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# EFFECT OF PYRIDOXAL PHOSPHATE AND CYSTEINE ON δ-AMINOLAEVULINATE SYNTHETASE AND DEHYDRATASE IN SOYA

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**Key Word Index**—*Glycine soya*; Leguminosae; soya callus; δ-aminolaevulinate synthetase (ALA-S); ALA dehydratase (ALA-D); -SH content.

**Abstract**—The specific activity of ALA-S extracts prepared from lyophilized soya callus growing in light or dark showed a striking increase when tissue cultures were grown in the presence of pyridoxal phosphate (PyP) in order to complex aminothiols. By contrast ALA-D specific activity in the light grown callus was reduced on incorporation of PyP into the growth medium. Cysteine (Cy) added to the culture medium did not influence the specific activities of either enzyme.

## INTRODUCTION

The effect of compounds inhibiting -SH groups on ALA-S and ALA-D activities from several biological sources has been widely studied [1,2]. In previous papers the author has studied the -SH content of bacteria, yeast and liver, in conditions which would affect ALA-S and ALA-D activities [3–5]. Callus of soya offered suitable material for investigating the behaviour of both enzymic activities in tissues grown in the presence of PyP or Cy respectively. PyP act as a trap of aminothiols [6] and it protected enzymes depending on it as a cofactor [7] in experiments in which penicillamine (an aminothiol compound) had inhibited those enzymes. In this paper ALA-S and ALA-D activities of extracts from callus of soya grown both in dark or light are presented and the -SH group contents are also determined to establish if any correlation exists with either enzyme.

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## RESULTS

Spectrophotometric measurement of extracts obtained with fresh tissue grown under light revealed two peaks at 664 and 618 nm respectively, the first being displaced to a lower value with respect to the peak obtained with a pigment extract of fresh soya leaves (680 nm). A at 664 nm were 0·82, 0·10 and 0·09 for extracts described as *L*, *L*PyP (0·25 mM) and *L*Cy (0·2 mM) respectively (see Experimental). If these values are expressed as a function of protein content of 10 mg, the corresponding A of 0·79, 0·19 and 0·08 for the same three treatments would show a rough correlation with ALA-S activity shown in Table 1.

The presence of Cy in Miller's medium (Table 1a) caused only a small increase of ALA-S activity in the tissue extracts but addition of PyP to that medium showed a striking effect on ALA-S activity. Those effects were lower but still noticeable in tissues grown in darkness. The enzymic assays were also performed after adding 1 mM PyP *in vitro* to the assay mixture. While this addition stimulated by 70% the ALA-S of extracts coming

Table 1. ALA S Activities and protein content in the different conditions assayed

| (a) Conditions of growing (light) | ALA S activities: μmol of ALA/g of dry wt/30′ | nmol of ALA/mg of protein/30′ | Protein content in extracts mg/g of dry wt | (b) Conditions of growing (dark) | ALA S activities: μmol of ALA/g of dry wt/30′ | nmol of ALA/mg of protein | Protein content in extracts mg/g of dry wt |
|---|---|---|---|---|---|---|---|
| *L* | 0·93 | 26 | 35 | *D* | 1·29 | 38 | 34 |
| *L* PyP (0·25 mM) | 1·79 | 78 | 23 | *D* PyP (0·25 mM) | 0·95 | 43 | 22 |
| *L* PyP (0·5 mM) | 2·63 | 202 | 13 | *D* PyP (0·5 mM) | 1·85 | 154 | 12 |
| *L* Cy (0·2 mM) | 1·49 | 36 | 41 | *D* Cy (0·2 mM) | 1·53 | 42 | 36 |
| *L* Cy (10 mM) | 1·20 | 26 | 47 | | | | |

The values presented in this table correspond to extracts prepared with lyophilized tissue grown in Miller's medium under two different conditions: *L* (light) and *D* (dark). *L* PyP (0·25 mM) and *L* PyP (0·5 mM) correspond to results obtained when Miller's medium was added with PyP at the indicated molarities. *L* Cy (0·2 mM) and *L* Cy (10 mM) represent values obtained when Cy was included in the growing medium at the two different concentrations. Analogous notation is used for dark conditions (*D* PyP (0·25 mM), *D* PyP (0·5 mM) and *D* Cy (0·2 mM) respectively.

from cultures grown under light in Miller's medium with no additions, it had no effect if tissue was grown in darkness and even inhibited those grown in the presence of PyP.

In Table 2 the -SH group content both in whole extracts and in precipitated protein coming from different extracts (see Experimental) are presented. PyP stimulated the -SH content in all conditions assayed, a result completely opposed to what was predictable according to the probable formation of thiazolidine compounds; otherwise it seems that Cy had no effect on cultures grown in darkness when values are expressed as a function of the protein content.

The -SH content of extracts prepared in 0·1 M Pi buffer pH 8 without urea was 0·17 and 0·0 nmol/mg of dry wt for *L* and *L*PyP (0·5 mM) conditions respectively. This was the only fact that favoured the hypothesis that PyP would complex aminothiols when measurements of -SH content were performed after maintaining the extracts 30 min at 37°, the lowest value corresponded to *L*PyP (0·5 mM) condition (0·3 nmol/mg of dry wt). It is necessary to point out that the highest activity of ALA-S was obtained in the last condition.

The presence of PyP in Miller's medium is related to a marked decrease of ALA-D activity.

Table 2. Sulphydryl groups in the different treatments assayed

| Growth conditions (light) | Whole -SH in extracts (μmol/g of dry wt) 24 hr | 48 hr | -SH in proteins (nmol/mg of protein) | Growth conditions (dark) | Whole -SH in extracts (μmol/g of dry wt) 24 hr | 48 hr | -SH in proteins (nmol/mg of protein) |
|---|---|---|---|---|---|---|---|
| *L* | 1·13 | 2·90 | 68 | *D* | 0·79 | | 45 |
| *L* PyP 0·25 mM | 0·59 | 3·00 | 119 | *D* PyP 0·25 mM | 1·96 | 2·17 | 61 |
| *L* PyP 0·5 mM | 0·40 | 3·51 | 116 | *D* PyP 0·5 mM | 0·88 | 1·37 | 78 |
| *L* Cy 0·2 mM | 1·45 | 1·50 | 90 | *D* Cy 0·2 mM | 1·82 | 2·12 | 42 |
| *L* Cy 10 mM | 1·73 | 1·80 | 98 | | | | |

For explanation see Table 1.

Table 3. ALA-D Activities in tissues grown under light

| Conditions of growing | nmol of PBG/g dry wt/hr | nmol of PBG/mg protein/hr |
|---|---|---|
| *L* | 143 | 4·1 |
| *L* Pyp 0·25 mM | 9 | 0·4 |
| *L* Pyp 0·5 mM | 13 | 1·1 |
| *L* Cy 0·2 mM | 172 | 4·3 |
| *L* Cy 10 mM | 82 | 1·7 |

For explanation see Table 1.

Tissue extracts prepared with soya callus grown with Cy in the culture medium did not exhibit an improved sp. act., on the contrary, it seemed to diminish when the concentration reached 10 mM Cy. While preincubations with mercaptoethanol enhanced ALA-D activity when working with *L* extracts, it had little activity on those containing PyP and no action on enzymic extracts from tissue cultures containing Cy (Table 3).

## DISCUSSION

The equilibrium reaction $2R\text{-}SH \rightleftharpoons R\text{-}S\text{-}S\text{-}R$ seems to be important for many biological activities such as cell division [8] ripening of wheat [9] and for sulphydryl enzymes or for those which require intact R-S-S-R bridges for their activities. Straub [10] postulated the existence of a family of enzymes catalyzing disulfide exchange reactions with different substrate specificities, with such enzymes having an important functional role in maintaining the dynamic equilibrium of the protein disulfide bond. Others have reported influences of disulfide–sulphydryl enzymic interchange reactions between different proteins [11–13]. The tetrapyrrole pathway beginning with ALA-S and ALA-D, the second enzyme being sulphydryl dependent, offers an interesting case in which the equilibrium cited could operate as a regulating mechanism *per se* or through specific catalytic proteins.

In *R. spheroides* ALA-S is activated by a compound identified as cystine catalyzed by a protein fraction in the same extracts [14]. In *R. spheroides* which are facultative bacteria whose behaviour is highly dependent on the oxidation-reduction potential of the medium of growing, Lascelles [15] suggested that -SH groups influence the tetrapyrrole pathway. A striking increase in -SH content in extracts was found in *R. spheroides* subjected to vigorous aeration [16] and this stimulated the present study on the behaviour of ALA-S and ALA-D from soya callus on trapping those -SH groups belonging to aminothiol compounds, with PyP [17,18]. Although the assays performed in order to identify such complexes in the extracts, both spectrophotometric, chromatographic and electrophoretic methods failed, there was an important effect of PyP on the -SH group contents of the callus tissue cultivated in its presence.

The results obtained do not allow general conclusions to be reached about the proposed control. In spite of the drastic fall in protein content, PyP seems to protect ALA-S activity probably by diminishing the free -SH content of the tissue. This could be related to the low -SH content measured in tissue extracts [*L*PyP (0·5 mM)] when they are prepared without urea. Otherwise it was logical to find a low ALA-D activity in soya tissue coming from the same treatment because of the sulphydryl character of this enzyme. No explanation can be offered for the fact that mercaptoethanol stimulated activity of ALA-D from light-grown tissues in the absence of PyP more than in its presence although this could be a consequence of the higher -SH content in protein in the last condition.

## EXPERIMENTAL

Tissue cultures of soya callus were grown in Miller's medium in darkness for 25 days at 25° ± 1° (indicated as *D*) in conical flasks. Another series, after 10 days in darkness was illuminated with 3200 lx of white light and 840 lx of incandescent light during 15 days, developing a greenish colour (indicated as *L*.) At the same time, cultures containing PyP at 0·25 or 0·5 mM or Cy at 0·2 and 10 mM in Miller's medium were performed under the same conditions; the last one (Cy 10 mM) failed to grow in darkness. Tissue was harvested, frozen, lyophilized and kept cold for use in all the measurements. Fr. tissue was only used for estimation of chlorophyll and pigment extracted according to ref. [19]. *A* values were obtained by extracting 5 g of fr. tissue grown as described above. PyP in extracts of lyophilized tissue (60 mg of dry wt/ml of 1 M Pi buffer pH 8) was detected by PC running the samples during 24 hr (solvent system *n*-BuOH–HOAc–$H_2O$, 12:3:5) [20]. Fluorescence at 366 nm revealed the presence of the compound in the corresponding extracts. The same method was used during 8 hr for determining presence of Cy in deproteinized extracts of callus tissues obtained from cultures containing Cy. Standards were run in parallel and detecting -SH groups with 5% ammoniacal $AgNO_3$. Enzymic extracts were prepared in the cold by grinding lyophilized tissue with 0·1 M Pi buffer pH 8 for assaying

ALA-S activity and for measuring -SH contents; 0·1 M Tris–HCl buffer pH 8·2 was used for determining ALA-D activity. After 15 min homogenate was centrifuged at 20000 *g* for 30 min and using a precooled pipette the supernatant was removed. For enzymic activities extract of dry tissue was prepared so that the protein content reached 0·7–0·8 mg in 0·25 ml and *ca* 1 mg in 0·5 ml both for ALA-S and ALA-D respectively. Incubation mixture for assaying ALA-S activity contained: 20 mM Pi buffer pH 8, 40 mM glycocoll, 2 mM $MgCl_2$, 0·2 mM ATP, 1·6 mM EDTA, 1 mM of succinyl CoA prepared according to Ref. [21], tissue extract 0·25 ml, final vol 0·5 ml. Incubation was carried out for 30 min at 34° and the reaction was stopped by the addition of 30% TCA. Enzymic activity is expressed both as μmol of ALA/g of dry wt or nmol of ALA/mg of protein formed in 30 min. The *in vivo* effect of 0·1 mM PyP was studied by including it in the incubation mixture. Blanks containing all components with the exception of succinyl-CoA were run in each expt. ALA formed was reacted with ethyl acetoacetate at pH 6·8 and resulting pyrroles were detected by reaction with modified Ehrlich reagent by the method of ref. [22]; standards of ALA were run in each colorimetric measurement. ALA-D assay was performed under vacuum in Thunberg tubes and the incubation mixture contained: 50 mM Tris–HCl buffer pH 8·2, 5 mM $MgCl_2$, enzyme extract 0·5 ml and 1 mM ALA soln which was in the side arm. The final vol was 1 ml. Preincubation with or without 5 mM of mercaptoethanol lasted 15 min. After mixing with the substrate the mixture was maintained for 1 hr at 37°. After adding 10 μl of half satd $CuSO_4$ soln the reaction was stopped by addition of 30% TCA. Porphobilinogen was determined by the method of Ref. [23].

The evaluation of the -SH groups (including those of protein and metabolites) in tissue extracts containing 8 M urea revealed different kinetics for the breakage of hydrogen bonds both for tissues grown in dark or light. In general, the first gave maximal values of -SH after 24 hr at 10° while the second required 48 hr of urea treatment. The assay was performed as follows: tissue extracts (0·1 M Pi buffer pH 8 containing 8 M urea and 20 mg of dry tissue/ml) was maintained at *ca* 10° under vacuum in Thunberg tubes for 24 or 48 hr protecting them from the light. Then one half of the vol was used as a blank and the other half was treated with 0·1 ml of 5,5′-dithio-bis (2 vitro benzoic acid) (DTNB) according to Ref. [24]. A was measured immediately at 412 nm. For measuring -SH content in proteins, 0·5 ml of tissue extract (*ca* 1 mg of protein) was pptd at 0° with 30% TCA. After 24 hr it was centrifuged, protein was washed with 5% TCA and then dissolved in 3 ml of 0·1 M Pi buffer pH 8 containing 8 M urea and 0·1 ml DTNB. *A* was measured immediately using as a blank the soln buffer–urea–DTNB (*E* at 412 nm: 13600). Protein in tissue extracts was pptd and washed with TCA and a micro-Kjeldahl method used for evaluation.

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