mechanism for regulation: at low substrate or cofactor concentrations, e.g. at low light intensities, an enzyme form with high affinity but low turnover rate is present. At increased cofactor and/or substrate concentrations, e.g. during bright illumination, an enzyme form with decreased affinity but elevated turnover capacity prevails. Medium substrate and/or cofactor concentrations, which cause the intermediary plateau, are present at average or rapidly changing light conditions, thus maintaining an equilibrium of both enzyme forms. By this, the ability for fast adaptation toward both sides is maintained.

The low activity of chloroplastic GPD with respect to NADH after preincubation with NADH further indicates that the dark-enzyme form does not take part in glycolytic processes. The trough regions in the kinetics vs PGA rather indicate that NADH pretreatment results in an enzyme form which differs from the normal one and is inactive *in vivo*. In the case of crystallized yeast GPD, cAMP induces such changes in kinetics. These are interpreted by Rock and Cook [11] as a conformational change in individual subunits, resulting in increased negative cooperativity under the action of cAMP. Assuming different enzyme species, an increase in binding sites (see [9]) also fits the experimental data. In general, sulphite causes non-competitive inhibition with respect to NADPH, NADH, and PGA. The same type of inhibition was also found with respect to substrate and cofactor in NAD-and NADP-dependent MDH [7,8]. The fully competitive type of inhibition seems to be restricted towards the HCO_3^- anion in CO_2 metabolizing enzymes, e.g. ribulosediphosphate carboxylase [16], PEP-carboxylase [17], and malic enzyme [18].

In the case of regulatory enzymes, sulphite exerts a different action: it changes the enzyme's conformation, as was found for NADP–MDH [8] and glutamate dehydrogenase [19]. It appears that, in the presence of sulphite during preincubation, the enzyme becomes unable to change into its $V_{max\,II}$ form, whereas the enzyme form with $V_{max\,I}$ retains almost all of its activity with respect to PGA or NADPH.

Once the enzyme is activated and its conformation established by preincubation with NADPH + DTT, the action of sulphite is different, and does not influence the NADPH or NADH binding sites, as the biphasic kinetics are unaffected; only $V_{max\,I}$ and $V_{max\,II}$ are equally reduced. With respect to PGA, the negative cooperativity is abolished, since the $V_{max\,I}$ form is suppressed and normal Michaelis–Menten kinetics of the enzyme form with $V_{max\,II}$ appears; even the maximum activity is reduced. A reduction in ligand induced conformational sites by sulphite is also indicated by the fact that the multiple peaked substrate kinetics vs PGA (with NADH used as cofactor) are converted to biphasic ones.

The action of inhibitors, which simultaneously change the conformation of regulated enzymes, seems further to be characterized by a biphasic inhibitor curve because after an initial drastic decrease in activity, about 10–20% remains even at elevated inhibitor concentrations. Apart from the above experiments, this was shown for sulphite action in NADP–MDH [8] and of cAMP action in yeast NAD–GPD [11]. For the organism, this may imply survival at low metabolic activities.

The competition of sulphite with HCO_3^- binding sites in CO_2 metabolizing enzymes (see [20]) seems to be most characteristic and specific. The action of sulphite on enzyme conformation, however, is shared with numerous other agents, e.g. cAMP [11], or even with external factors like ionic strength, temperature, or pH (see [9]). The interference of sulphite with regulatory processes in plant metabolism, governed by environmental changes, may contribute a good deal to the various actions of SO_2 under field conditions.

EXPERIMENTAL

Spinach was grown at 17° in a Heraeus incubator (20 000 lx; 10 hr light, 14 hr dark). Chloroplasts were isolated according to ref. [21]. For preincubation, the chloroplasts were resuspended in Tris–buffer (50 mM, pH 7.9; containing 0.1% mercaptoethanol) and sonicated for 3 sec (Branson Sonifier B 12; step 3). For activation studies the sample was divided into 2 parts: one was assayed immediately, the other was supplied with NADPH + DTT (or with NADH + DTT, respectively) to the concns given and incubated for 40 min at 23°. In order to study the action of sulphite on the activation process, Na_2SO_3 was added (with 0.1 ml Tris–buffer pH 7.9) to the incubation medium. In all sulphite expts, complete activation was involved (achieved by 2.7 mM NADPH + 50 mM DTT). Action of sulphite on activity after full activation was followed by addition of sulphite (in 0.1 ml Tris-buffer pH 7.6) to the assay medium. The standard assay medium contained: 8.6 mM PGA, 0.16 mM NADPH or NADH; 1 mM ATP; 5 mM cysteine, 7.8 mM $MgSO_4$; 1 mM glutathione red. For kinetics, the component studied was varied according to the values indicated. The reaction was started by addition of the chloroplast extract (0.1 ml with about 10 μg chlorophyll). The A was measured at 340 nm every 30 sec using an automatic spectrophotometer. Due to the unusual kinetics found, each series was measured at least 5 times. Performance of the assays by 3 different persons further eliminated the possibility that the

unusual kinetics are due to human bias. The initial velocity was determined graphically. Chlorophyll determination was carried out according to ref. [22]. Glycerate-3-phosphate (tricyclohexylammonium salt), ATP, NADPH, NADH, glutathione red were obtained from Boehringer, cysteine HCl, DTT and mercaptoethanol from Sigma. All other chemicals were analytical grade from Merck.

REFERENCES

1. Ziegler, H. and Ziegler, I. (1965) *Planta* **65,** 369.
2. Johnson, H. S. and Hatch, M. D. (1970) *Biochem. J.* **119,** 273.
3. Melandri, B. A. (1970) *Biochim. Biophys. Acta* **220,** 178.
4. Müller, B., Ziegler, I. and Ziegler, H. (1969) *European J. Biochem.* **9,** 101.
5. Pupillo, P. and Piccari, G. G. (1973) *Arch. Biochem. Biophys.* **154,** 324.
6. Ziegler, I. unpublished.
7. Ziegler, I. (1974a) *Phytochemistry* **13,** 2403.
8. Ziegler, I. (1974b) *Phytochemistry* **13,** 2411.
9. Hollander, P. M., Bartfai, F. and Gatt, Sh. (1975) *Arch. Biochem. Biophys.* **169,** 568.
10. Gelb, W. G., Olivier, E. J., Brandts, J. F. and Nordin, J. H. (1970) *Biochemistry* **9,** 3228.
11. Rock, M. G. and Cook, R. A. (1974) *Biochemistry* **13,** 4200.
12. Saski, R. and Pizer, L. J. (1975) *European J. Biochem.* **51,** 415.
13. Pawlitzki, K. and Latzko, E. (1974) *FEBS Letters* **42,** 285.
14. Bahr, J. T. and Jensen, R. G. (1974) *Plant Physiol.* **53,** 39.
15. Levitzky, A. and Koshland, Jr. D. E. (1969) *Proc. Natl Acad. Sci. (Wash)* **62,** 1121.
16. Ziegler, I. (1972) *Planta* **103,** 155.
17. Ziegler, I. (1973) *Phytochemistry* **12,** 1027.
18. Ziegler, I. (1974) *Biochim. Biophys. Acta* **364,** 28.
19. Pahlich, E. (1972) *Planta* **104,** 78.
20. Ziegler, I. (1975) *Residue Rev.* **56,** 79.
21. Jensen, R. G. and Bassham, J. A. (1966) *Proc. Natl Acad. Sci. (Wash)* **56,** 1095.
22. Arnon, D. L. (1949) *Plant Physiol.* **24,** 1.