INTRODUCTION

Chlorophylls are the end products of a complex multienzymic biosynthetic pathway, which in some organisms can also produce hemes, bile pigments and corrinoids (Granick, 1967).

The biosynthesis of the magnesium, iron and cobalt tetrapyrroles, is regulated through several control mechanisms which are not yet known so well (Lascelles, 1964). These controls are very efficient, since under normal conditions only traces of precursors and porphyrins are found, and it is accepted that their abnormal accumulation may be due to the disorder of such control mechanisms.

In photosynthetic organisms, the formation of hemes and chlorophylls follow a common path which diverges at the stage of protoporphyrin into the iron and magnesium branches.

A number of workers have contributed to our knowledge on pyrrole, porphyrin and phorbin chemistry and biochemistry, and many papers on chlorophyll biosynthesis in lower and higher plants have been published (for reviews see Lascelles, 1964; Bogorad, 1965, 1966; Granick, 1967; Jones, 1968; Marks, 1969; Kirk, 1971; Rebeiz & Castelfranco, 1973); however, our understanding of the step-by-step enzymology of chlorophyll biosynthesis is still primitive.
Although the conversion of delta aminolevulic acid (ALA) to Protoporphyrin 9, has been investigated in considerable detail in cell-free preparations from higher plants, both the formation of ALA and the steps involved in the conversion of Protoporphyrin 9 to chlorophyll are still greatly unknown.

Progress in understanding the metabolic control of chlorophyll synthesis, has been held back by the difficulty of isolating cell free systems able to catalyze some of the reactions involved.

Synthesis of tetrapyrroles by different cells is significantly influenced by the environment, particularly light intensity and oxygen pressure.

It has been proposed, that light regulates chlorophyll biosynthesis by mediating the formation of the enzymes of ALA synthesis (Gassman & Bogorad, 1967a, b). The light dependence of the cultures, a known highly dividing system, which indicate that such inability could be due to the absence or low activity of some of the enzymes involved in the earlier stages of chlorophyll synthesis.

For some time we have been interested in the study of tetrapyrrole biosynthesis in soybean callus tissue cultures, a known highly dividing system, which either dark- or light-grown, fails to synthesize chlorophyll in amounts equivalent to that found in mature leaves (Tigier et al., 1968, 1970; Wider de Xifra & Tigier, 1970, 1971; Llambias & Batlle, 1970a, b, c, 1971; Stella et al., 1971; Wider de Xifra et al., 1971; Wider de Xifra et al., 1972; Wider de Xifra & Batlle, 1973, 1974). Previous results would indicate that such inability could be due to the absence or low activity of some of the enzymes involved in the earlier stages of chlorophyll synthesis.

To obtain more information both on the regulation and the biosynthesis of porphyrins in these tissues we have studied the influence of growing the callus under different light conditions, and also the effect of the addition or omission of various compounds to the culture media, to see whether they produce any significant change in enzymic activities, porphyrin and chlorophyll synthesis and growth, and whether there is any correlation among the changes observed.

All other reagents were of AR grade obtained from several commercial sources. All solutions were made up in ion-free three times glass distilled water. Source material of enzyme: undifferentiated callus cultures from soybean seeds were obtained and grown according to Miller (1963); growth medium and culture conditions have already been reported (Tigier et al., 1970) and any additional component is indicated in the text or in the tables.

"Dark-grown callus", refers to callus cultures which were grown in the dark for the periods of time and conditions indicated.

"Light-grown callus", refers to callus cultures that were subcultured from dark grown callus and then grown under 100 ft-c of white light, for the periods of time and conditions indicated. Illumination was supplied by 60 W bulbs at an appropriate distance from the flasks.

Growth was measured by weighing the total amount of wound fresh tissue. Protein concentrations were determined by the method of Lowry et al. (1951), and chlorophyll was determined spectrophotometrically in 80% (v/v) acetone (Arnon, 1949).

Extraction and determination of porphyrins from callus cultures: the wound callus cells were harvested and the fluorescent pink material was extracted from them with either ethyl acetate-acetic acid (3:1, v/v) or acetic acid-HCl (9:1, v/v), total porphyrins were estimated spectrophotometrically in the fluid supernatant and then processed by a method similar to that described by Falk (1964); in this way, uroporphyrins and protoporphyrins fractions were obtained; all three fractions in HCl solution were measured at the Soret maxima using the correction formula and extinction coefficients of Rimington (1960). Porphyrins in acid solutions were esterified by the procedure described by Batlle & Grinstein (1964). In some experiments, porphyrins were further characterized by paper or thin layer chromatography (Eriksen, 1958; Jensen, 1963) and after esterification by the dioxan method (Cornford & Benson, 1963; Batlle & Benson, 1966).

**Assay of enzymic activities**

Succinyl CoA Synthetase (Suc-CoA-S) was determined in homogenates of wound callus in 0.1 M glycine-NaOH buffer, pH 9.0 and assayed as described by Wider de Xifra & Tigier (1971).

Delta Aminolevulinate Synthetase (ALA-S) was assayed in crude extracts according to Wider de Xifra et al. (1971).

Delta Aminolevulinate Dehydratase (ALA-D) was assayed in crude extracts as described by Tigier et al. (1970).

Porphobilinogenase (PBGase) the trivial name PBGase, suggested by Lockwood & Rimington (1957) was used to designate the two enzyme system of Porphobilinogen Deaminase-Uroporphyrinogen III cosynthetase. PBGase was estimated in the 24,000 g supernatants according to Llambias & Batlle (1971), for the activity in the crude homogenates was three times lower.

Decarboxylase (Dcase) was estimated on the basis of the sum of porphyrins with 7,6,5 and 4 carboxyls formed, when 8 ml of 24,000 g supernatants obtained from crude homogenates as described by Llambias & Batlle (1971), were incubated in 0.05 M phosphate buffer, pH 7.2 with 250 μg of PBG, at 38°C, anaerobically, in the dark, with

**MATERIALS AND METHODS**

Porphobilinogen (PBG), was obtained enzymatically (Sancovich et al., 1970). CoA, ATP, ALA, Sucinate, Puromycin, Mitomycin and buffers were purchased from Sigma Chemical Co. Gibberelic acid and carbonyl cyanide m-chlorophenyl hydrizine (CCCP uncoupler), were a generous gift from Dr. M. Grinstein.
shaking, for 6 hr. After incubation, the system was treated as with the PBGase.

Coproporphyrinogenase (CPGase) was tested by a slightly modified version of the method of Batlle et al. (1965). Incubations were carried out aerobically in 5 ml conical flasks for 2 and 4 hr at 38°C with shaking. The standard system used was: 1 ml or 1.5 ml homogenate or 24,000 g supernatant obtained as described by Llambias & Batlle (1971), in 0.05 M Tris buffer pH 7.4, to which it was added, under red light, 0.5 ml of freshly prepared Coproporphyrinogen III (approx. 0.1 mM) in a final volume of 2 ml. After incubation the system was tested as described by Batlle et al. (1965).

Enzyme units: 1 unit of enzyme activity is defined as the amount of enzyme which catalyses the formation of 1 nmole of product in 60 min under the standard incubation conditions. Enzymic activities are expressed as units per mg of protein. In some cases, the total amount of tissue harvested, was not sufficient to allow us to carry out all the determinations, this is shown by a hyphen (-) in Tables; while N.D. means “not detectable”, i.e. the levels were too low to be measurable with the methods employed.

Spectrophotometric measurements were carried out in a Beckman DU or DB spectrophotometer and a Spectronic 20 Bausch & Lomb.

All other materials and methods not specified here, are described in Batlle et al. (1965); Tigier et al. (1970); Llambias & Batlle (1971) and Wider de Xifra et al. (1971).

RESULTS

It has been found that when dark-grown callus was exposed to light, PBGase and ALA-S activities increased being paralleled by an increase in chlorophyll content (Llambias & Batlle, 1971; Wider de Xifra et al., 1971). Preliminary determinations of other enzymic activities had shown that both ALA-D and Suc. CoA-S were almost the same in callus light- or dark-grown, however, it is a fact that initial activities varied with different batches of callus culture and with the physiological age of them; therefore, the effect of light and simultaneously the addition of various compounds to the culture media, on porphyrin and chlorophyll synthesis, growth and different enzymic activities was re-examined.

It must be noted that within each set of experiments, the data are directly comparable, whereas some caution must be taken when comparing data between experiments.

Effect of light, ALA and iron

Soybean callus precultured in darkness were subcultured to fresh normal medium and modified media, containing either 0.1 or 0.3 mM ALA, iron deficient media and iron deficient media supplemented with 0.1 mM ALA and then grown in the light. Also medium containing 0.1 mM PBG was tested but in such conditions the cells did not grow and died in 2–3 days. Results are shown in Tables 1 and 2.

Although determinations were planned to be performed at day 9th, they had to be carried out at day 5th, for it was observed that in iron deficiency and with 0.3 mM ALA supplemented media growth was prevented; within a few hours after subculturing, the callus showed the typical red fluorescence of porphyrins and soon acquired a greenish-brown or brownish colour. These changes were consistently observed in a number of experiments; so, as porphyrin content increased, growth decreased.

It was also always found, that porphyrins, and probably porphyrinogens too, were bound to the tissue, for in none case porphyrins were detected in the culture media.

The content of porphyrins in light-brown colourless callus was very low, both in 5 or 14 days old callus; however iron deficiency and addition of

| Table 1. Effect of ALA and iron on growth, porphyrin, protein and chlorophyll synthesis in light-grown callus |
|---------------------------------------------------------------|
| Expt. | Additives | Total porphyrins (nmol/g wt) | Uro (%) | Copro (%) | Proto (%) | Growth | mg protein | µg chlorophyll | Fluoresc. of tissue |
|-------|------------|-------------------------------|---------|-----------|-----------|--------|------------|---------------|-------------------|
|       |            | 5 | 14 | 5 | 14 | 5 | 14 | 5 | 14 | 5 | 14 | 5 | 14 | 5 | 14 |
| 1     | None       | 0.068 | 0.050 | — | — | — | — | 4.76 | 100 | 8.5 | 100 | 8 | 6.8 | 12.6 | 13.0 | nf | nf |
| 2     | ALA 0.1 mM | 0.180 | 0.050 | 20 | 60 | — | — | 2.70 | 57 | 54 | 40 | 6.9 | 21.5 | 25 | + | + |
| 3     | ALA 0.3 mM | 0.350 | 0.090 | 30 | 25 | 54 | 55 | 16 | 20 | 1.45 | 30 | 40 | 47 | 8.6 | 10.2 | + | + |
| 4     | Without Fe | 0.252 | 0.070 | 20 | 50 | — | — | 2.45 | 51 | 27 | 31 | 5.9 | 21.5 | 25 | + | + |
| 5     | Without Fe | 0.256 | 0.080 | 32 | 35 | — | — | 1.48 | 30 | 2.2 | 25 | 7.5 | 15.6 | — | + | nf |

Soybean callus tissues were grown in the light under standard conditions, in the presence of ALA or in the absence of iron, and harvested at 5 and 14 days of growth. Concentrations of ALA shown are the final concentrations in the media. Determinations of porphyrins, chlorophyll and protein content and growth were carried out as described in methods. (—): not determined; ( ): indicates intensity of red fluorescence of callus tissues viewed under ultraviolet light; nf: non fluorescent.
Table 2. Effect of ALA and iron on enzymic activities in extracts from light-grown callus

| Expt. | Additives | Suc. CoA-S | ALA-S | ALA-D | PBGase | Dcase | CPGase |
|-------|-----------|------------|-------|-------|--------|-------|--------|
|       |           | 5 | 14 | 5 | 14 | 5 | 14 | 5 | 14 | 5 | 14 | 5 | 14 |
| 1    | None      | 402 | 314 | 8.58 | 8.10 | 3.7 | 6.9 | 0.130 | ND | ND | ND | ND |
| 2    | ALA 0.1 mM| 228 | 182 | 8.27 | 4.36 | 3.7 | 6.8 | 0.170 | 0.004 | ND | ND | ND |
| 3    | ALA 0.3 mM| 96  | 156 | 2.84 | 4.99 | 4.7 | 7.6 | 0.175 | ND | ND | ND | ND |
| 4    | Without Fe| 432 | 240 | 7.0  | 1.87 | 3.9 | 4.5 | 0.110 | ND | ND | ND | ND |
| 5    | Without Fe+ALA 0.1 mM| 104 | 212 | 1.55 | 5.55 | 3.7 | 4.3 | 0.099 | 0.007 | ND | ND | ND |

Extracts were prepared from 5 and 14 days old callus, grown in the light, under standard conditions, in the presence of ALA or in the absence of iron and enzymes were assayed as described in the text. All activities are expressed as nmoles of product formed per hour per milligram of protein. ND: undetectable; (-): not determined.

ALA, stimulated porphyrin accumulation when it was measured at day 5th, although porphyrin formation in iron deficiency was about 70% of that in media supplemented with 0.3 mM ALA.

Either destruction and/or consumption of porphyrins should then have occurred, for their levels decreased to nearly normal values after 14 days of growth.

An analysis of the porphyrins formed, showed coproporphyrin to be the main component, except in experiment 5; unexpectedly, in iron deficiency the proportion of protoporphyrin increased. In the uroporphyrin fraction, most of the uroporphyrin behaved as uroporphyrin III; phyriaporphyrin was also present in this fraction.

In experiment 3, was not found much difference in the composition of the porphyrins formed in 5 and 14 days old callus.

As stated above, growth was prevented when callus was grown in the light and in the presence of 0.3 mM ALA, 0.1 mM PBG or in iron deficiency; however, protein content, expressed on fresh weight basis, was not significantly modified, except under iron deficiency (experiment 4). More or less the same picture emerged for the chlorophyll content, also expressed on a fresh-weight basis, but again, unexpectedly, a measurable increase was found when iron was absent in the media.

As to the levels of the different enzymic activities, it must be remembered that they are all expressed in terms of their specific activities (units/mg protein).

Suc.CoA-S was slightly less active in 14 days old callus than in 5 days old, approximately the same values of the control were found in iron deficiency (experiment 4); the presence of ALA, either alone or added to iron deficient media, seemed to decrease Suc.CoA-S activity.

The presence of ALA or the absence of iron,

Table 3. Effect of ALA, PBG and iron on growth, porphyrin, protein and chlorophyll synthesis in dark-grown callus

| Expt. | Additives | Total porphyrins | Uro (%) | Copro (%) | Proto (%) | Growth | mg protein | Fluorescence |
|-------|-----------|------------------|--------|-----------|----------|--------|------------|-------------|
|       |           | nmoles/g wt |       |          |          | 9 | 14 | g fresh wt | g g fresh wt |
|       |           | 9 | 14 | 9 | 14 | 9 | 14 | 9 | 14 | 9 | 14 | 9 | 14 |
| 1    | None      | 0.070 | 0.065 | —      | —      | —      | 8 | 0 | 100 | 100 | 8.7 | 6.5 | 6.7 | 7 | nf | nf |
| 2    | ALA 0.1 mM| 0.115 | 0.070 | 22     | 60     | 18     | 8 | 1 | 100 | 95 | 9.5 | 7.7 | 5.1 | 6 | + | nf |
| 3    | ALA 0.3 mM| 0.500 | 0.127 | 20     | 70     | 10     | 5 | 1 | 63 | 90 | 7.4 | 6.0 | 7.4 | 5.5 | + + + + |
| 4    | Without Fe| 0.538 | 0.155 | 27     | 46     | 27     | 4 | 4 | 55 | 46 | 8.6 | 8.6 | 10.8 | 7 | + + + + |
| 5    | Without Fe+ALA 0.1 mM| 0.500 | 0.220 | 24     | 13     | 43     | 33 | 43 | 5.0 | 63 | 3.9 | 35 | 7.9 | 6.6 | 6.1 | 8.4 | + + + + + + + + |
| 6    | PBG 0.1 mM| 0.280 | 0.273 | 88     | 80     | 14     | 10 | 0 | 7.2 | 90 | 3.0 | 33 | 8.2 | 6.5 | 6.1 | 6 | + + + + + + + + |

Soybean callus tissues were grown in the dark under standard conditions, in the presence of ALA, PBG or in the absence of iron, and harvested at 9 and 14 days of growth. Concentration of ALA and PBG shown are the final concentrations in the media. Determinations of porphyrins, chlorophyll and protein content and growth were carried out as described in methods; (—): not determined; (+): indicates intensity of red fluorescence of callus tissues viewed under ultraviolet light; nf: non fluorescent.
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Table 4. Effect of ALA, PBG and iron on enzyme activities in extracts from dark-grown callus

| Expt. | Additives | Suc. CoA-S | ALA-S | ALA-D | PBGase | Dcase | CPGase |
|-------|-----------|------------|-------|-------|--------|-------|--------|
|       |           | 9 14       | 9 14  | 9 14  | 9 14   | 9 14  | 9 14   |
| 1     | None      | 268 260    | 8.2 4.3 | 3.5 4.7 | 0.010 0.042 | 0.004 0.007 | ND 0.0005 |
| 2     | ALA 0.1 mM | 244 240    | 5.6 4.3 | 6.7 7.3 | 0.008 0.042 | 0.001 ND | ND |
| 3     | ALA 0.3 mM | 214 240    | 5.0 4.5 | 7.4 10  | 0.010 0.080 | ND ND | ND |
| 4     | Without Fe | 340 288    | 6.0 3.4 | 1.3 4.0 | 0.030 0.040 | 0.010 ND | ND |
| 5     | Without Fe+ALA 0.1 mM | 236 194    | 1.3 4.4 | 2.0 2.8 | 0.050 0.081 | ND ND | ND 0.0003 |
| 6     | PBG 0.1 mM | 240 —      | 1.8 5.4 | 3.3 4.3 | 0.008 0.040 | — — | — — |

Extracts were prepared from 9 and 14 days old callus, grown in the light, under standard conditions, in the presence of ALA and PBG or in the absence of iron, and enzymes were assayed as described in the text. All activities are expressed as nmoles of product formed per hour per milligram of protein. ND: undetectable; (—): not determined.

significantly inhibited ALA-S, while ALA-D was not greatly affected by any of these conditions in light-grown callus, except for 14 days old tissues in iron deficiency.

Only ALA produced some increase in PBGase activity which was not much affected by iron deficiency.

Dcase could have only been detected in experiment 2 and 5. Finally, CPGase was detected in none experiment.

Effect of ALA, iron and PBG in dark-grown callus

Although it has been found that light seems to increase some enzymic activities, we have shown that when we studied the effect of the addition of ALA or the omission of iron, under such light conditions, growth was prevented; therefore, the same set of experiments was carried out with dark-grown callus; results obtained are shown in Tables 3 and 4.

Growth was again prevented in the presence of 0.3 mM ALA and iron deficiency but not to the same extent as with light-grown callus. When PBG was supplemented to the medium, 9 days old callus grew nearly as well as the controls, but then the tissue became rapidly brownish, dry and died; the same happened in experiment 5 (iron omitted + 0.1 mM ALA).

As expected, the content of porphyrins in the controls was low, slightly increased in 0.1 mM ALA and significantly greater in the presence of 0.3 mM ALA, iron deficiency and 0.1 mM PBG; even at day 9th, values were higher than those obtained with light-grown callus 5 days old; again, as callus grows older, the corresponding levels of porphyrins decrease. More or less the same composition of porphyrins as that shown in Table 1 was obtained, for dark-grown callus with ALA or without iron; however, with PBG, nearly 90% of the porphyrin detected in the callus was uroporphyrin and it was the only case where some fluorescence was also observed in the culture media although attempts to extract the porphyrins from the media were unsuccessful.

In general, protein and chlorophyll content was not greatly modified, except in iron deficiency, which was again higher than that in the controls.

Suc-CoA-S did not vary much in 9 or 14 days old callus; only was slightly decreased in older callus as already observed in Table 2; in the presence of 0.3 mM ALA, iron deficiency plus 0.1 mM ALA and with 0.1 mM PBG, it was somewhat decreased and increased again in iron deficiency (experiment 4).

In all cases, ALA-S in 9 days old callus was inhibited, as compared to the controls.

In dark-grown callus, ALA-D was modified to a greater extent than in the previous experiments in the light, it was now found that ALA seemed to stimulate its activity and, on the contrary, in iron deficiency ALA-D was diminished, while PBG did not make much difference.

Only 0.3 mM ALA produced some measurable increase of PBGase activity in older callus; in the dark, iron deficiency stimulated its activity in 9 days old callus and PBG did not produce much change.

Dcase was detected in the controls and in experiment 4 (iron deficiency) also CPGase was detected in 14 days old controls, but as for Dcase, their activities were very low.

Effect of subculturing dark-growing soybean cells, previously grown in modified media, to the same media and normal media

To see whether or not the observed changes shown in Tables 3 and 4, reflect a permanent modification in the properties analysed, callus which had been first grown in the dark and in the presence
Table 5. Effect of transferring callus previously grown in the dark in ALA or PBG supplemented media to standard or modified media

| Expt. | Additives | Total chlorophyll | Uroplastins | Coprophyrins | Protochlorophyll | Growth | protein | Chlorophyll | Secondary metabolites |
|-------|-----------|------------------|-------------|--------------|-----------------|--------|---------|-------------|----------------------|
|       |           | nmoles fr wt %    | %           | %            | %               | %     | g fr wt | %           | nmoles fr wt %       |
| 1     | None      | 0.035            | 100         | 74           | 42              | 34     | 67      | 5.3         | 268                  |
| 2a    | from ALA 0.1 mM to ALA 0.1 mM | 0.045 | 100 | 74 | 67 | 42 | 34 | 67 | 5.3 | 268 |
| 2b    | from ALA 0.1 mM to standard | 0.033 | 100 | 74 | 67 | 42 | 34 | 67 | 5.3 | 268 |
| 3a    | from ALA 0.3 mM to ALA 0.3 mM | 0.034 | 100 | 74 | 67 | 42 | 34 | 67 | 5.3 | 268 |
| 3b    | from ALA 0.3 mM to standard | 0.032 | 100 | 74 | 67 | 42 | 34 | 67 | 5.3 | 268 |
| 4a    | from iron deficient to iron deficient | 0.056 | 100 | 74 | 67 | 42 | 34 | 67 | 5.3 | 268 |
| 4b    | from iron deficient to standard | 0.055 | 100 | 74 | 67 | 42 | 34 | 67 | 5.3 | 268 |
| 5a    | from iron deficient + ALA 0.1 mM to iron deficient + ALA 0.1 mM | 0.054 | 100 | 74 | 67 | 42 | 34 | 67 | 5.3 | 268 |
| 5b    | from iron deficient + ALA 0.1 mM to standard | 0.053 | 100 | 74 | 67 | 42 | 34 | 67 | 5.3 | 268 |
| 6b    | from PBG 0.1 mM to standard | 0.052 | 100 | 74 | 67 | 42 | 34 | 67 | 5.3 | 268 |

Soybean callus tissues were previously grown in the dark for 9th of growth in the dark, concentrations of porphyrins, chlorophyll, and protein were carried out as described in Methods. Extracts were prepared from callus grown under iron deficient conditions, and enzyme activities were assayed as described in the text. All activities are expressed as nmoles of product formed per hour per milligram of protein. ND: undetectable; (-): not determined.
### Table 6. Effect of puromycin and mitomycin added along with ALA

| Expt. | Additives       | Total porphyrins | Uro | Copro | Proto | Growth | mg protein | µg chlorophyll | Enzymic specific activities |
|-------|-----------------|------------------|-----|-------|-------|--------|------------|----------------|-----------------|---------------------------|
|       |                 | nmoles/g fr wt | %   | %     | %     | g fr wt | g fr wt | Suc. CoA-S | ALA-S | ALA-S | PBGase | Dcase |
| 1     | None            | 0.070            | —   | —     | —     | 4.8    | 100      | 8.7          | 6.8             | 240 | 8.7 | 5.5 | 0.015 | 0.007 |
| 2     | ALA 0.1 mM      | 0.106            | 20  | 60    | 20    | 4.4    | 96       | 7.2          | 9.0             | 194 | 3.7 | 5.8 | 0.022 | ND   |
| 3     | ALA 0.1 mM + puromycin 0.1 mg/ml | 0.175 | 33  | 33    | 33    | 2.8    | 60       | 7.2          | 8.2             | 148 | 3.8 | 6.6 | 0.069 | ND   |
| 4     | ALA 0.1 mM + mitomycin 0.3 µg/ml | 0.108 | 20  | 60    | 20    | 3.7    | 80       | 7.4          | 4.2             | 280 | 2.5 | 5.8 | 0.011 | ND   |
| 5     | ALA 0.3 mM      | 0.165            | 20  | 60    | 20    | 4.8    | 100      | 7.2          | 6.9             | 136 | 2.7 | 6.6 | 0.032 | 0.003 |
| 6     | ALA 0.3 mM + puromycin 0.1 mg/ml | 0.262 | 30  | 40    | 30    | 3.3    | 70       | 6.0          | 7.6             | 160 | 2.9 | 6.6 | 0.041 | 0.003 |
| 7     | ALA 0.3 mM + mitomycin 0.3 µg/ml | 0.114 | —   | —     | —     | 5.1    | 104      | 7.8          | 4.4             | 242 | 3.1 | 7.7 | 0.019 | ND   |

Soybean callus tissues were grown in the dark, under standard conditions, in the presence of ALA alone or either puromycin or mitomycin added along with, and harvested at day 8th of growth. Concentrations of ALA shown are the final concentrations in the media. Determinations of porphyrins, chlorophyll and protein content, growth and enzymes activities were carried out as described in the text. All activities are expressed as nmoles of product formed per hour per milligram of protein. ND: undetectable; (—): not determined.
of ALA, PBG or iron deficiency, were subcultured to the same medium and to normal medium. Results obtained are shown in Table 5.

Again, when porphyrin content was higher than that in controls, growth was inhibited, and in this case, except in experiments 4b and 5a, a parallel decrease in protein content was also observed.

As it has already been found, in the presence of ALA or iron deficiency, porphyrin accumulated and in general, except in experiment 3b and 6, porphyrin content was in the order of that in the controls, when callus was transferred to normal medium. The same composition of porphyrins as that previously shown, was found.

As can be seen, Suc-CoA-S was unchanged.

ALA-D was inhibited, even after being transferred to normal medium.

ALA was again increased in the presence of ALA; it was inhibited when grown in iron deficiency, and some recovery of this inhibition occurred, when transferred to normal medium.

Callus first grown in iron deficiency plus ALA and then transferred to normal media, recovered completely.

PBGase was slightly increased when grown in ALA supplemented media, still after being transferred to normal media, and also when previously grown in iron deficient media and then transferred to normal media.

Both Dcase and CPGase could not be detected at all.

Effect of ALA, puromycin and mitomycin

To see whether the formation of porphyrins, chlorophyll or enzymic activities can be modified by inhibitors of protein and nucleic acid synthesis and, in the case that changes were produced, if they could be overcome by ALA, the set of experiments shown in Table 2 was performed.

Growth was only 30-40% inhibited in the presence of puromycin, however protein and chlorophyll content was not parallelly modified by this protein inhibitor. Porphyrins were accumulated in the presence of ALA, puromycin further increased this accumulation, while mitomycin seemed not to significantly modify the effect of ALA.

Porphyrin content in the control and in the presence of puromycin or mitomycin was low.

As shown before, in the presence of ALA, Coproporphyrin III was the main porphyrin accumulated, the presence of puromycin seemed to modify the proportions of uro, copro and protoporphyrin.

Suc-CoA-S activity was about the same in the presence of any of the inhibitors added alone, 0.3 mM ALA or ALA plus mitomycin, slightly decreased in the presence of 0.1 mM ALA and ALA plus puromycin and slightly increased with ALA plus mitomycin. Instead, ALA-S activity diminished in all cases.

As to the PBGase, low initial activities were measured in controls in the presence of 0.1 mM ALA or 0.3 mM ALA, with or without mitomycin; only in the presence of 0.1 mM ALA or 0.3 mM ALA plus puromycin, a 3-4 fold increase was observed.

Dcase was only detected in controls and with ALA plus puromycin.

CPGase was detected in none of these experiments.

It must be noted that in the presence of ALA plus puromycin, the callus was deeply greenish.

Effect of dark and light, ATP, gibberellic acid, succinate and CCCP

Callus was grown in the dark and in the light, and

| Light conditions | Additives            | Total porphyrins (nmol/g fresh wt) | Growth | Chlorophyll (mg/g fresh wt) |
|-----------------|----------------------|------------------------------------|--------|---------------------------|
|                 |                      | 13  | 20  | 13  | 20  |
| Light           | None                 | 0.065 | 0.015 | 10  | 100  | 9   | 100  | 8   | 10   |
| Dark            | None                 | 0.030 | 0.030 | 9   | 100  | 9.5 | 106  | 6.8 | 3.5  |
| Light           | ATP 2 mM             | 0.040 | 0.085 | 7.1 | 85   | 8.5 | 95   | 6.6 | 7.3  |
| Dark            | ATP 2 mM             | 0.050 | 0.050 | 9.4 | 100  | 9.5 | 106  | 7.4 | 4.1  |
| Light           | Gibberellic acid 3-3 µg/ml | 0.040 | 0.110 | 9.8 | 100  | 5.1 | 55   | 10  | 10   |
| Dark            | Gibberellic acid 3-3 µg/ml | 0.050 | 0.028 | 7.5 | 82   | 4.5 | 50   | 6.6 | 6.7  |
| Dark            | Succinate 0-5 mM     | 0.049 | —     | 15  | 150  | 15.3 | 165  | 5.4 | 8.0  |
| Dark            | CCCP 0-01 mg/ml      | 0.196 | —     | 7   | 70   | —   | —    | 5.0 | 5.0  |

Soybean callus tissues were grown in the light or in the dark as indicated, under standard conditions, in the presence of ATP, gibberellic acid, succinate and CCCP, and harvested at days 13th and 20th of growth. Concentrations of ATP and succinate shown are the final concentrations in the media.

Determinations of porphyrins, chlorophyll and protein content and growth were carried out as described in Methods. (—): not determined.
Porphyric biosynthesis in the soybean callus tissue system

Table 8. Effect of ATP, gibberelic acid, succinate and CCCP on enzymic activities

| Light conditions | Additives | Enzymic specific activities |
|------------------|-----------|-----------------------------|
|                  |           | Suc-CoA-S | ALA-S | ALA-D | PBGase | Ccase | CPGase |
| Light None       |           | 316       | 8:0   | 6:9   | 0:096  | 0:002 | 0:0005 |
| Dark None        |           | 240       | 4:0   | 4:7   | 0:053  | ND    | ND     |
| Light ATP 2 mM   |           | 192       | 3:86  | 6:6   | 0:004  | ND    | ND     |
| Dark ATP 2 mM    |           | 114       | 2:06  | 4:3   | 0:004  | ND    | ND     |
| Light Gibberelic acid 3:3 µg/ml |   | 218       | 2:80  | 6:7   | 0:222  | 0:016 | 0:0008 |
| Dark Gibberelic acid 3:3 µg/ml |   | 204       | 2:50  | 5:2   | 0:202  | ND    | ND     |
| Dark Succinate 0:5 mM |   | 242       | 2:89  | 6:0   | 0:040  | ND    | ND     |
| Dark CCCP 0:01 mg/ml |       | 236       | 0:17  | 6:0   | 0:040  | ND    | ND     |

Extracts were prepared from 13 days old callus grown in the light or in the dark as indicated, under standard conditions, in the presence of ATP, gibberelic acid, succinate or CCCP and enzymes were assayed as described in the text. ALA-S was measured at day 11th of growth. All activities are expressed as nmoles of product formed per hour per milligram of protein. ND: undetectable. (—): not determined.

in the presence of ATP, gibberelic acid, Succinate and CCCP. Results are shown in Tables 7 and 8.

In 13 days old callus, growth was not greatly changed under these conditions; protein content was slightly lower in dark-grown cells, light–ATP and dark–gibberelic grown callus. In older callus (20 days), growth was inhibited in the presence of gibberelic acid.

Chlorophyll was always higher in light-grown cultures than in those grown in the dark.

Growth, protein and chlorophyll content was diminished in the presence of the CCCP uncoupler. In all cases, except with CCCP, porphyrin content was very low, it was found that the medium supplemented with CCCP accumulated great amounts of uroporphyrinogens (83%), some coproporphyrinogens (17%) and none protoporphyrin.

The activity of Suc-CoA-S in these experiments was about the same in light or dark grown controls and it was somewhat inhibited in the presence of ATP, and gibberelic acid.

As already reported, when dark-grown callus was illuminated, ALA-S increased; the presence of ATP, gibberelic acid, succinate and CCCP produced a significant decrease in activity.

ALA-D seemed also to be enhanced by light while ATP, gibberelic acid, succinate and CCCP did not make much difference.

Also PBGase was higher in light-grown callus, ATP greatly inhibited its activity; gibberelic acid produced a measurable increase and succinate or CCCP had no effect.

Dcase was only detected in light-grown controls and in light-gibberelic grown callus; correspondingly CPGase was also detected in the same experiment.

DISCUSSION

In chlorophyll synthesizing organisms, control of porphyrin synthesis must be correlated to control of chlorophyll synthesis. Colourless soybean callus can grow either in the dark or in the light forming low amounts of chlorophyll (Llambias & Batlle, 1971). It was previously supposed that this inability could be due to the low activity or to the absence of the enzymes involved in chlorophyll synthesis; another probable explanation is that the production of lipids, essential for the formation of membranes in the chloroplasts, in the soybean cells, would be very low. We have only examined the former possibility so far.

Stobart et al., (1967), have found that, when freshly inoculated colourless callus cultures of Kalanchoe crenata were transferred to a light cabinet and were subcultured at monthly intervals, they became green. Stobart et al. (1967) also suggested that in order to produce an autotrophic green callus it would be necessary to enhance chlorophyll production. Although the conditions of growth of soybean callus are different from those of Kalanchoe crenata callus, we tried to modify the composition of the growing medium, to get an increase in chlorophyll synthesis, with the hope of obtaining green soybean callus and knowing if the activity of the enzymes involved in the early stages of tetrapyrrole synthesis was correspondingly modified; however, it is important to state that, so far, all attempts to obtain green soybean callus, by growing several generations of callus in the light, were unsuccessful. Some evidence would indicate that these cells, are unable to develop mature chloroplasts, so, it is possible that the ability of the callus to produce chlorophyll is related to its ability to produce vital genetic or hormonal regulators of plastid growth, which seems to be unpaired or deleted out in these tissues. Further studies on the synthesis of lipids, plastids and chloroplasts development, and electron microscopy are necessary to support any alternative proposed.
Effect of light

When dark-grown callus was exposed to light, it was found that chlorophyll content increased, together with ALA-S, ALA-D and PBGase activities, although the amount of porphyrins formed was not modified.

Wider de Xifra & Tigier (1971), have shown that Suc-CoA-S is involved in porphyrin biosynthesis in soybean callus; Stobart & Pinfield (1970) and Steer & Gibbs (1969a) have reported that light induced increases of Suc-CoA-S after illumination of etiolated barley seedlings and bean leaves respectively. Although the values reported in the typical set of experiments of Table 8, show an increase of only about 15% and it has already been stated, that caution must be taken to compare results from different sets of experiments, data from Tables 2 and 4 appear to indicate that the activity of Suc-CoA-S was also higher in light-grown than in dark-grown callus. On the other hand, additional evidence would indicate that greater differences are obtained with callus between 7 and 10 days old than with older callus; nevertheless, the significance of the nature of the changes in Suc-CoA-S awaits further investigation.

An increase, in both activity of ALA-S and chlorophyll content was found when dark-grown callus was exposed to light, it was suggested (Wider de Xifra et al., 1971), that light could have induced the new synthesis of the enzyme.

Also ALA-D activity increased when callus was grown in the light, but not as much as ALA-S and PBGase did. A parallel increase in chlorophyll synthesis and ALA-D has been reported for bean leaves (Steer & Gibbs, 1969b), tissue cultures of Kalanchöe crenata (Stobart & Thomas, 1968), greening Euglena gracilis (Ebbot & Tait, 1969; Stella & Batlle, unpublished results) and tissue cultures of tobacco (Schneider, 1971).

PBGase activity was also enhanced when callus was grown in the light; it was found (Llambías & Batlle, 1971) that the enzyme activity, on a protein basis, increased in greening callus, probably owing to synthesis of a new protein rather than the activation of existing protein, and it has already been proposed that light induces the synthesis of enzymes necessary for chlorophyll production (Stobart & Thomas, 1968).

Both Dcase and CPGase activities were frequently undetectable, either in light- or dark-grown callus, therefore, no conclusions may be drawn about them.

It is also important to recall here, that it was consistently observed, that the activities of the enzymes varied with the age of the callus, this being most critical for ALA-S, which shows its maximum on the 11th day of growth (Wider de Xifra et al., 1971); nevertheless, the general picture so far obtained, would suggest a common control over porphyrin and chlorophyll production, as proposed by Steer & Gibbs (1969b).

Effect of ALA

As it has already been observed with cultures of Rhodopseudomonas spheroides (Lascelles, 1959, 1968), addition of ALA to the medium culture, was found to stimulate porphyrin accumulation, 0.3 mM ALA produced a 5–7 times increase; such accumulation was greater when callus were only 5–9 days old and grown in the dark.

In the presence of light, porphyrin content was lower and the amount of porphyrins diminished when callus was 14 days old. Under our experimental conditions, the ALA added to the medium, was rapidly utilized by the tissue, partly being converted to porphyrinogens and partly being metabolized through other unidentified ways. Some of the porphyrinogens were oxidized to porphyrins, as callus showed the typical red fluorescence under u.v. light, this oxidation was even more rapid when callus was grown in the light, the rest of the porphyrinogens were probably metabolized by the tissue.

Inhibition of growth by addition of ALA was observed, such effect occurred with 0.3 mM ALA in 5 or 9 days old callus, however if callus was 14 days old and grown in the dark, this action seemed to be overcome. When the callus was 5 or 14 days old and light-grown, the tissue was dry and partly dying. It must be emphasized that the greater the amount of porphyrins formed, the less the callus growth.

Inhibition of bacteriochlorophyll synthesis and growth by added ALA in Rhodopseudomonas spheroides has been observed by Lascelles (1966). Shemin et al. (1960) reported that ALA had a strong bacteriociidal action on Rhodopseudomonas spheroides, but it was unclear whether the observed inhibition of growth by ALA was due to a direct action of ALA or caused by some metabolic product or products. According to our findings, it is the accumulation of porphyrins, rapidly formed from ALA, which inhibits growth, consequently producing the death of the tissue, although it is not possible at present, to clearly distinguish between parallel effects and causative reactions.

In some porphyrias, failure of the control mechanisms, are responsible for the over-production or accumulation of porphyrins, which are thought to be one of the reasons of skin photosensitivity. Results here reported and those described by Tigier et al. (1970), show that there are parallels between the soybean callus and some porphyria, and that we could obtain interesting experimental models with the soybean cultures, to study and help to clarify problems related to this disease.

Although porphyrins were readily formed from ALA, chlorophyll content was not significantly modified, these results suggest that porphyrin synthesis could be dissociated from chlorophyll formation, perhaps these cultures contain low or no
precursors of the chlorophyll path, such as those of the methyl ester or phytol groups.

The supply of ALA has been proposed to be the most important factor in the control of chlorophyll synthesis in plant and bacteria (Gassman & Bogorad, 1967a; Lascelles, 1968). The rate of ALA formation would be influenced by the activity of ALA-S and by the availability of the substrates used by the enzyme. ALA-S should play a significant role in the regulation of tetrapyrroles synthesis in plants; however, ALA-S in extracts from higher plants had, so far, only been detected in soybean callus in our laboratory (Wider de Xifra et al., 1971) and more recently in cold stored potatoes (Ramaswamy & Nair, 1973). It was then interesting to measure the levels of activity of ALA-S as well as other activities in media supplemented with ALA.

From the results already discussed, it is evident that the activity of Suc-CoA-S in soybean callus, is clearly in excess and could not be limiting ALA production.

Gassman, Pluscsec and Bogorad (1968), have suggested another route for ALA synthesis, by transamination of γ, δ-dioxovaleric acid, ALA can be formed; however all attempts to detect ALA-transaminase in soybean callus have failed (Tigier, unpublished results). From the above considerations, it is clear that metabolic precursors for ALA synthesis would probably not be limiting. On the other hand, ALA-S seems to be as active as ALA-D, while PBGase was 150-300 times less active than the synthetase or the dehydratase.

Suc-CoA-S was slightly diminished when ALA was added to the media, this effect being more pronounced with light-grown callus. ALA-S was significantly inhibited in ALA supplemented media, differences were greater with 0.3 mM ALA and light-grown callus; it has been proposed that the porphyrins or related intermediates accumulated under these conditions could interfere with the activity of ALA-S (Wider de Xifra et al., 1971), simply operating by a negative feedback control as proposed by Lascelles (1964); however this only event cannot account for the strong inhibition of ALA-S by ALA, still observed, when callus previously grown with ALA, were transferred to normal media.

On the other hand, ALA produced an increase of both ALA-D and PBGase activities, it has been suggested (Llambias & Batlle, 1971) that ALA could have induced or activated ALA-D, which then would form greater amounts of PBG, this resulting, in turn, in an increased activity of PBGase. No PBG was detected in 5 or 9 days old callus, light- or dark-grown, but the possibility remains that it could have been rapidly formed and metabolized, concurrently with porphyrin formation, during the first hours after subculturing.

No conclusions can be drawn again, as to the Dcase and CPGase, which are in fact limiting, so it is difficult to explain how the porphyrins accumulated under these conditions contain mainly coproporphyrin, the Dcase being generally undetectable.

Effect of ALA, puromycin and mitomycin

It has been found that chlorophyll synthesis can be blocked by inhibitors of protein synthesis, but inhibition was overcome when ALA was simultaneously administered (Nadler & Granick, 1970). It was of interest to study the effect of puromycin, which inhibits chlorophyll development and mitomycin a known nucleic acid inhibitor, when added along with ALA to the media.

It was observed that ALA-puromycin treated callus was rather green, porphyrin content was correspondingly increased, but chlorophyll was not enhanced; the composition of the porphyrins formed was somewhat modified, and growth was 30-40% inhibited. Suc-CoA-S, ALA-S, already inhibited in the presence of ALA were not altered by the simultaneous addition of puromycin and the same happened with the other enzymes; in conclusion, it seems that puromycin did not much change the effects produced by ALA, which were then not dependent on the synthesis of proteins.

Neither mitomycin produced great differences on the action of ALA, only slightly less chlorophyll was formed. From these data, it appears that the inhibitor of RNA synthesis did not appreciably affect synthesis of tetrapyrroles already functioning in the callus, but it is also possible that the concentration of inhibitor used was not enough to produce observable changes.

Effect of PBG

The monopyrrole is unstable and easily decomposes on standing, one of the products is uroporphyrin which can account for about 20% of the disappeared PBG; therefore, although we have added PBG to get a final concentration of 0.1 mM in the medium, the actual concentration cannot be known; furthermore, the quantity absorbed by the callus, if any, cannot be recorded either.

The callus very rapidly died when grown in the light, in media supplemented with PBG; on the contrary, if the callus was grown in the dark, the content of porphyrins was about the same and most of it was uroporphyrin, both in 9 or 14 days old callus; when the callus was 14 days old, it died.

Protein and chlorophyll content, Suc-CoA-S, ALA-D and PBGase activities were not modified as compared with the controls, only ALA-S was inhibited, and we assume that inhibition was simply due to the presence of porphyrins in the extract. We also assume that, as expected, the callus was not permeable to PBG, that added PBG was not converted into porphyrins by the enzyme system of the callus but the formation of uroporphyrins...
may have been due to non-enzymic decomposition of PBG, probably on the surface of the tissue.

Effect of iron

Iron deficiency is known to stimulate porphyrin excretion by growing cultures of *Rhodopseudomonas spheroides* and related organisms, under these conditions, formation of bacteriochlorophyll is limited (Lascelles, 1956, 1959, 1968). It was suggested that iron participates in the conversion of porphyrins or derivatives into chlorophyll and there is evidence that the metal is required for the conversion of coproporphyrinogen to protoporphyrin (Lascelles, 1956; Batlle et al., 1965). A similar situation is found in *C. diphteriae*, where porphyrin excretion is increased while intracellular haems are decreased by iron (Rawlinson & Hale, 1949).

One of the components of the soybean callus culture medium is iron-EDTA (Miller, 1963). Therefore, it was of interest to examine the effect of the omission of iron in the media.

As it was observed with ALA supplemented media, under iron deficiency, a rapid accumulation of porphyrins was found, being greater in 9 days old dark-grown callus; unexpectedly, the amount of chlorophyll also increased, this is very difficult to explain and we suspect that some other pigment might have been extracted along with chlorophyll.

When 0.1 mM ALA was simultaneously added to iron deficient media, porphyrins also accumulated but chlorophyll values were about the same as those in the controls.

As to the type of porphyrins formed, uroporphyrin and protoporphyrin fractions increased and so coproporphyrin decreased.

Growth was strongly inhibited under iron deficiency, as stated above, when we examined the effect of adding ALA to the media; qualitatively, the reverse association between growth and formation of porphyrins has been consistently found in a number of experiments, although the quantitative changes might vary from one set of experiments to the other.

Suc.CoA-S was not much affected by omitting iron, although it was inhibited when ALA was simultaneously added, the action being greater in light-grown callus. ALA-S activity was reduced in iron deficient media, porphyrins accumulated might have inhibited the synthetase as already discussed, however it has also been suggested that iron is involved in ALA-S synthesis (Stein 1970; Wider de Xifia et al., 1971).

ALA-D activity was also lower in callus grown without iron, although the results were more variable than those for the ALA-S and it can be seen that the differences were dependent on the light conditions and the age of cultures. It has already been found that soybean callus Dehydratase activity was dependent on the concentration of Zn in the culture media (Tigier et al., 1970) and there have been reports (Nandi & Waygood, 1967; Komai & Neilands, 1968) about the existence of a metal necessary for maximal ALA-D activity. According to these results, iron requirement on the activity of soybean callus dehydratase would also be envisaged.

Effect of ATP, gibberellic acid, succinate and CCCP

Gajdos et al., (1967) have shown that nucleoside triphosphates diminished porphyrin excretion by *Rhodopseudomonas spheroides*, they proposed that ATP could act through a mechanism which can control porphyrin excretion by modifying the activity of the enzymes involved in the porphyrin pathway. The addition of ATP to the media, did not significantly affect porphyrin, protein and chlorophyll synthesis, callus growth, or ALA-D activity; however, Suc.CoA-S, ALA-S and PBGase activities diminished; it has already been suggested (Llambias & Batlle, 1971) that in this case ATP could be functioning by repressing enzyme formation.

Several authors have found that plants treated with gibberellic acid become more or less chlorotic and chlorophyll decreases (Brian & Grove, 1957; Milet et al., 1962; Szalai, 1969). The addition of gibberellic acid to the soybean callus culture medium, did not change chlorophyll content, slightly diminished Suc.CoA-S and ALA-S, while ALA-D was not affected and PBGase was unexpectedly higher; we cannot offer a good explanation for the results observed.

Succinate was added to the medium to see if it had any effect on Suc.CoA-S and the other enzymes as well. As shown in Table 8, the only significant changes observed were an increase in growth and an inhibition of ALA-S and ALA-D.

Recently, a variety of uncouplers, including newly synthesized carbonyl cyanide m-chlorophenyl hydrazine (CCCP) have been developed. A strong inhibitory action of ring substituted phenylhidrazines of carbonyl cyanide, on the oxidative phosphorylation in mitochondria and on the light induced phosphorylation in chloroplasts has been described (Heytler & Prichard, 1962; Heytler, 1963; Kimimura et al., 1971). The effect of CCCP on the soybean callus was examined; as expected, accumulation of porphyrins occurred, but uroporphyrin was the main porphyrin component of the mixture extracted, correspondingly, growth was prevented, chlorophyll reduced and ALA-S greatly inhibited. PBGase activity was also diminished, so uroporphyrin accumulated could have been formed non enzymically from PBG.

A great number of compounds can induce experimental porphyria in animals, but such an effect had not been observed in plants tissues, however it has already been found that the addition of allil isopropyl acetamide (AIA) to the soybean callus cultures (Tigier et al., 1970), produced enhancement of ALA-D, and it was suggested that the drug could act through a mechanism
similar to that proposed for experimental porphyria. The action of CCCP has so far only been tested in rats (Batlle, unpublished results) and preliminary results would indicate that it can induce a form of porphyria of the hepatic type. We cannot know if the action of CCCP on the callus follows the same pattern as in animals, but it is very likely. It is perhaps necessary to emphasize again, that the soybean callus system, could probably be used in order to experimentally produce disorders in porphyrin metabolism, which can resemble different types of porphyria.

Although the experiments here reported do not give any idea of the mechanism by which the levels of the enzymes studied are controlled and modified, it is noteworthy that in some conditions, when chlorophyll content was increased a parallel increase in the activity of the enzymes involved in the early stages of the pathway was observed. On the other hand, it has been found that the inhibition of ALA-S was not always associated with the concomitant decrease in chlorophyll formation and although ALA-S plays an important role in this pathway, the other enzymes, specially PBGase, would also be responsible for the changes observed. However, the complex pattern of changes observed, in the levels of porphyrin and chlorophyll content, growth and enzymic activities, would indicate that the regulation mechanism of porphyrin synthesis in soybean callus is indeed manyfold, and additional experiments are necessary to get a further insight into this problem.

Finally, it is known, that morphological, cytological and metabolic changes could occur in plant tissues cultures after prolonged culture (Street, 1966); in connection with this, it is important to mention, that the properties of the soybean callus cultures were modified as the clone of callus was physiologically older. Most of the experiments reported in this work were carried out with the clone originally obtained from soybean seeds (Tigier et al., 1968) which had been subcultured at 14 days intervals and grown in the dark for about 4 years. It was observed, that the longer the time colourless callus was cultured in the dark, the less was the activity of the enzymes involved in porphyrin biosynthesis.

The callus used in the earlier studies (Tigier et al., 1968, 1970; Llambias & Batlle, 1970a, b, c; 1971), showed greater differences in chlorophyll formation when they were transferred from the dark to the light. PBGase activity was easily measured and we were able to detect Dcase and CPGase. In 6 years old callus, activity of PBGase was very much lower than in younger callus.

Therefore, it would seem that the length of time previously spent by the callus undergoing subculture, diminishes its capacity to synthesize the enzymes responsible for porphyrin synthesis. It could be interesting then, to reinvestigate, in older callus, some of the phenomena already studied and compare with results obtained in early work.

**SUMMARY**

Colourless soybean callus can grow either in the dark or in the light forming low amounts of chlorophyll.

The effect of light and darkness, and simultaneously the addition or omission of various compounds to the culture media, on chlorophyll, porphyrin and protein content, growth, and on the activity of the enzymes involved in the earlier stages of chlorophyll synthesis, in cultured soybean cells, was studied.

When dark-grown soybean callus cultures were transferred to a light cabinet, it was found that porphyrin content was not modified; however, chlorophyll content was increased and a parallel increase in the activities of SucCoA-S, ALA-S, ALA-D and PBGases was observed, suggesting a common control over porphyrin and chlorophyll production; it has also been proposed that light could have induced the synthesis of enzymes necessary for chlorophyll production.

Addition of ALA to the medium culture, was found to stimulate porphyrin accumulation and to prevent growth; however, chlorophyll content was not greatly modified, suggesting that porphyrin synthesis could be dissociated from chlorophyll formation under these conditions. ALA-S was inhibited, it was proposed that porphyrins accumulated could interfere with ALA-S activity. Both ALA-D and PBGase activities were enhanced.

The action of puromycin and mitomycin added along with ALA to the media, was also studied, but it was found that neither of these inhibitors modified much the effects produced by ALA.

In media supplemented with PBG, content of porphyrins was increased, most of it was uroporphyrin and both growth and ALA-S activity were inhibited. The other enzymes, protein and chlorophyll content were not changed. It was proposed that formation of uroporphyrin may have been due to non-enzymic decomposition of PBG.

Omission of iron from the culture medium, produced porphyrin accumulation and inhibited growth. It has been consistently found that, the greater the amount of porphyrins formed, the less the callus growth, and it was proposed that is the accumulation of porphyrins, either in ALA supplemented media or under iron deficiency, which inhibits growth. In any case, Coproporphyrin was the major component of the porphyrins formed.

ALA-S and ALA-D activities were reduced under iron deficiency, indicating that iron could be involved in the synthesis of these enzymes.

The effect of ATP, gibberellic acid, succinate and CCCP uncoupler, added to the media was also studied.
In the presence of ATP, only Suc-CoA-S, ALA-S and PBGases activities were diminished, repression of enzyme formation by ATP could have occurred. The addition of gibberellic acid, slightly inhibited Suc-CoA-S and ALA-S and unexpectedly enhanced activity of PBGase was observed.

Sucinate added to the medium, increased growth and inhibited ALA-S and ALA-D. CCCP produced accumulation of porphyrins, uroporphyrin being the major porphyrin component, consequently, growth was prevented and ALA-S greatly inhibited. Chlorophyll content and PBGase were also diminished. 

Dcase and CPase were hardly detected in most experiments, and no conclusion can be drawn about them, except that are limiting.

The complex pattern of changes observed, would suggest that the regulation mechanism of tetrapyrrole synthesis in soybean cultured cells is manifold.

It has also been stated that, it would appear, that the length of time previously spent by the callus undergoing subculture in the dark, diminished its capacity to synthesize the enzymes involved in porphyrin biosynthesis.

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Key Word Index—Porphyrin biosynthesis; soybean callus tissue; chlorophyll; growth; succinyl CoA synthetase; Aminolevulic Acid Synthetase; Aminolevulic Acid Dehydratase; Porphobilinogenase; Decarboxylase; Coproporphyrinogenase; regulation.