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PORPHYRIN BIOSYNTHESIS IN *EUGLENA GRACILIS*—IV. AN ENDOGENOUS FACTOR CONTROLLING THE ENZYMIC SYNTHESIS OF PORPHYRINOGENS AND ITS POSSIBLE ROLE IN THE TREATMENT OF SOME PORPHYRIAS

ADELA ANA JUKNAT DE GERALNIK, MARÍA VICTORIA ROSSETTI and ALCIRA MARÍA DEL CARMEN BATLLE

Centro de Investigaciones sobre Porfirinas y Porfirias—CIPYP, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires y Consejo Nacional de Investigaciones Científicas y Técnicas—CONICET, Casilla de Correo N° 53, Sucursal 48, 1448 Buenos Aires, Argentina

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Abstract—1. Preliminary experiments with *Euglena gracilis* indicated that an endogenous factor which modified enzymic synthesis of porphyrinogens from PBG, was present in Homogenates (H) and Supernatant (S) fractions.

2. When H or S was stored at 4–6°C, enzymic activity underwent an apparent spontaneous activation, increasing by as much as 7.5–8 times after 14 and 22 days of aging respectively.

3. Experiments were carried out to detect, isolate and identify this factor. S and H were heated and the effect of the protein free supernatants (Hϕ,Sϕ) on activity were tested. By gel filtration of H and S, a low molecular weight compound (FH, FS) was separated, and the activity of the eluated protein (PrH, PrS) was enhanced 10–12 times.

4. Addition of either Hϕ, Sϕ, FH or FS to different *E. gracilis* preparations increased their activities, suggesting the existence of a low molecular weight, heat-stable factor which would act stimulating enzymic synthesis of porphyrinogens. However some differences in the properties of the factor present in Hϕ or Sϕ and that present in FH or FS were observed.

5. Studies on the FH and FS, confirmed that the factor was heat-stable, upon storage at 4°C its activation properties were not modified; but they were destroyed by basic or acid treatment.

6. The same degree of activation as that produced by FH and FS on H, S, PrH and PrS, was obtained by replacing the factor solution by 10^{-7} M folic acid or 10^{-3}–10^{-2} M Gluthation (GSH); however, neither the factor nor folic acid or GSH has any effect on the pellet enzyme, either bound to the membrane or solubilized by means of a chaothropic agent.

7. The potential use of this factor in the treatment of acute porphyrias was indirectly investigated, by treating acute intermittent porphyria patients in early or acute attack with folic acid; after its oral administration at a dose of 30 mg daily for not longer than 10 days, both biochemical and clinical recovery followed.

8. A scheme to explain the role of this factor in acting and controlling porphobilinogenase activity in *E. gracilis* is proposed.

INTRODUCTION

Hemes, chlorophylls, cytochromes and corrins, the so-called pigments of life are biosynthetically derived from Uroporphyrinogen III (Urogen III), which therefore plays a central role in this family of vital compounds. Urogen III is the product of the cyclotetramerization of porphobilinogen (PBG) by the dual enzymic system of the deaminase and isomerase, known as porphobilinogenase (PBGase) (Lockwood & Rimington, 1957).

In the absence of isomerase, deaminase converts PBG into uroporphyrinogen I (Urogen I), the non-physiological intermediate. Isomerase has not any action on Urogen I, nor consumes PBG; however it had been demonstrated 10 years ago, that either soybean callus or avian erythrocyte isomerase were able to bring about 80 and 100% Urogen III synthesis from a natural polypyrrole intermediate, formed by the action of soybean callus PBGase on PBG (Llambías & Batlle, 1970). Later, the occurrence of tetrapyrrlmethane intermediates synthesized by the *E. gracilis* enzymes has also been reported (Rossetti & Batlle, 1977; Rossetti *et al.*, 1977) and more recently evidence on the formation of a transient free-unrearranged bilane, which can be converted in Urogen III by the isomerase has been obtained (Burton *et al.*, 1979; Jordan *et al.*, 1979; Battersby *et al.*, 1979); therefore, the mode of action of isomerase, deaminase and their complex PBGase, needs to be re-examined, and the possibility of the existance of other factors or coenzymes regulating their activities must also be investigated.

In 1969, the detection of a factor, obtained by either ultrafiltration or dialysis of bovine liver preparations, which modified the activity of both PBGase and deaminase was reported (Sancovich *et al.*, 1969). A dializable factor, stimulating deaminase activity has also been detected and purified from rat hepatic cytosol; its action was antagonized by folic acid and it was

suggested that this compound was a pteridine derivate (Tephly, 1975; Piper & Van Lier, 1977).

On the other hand, in early experiments with *E. gracilis* we had observed, that under anaerobic incubations, the activity of the initial homogenate (H) was much lower than the activity of either the supernatant (S) or the pellet (P) fractions (Rossetti & Batlle, 1977). By standing H or S at 4–6°C, the activity of any of these preparations was several times higher, 2 or 3 days after, as compared with their initial values. This apparent spontaneous time-dependent activation, suggested us that some endogenous compound, which was able to modify enzymic synthesis of porphyrinogens from PBG was liberated after disruption of the cells, being mostly present in the H and S fractions.

These findings prompted us to investigate further the nature of this possible factor, that might control porphyrin synthesis in *E. gracilis* and correlate our results with previous reports on the existence of such an activity modulator in other tissues.

MATERIALS AND METHODS

PBG source and its estimation; growth and harvesting of *E. gracilis* Z strain; preparation of H, S and P; measurement of enzymic activity as well as all other methods not specified here or in legends to tables and figures, were those already described by Rossetti & Batlle (1977).

All chemicals were purchased from Sigma Chemical Co., unless otherwise stated. Sephadex gels were from Pharmacia Fine Chemicals, Uppsala.

Gel columns were prepared and run following usual techniques (Batlle *et al.*, 1965).

Solubilization of particulate PGBase, from the *E. gracilis* pellet fraction has been achieved, using a chaothropic agent and partially purified according to Rossetti *et al.* (1980).

RESULTS AND DISCUSSION

Spontaneous activation on aging

The effect of aging on enzymic synthesis of porphyrinogens was investigated in *E. gracilis*. As shown in Fig. 1, activity was found to change markedly when different fractions were stored at 4°C for various periods of time. After 22 days of aging the enzyme activity in S and P was about 8 and 2 times higher than that measured immediately after disruption and centrifugation, while a 7.5 times activation for H was reached on the 14th day of aging, after that initial rise the activity began to decrease more rapidly in H and S than in P preparations. It was later found that after thoroughly washing P fractions with buffer, its apparent spontaneous activation was greatly reduced; we could attribute the changes first observed to supernatant still ocluded in the pellet, suggesting that no modifying factor is present in P fraction.

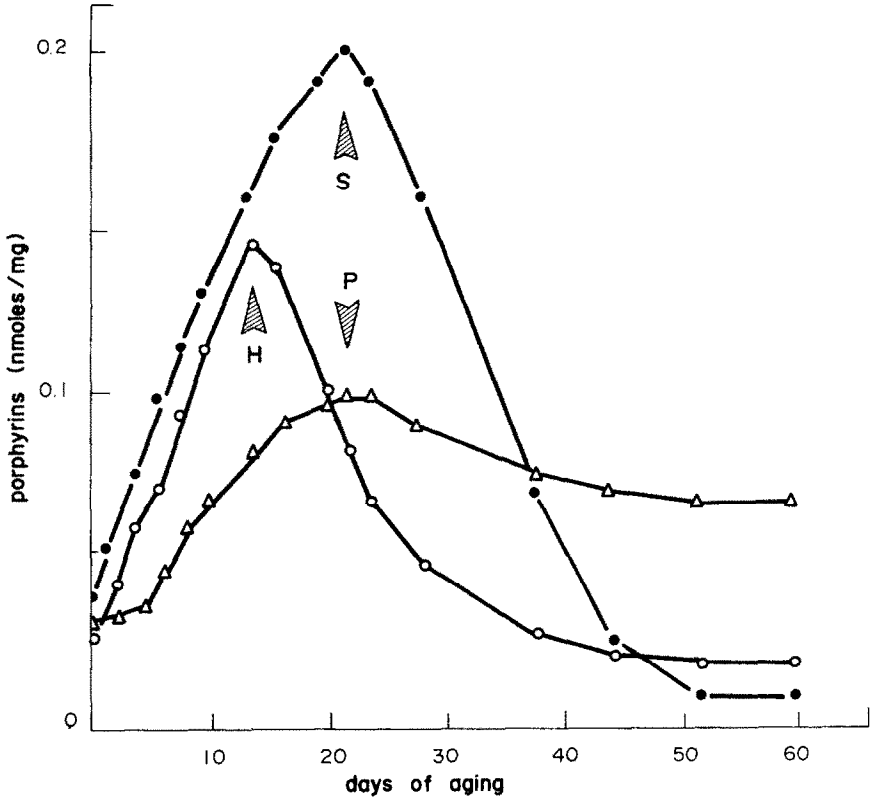

Fig. 1. Effect of aging on porphyrin synthesis activity in Homogenate H (○), Supernatant (S) (●) and pellet (P) (△) fractions, obtained from *Euglena gracilis*. H, S and P in 0.05 M phosphate buffer pH 7.4 were stored at 4–6°C. At different days, portions were removed enzyme activity was measured and expressed as nmoles of porphyrins formed per mg of protein. Incubation conditions, controls run and methodology was as described by Rossetti & Batlle (1977).

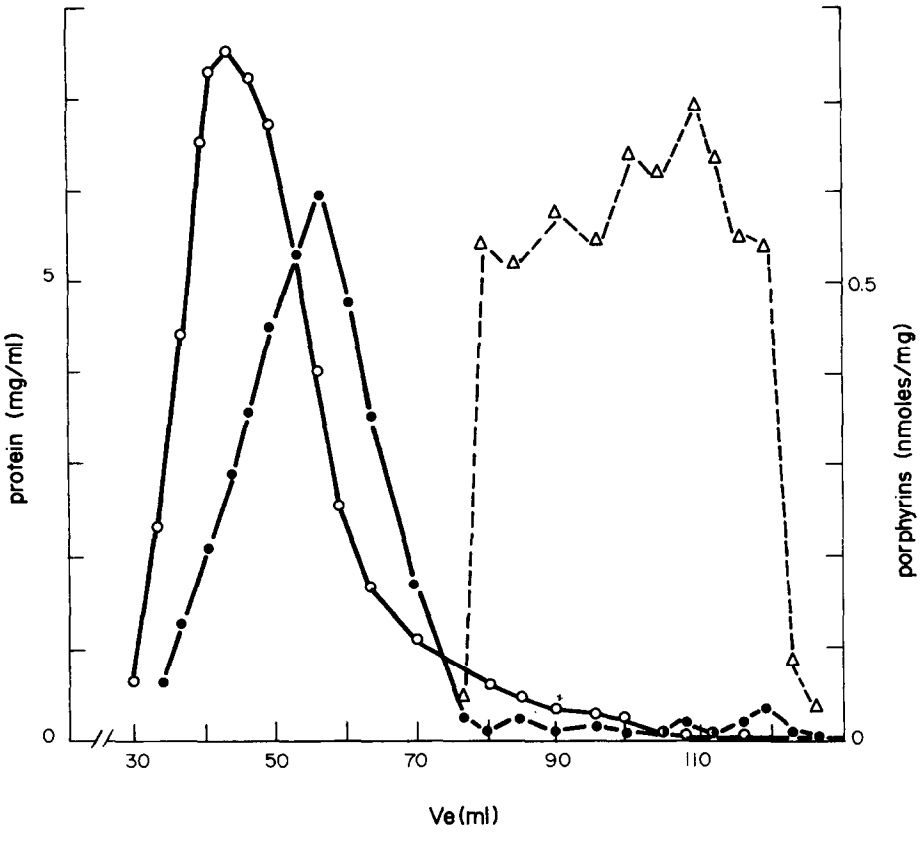

Fig. 2. Separation of a low molecular weight factor (FH) stimulating enzyme activity, from homogenates (H) obtained from *E. gracilis*. 10 ml of H (300 mg protein, 0.028 nmol porphyrins/mg) were passed through a Sephadex G-25 column (1.8 × 50 cm), at 4–6°C, equilibrated and eluted with 0.05 M phosphate buffer pH 7.4. Protein content (O——O) and enzymic activity (●——●) were determined in each eluate. Protein eluted between 35 and 70 ml was pooled and used and designated as PrH, its activity was 0.308 nmol porphyrins/mg. One ml of PrH was added to 1 ml of the retarded column fractions (FH) and the activity was measured (△---△). FH eluted between 74 and 115 ml. Blanks, controls and methodology were the same as indicated in Fig. 1.

These results confirmed the assumption that *E. gracilis* cells contain some compound which modifies enzyme activity and can be released after disruption.

Effect of gel filtration and heating

Preliminary experiments had shown that PGBase activity from H and S preparations greatly increased by gel filtration. To determine if such increase was due to the loss of some inhibitor, H and S fractions were passed through a Sephadex G-25 column (Figs 2 & 3).

Protein was practically excluded with the void volume; the activity of the enzyme in the protein eluates, which we call PrH and PrS was several times higher (Table 1) than that corresponding to H and S respectively, suggesting that, apparently, a low molecular inhibitor had been separated. However, by adding back retarded column fractions, designated as FH and FS, to the eluted enzyme, a further 2-fold increase in activity of the excluded enzyme was obtained, indicative of the existance of a low molecular weight activator in both H and S preparations.

H and S fractions were heated at 100°C for 10 min, the denatured protein separated by centrifugation and the clear resulting supernatants, called Hϕ and Sϕ respectively were then tested for its effect on activity. As can be seen in Table 1 addition of either Hϕ, Sϕ, FH or FS to different *E. gracilis* enzyme preparations enhanced their activities, suggesting that they contain a low molecular weight, heat stable factor which would act stimulating the enzyme synthesis of porphyrinogens.

Effect of the factor on the activity of different enzyme fractions

So far, only the presence of a low molecular weight, heat stable factor on both H and S, which would enhance and possibly control *in vivo* PGBase activity had been confirmed. Apparently, the compound obtained after heating H or S, that is Hϕ and Sϕ, is the same molecule and a similar reasoning applies to FH and FS. However, in order to gain further information as to the properties of the factor, we decided to comparatively study the effects of these four sources of factor on the activity of different enzyme preparations. Results are shown in Table 1.

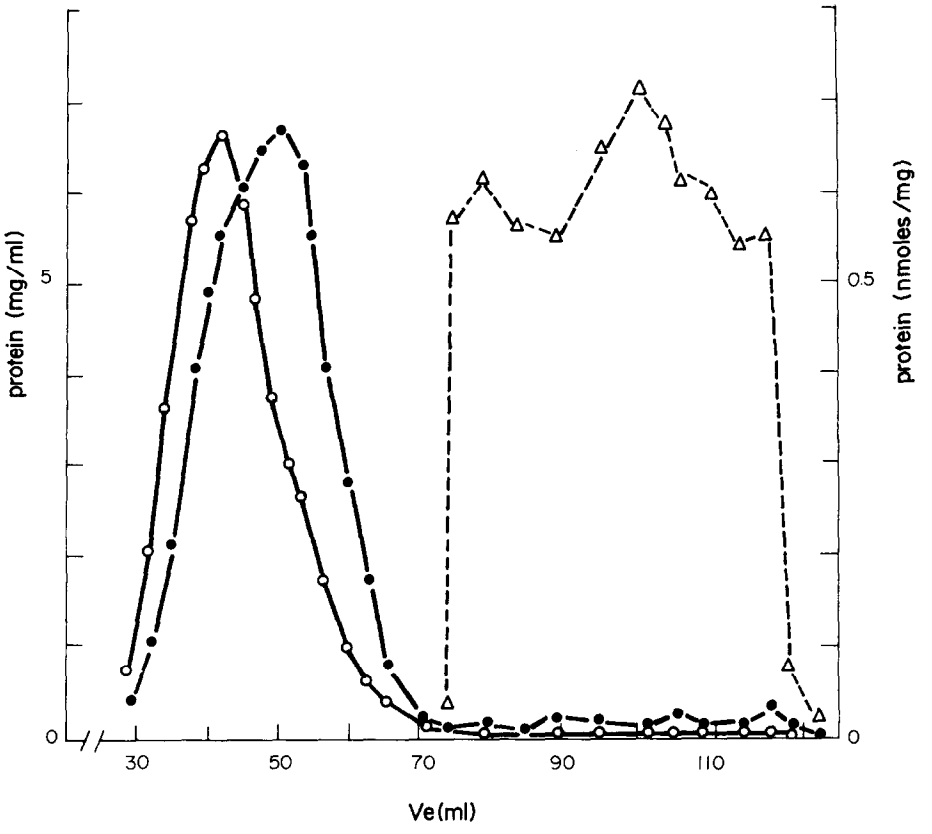

Fig. 3. Separation of a low molecular weight activating factor (FS) from *E. gracilis* supernatant (S). Ten ml of S (200 mg protein, 0.043 nmol porphyrins/mg) were passed through a Sephadex G-25 column, under the same conditions as described in Fig. 2. Protein content (○——○) and enzymic activity (●——●) were determined in each eluate. Protein eluted between 35 and 65 ml was pooled and designated as PrS. One ml of PrS (0.430 nmol porphyrins/mg) was added to 1 ml of the retarded column fractions (FS) (72 to 125 ml), and the activity was measured (△- - -△). Other experimental conditions were as indicated in Fig. 1.

As already stated, by gel filtration of H and S, the activity of the resulting enzyme preparation, PrH or PrS was 10–12 times higher. It is interesting that the effect of Hϕ and Sϕ or FH and FS on equal fractions was the same. If we arbitrarily define 1 unit of factor as the amount of this compound which produces a 2-fold increase in the activity of the corresponding control, we can observe that, even using the same volume and source of factor, its stimulating capacity depends too on the activity of the enzyme preparation upon it acts; for instance the effect of Hϕ or Sϕ is always 14% greater on S than on H and between 2.1 and 2.5 units of active factor would be present in 1 ml; we should also note that these same sources of factor, Hϕ and Sϕ seem not to have any action on the enzyme protein PrH or PrS.

As far as FH and FS are concerned, a similar effect has been observed, 1 ml of FH and FS would contain 2.4–3.3 units of factor and again its activating capacity is 14% higher on S than on H. Another difference between the factor present in the heated fractions and that obtained by gel filtration, is that the latter stimulated 2-fold the activity of PrH and PrS; its effect being again 12–14% greater on PrS than on PrH.

It must also be noted that, independent of its source, the factor has no action whatsoever on the P enzyme.

So far, these findings would indicate that very likely, Hϕ, Sϕ, FH and FS contain the same activating factor; however, the initial heating treatment of H and S might have produced some structural changes and correspondingly diminished its stimulating capacity, as a consequence, the so-altered factor is unable to express itself on certain enzyme fractions which already exhibit highest activity, as it is the case of PrH and PrS. Therefore, the properties of this factor were further investigated in the FS or FH preparations.

Stability of the factor

From the preceding experiments, it was evident that the factor was heat stable; although some changes resulted when obtained by heating crude H and S preparations, it was then decided to examine the effect of directly heating FH and FS, on its stimulating capacity. It was found (Table 2) that the factor present in FH and FS was also heat-stable (100° for 10 and 20 min) and no appreciable changes as those appear to occur on H and S were observed.

Table 1. Effect of the factor on the activity of different enzyme preparations

Incubation mixture	Activity porphyrins formed nmol/mg	PBG consumed μg/mg	Relative activity (*)	Units of factor (**)
H	0.0254	0.651	—	—
S	0.0266	0.804	—	—
P	0.1467	1.303	—	—
PrH	0.2473	1.849	9.73	—
PrS	0.3128	2.231	11.72	—
H + Hφ	0.1095	1.033	4.30	2.15
H + Sφ	0.1093	1.037	4.30	2.15
H + FH	0.1208	1.219	4.76	2.38
H + FS	0.1278	1.223	5.03	2.50
S + Hφ	0.1300	1.222	4.87	2.43
S + Sφ	0.1320	1.245	4.94	2.47
S + FH	0.1780	1.539	6.67	3.33
S + FS	0.1775	1.519	6.65	3.32
P + Hφ	0.1466	1.670	1.00	—
P + Sφ	0.1430	1.680	0.97	—
P + FH	0.1440	1.672	0.98	—
P + FS	0.1430	1.678	0.97	—
PrH + Hφ	0.2899	2.166	1.17	—
PrH + Sφ	0.3036	1.995	1.22	—
PrH + FH	0.4992	2.777	2.02	1.01
PrH + FS	0.5429	2.796	2.09	1.04
PrS + Hφ	0.3614	2.330	1.15	—
PrS + Sφ	0.4211	2.149	1.30	—
PrS + FH	0.7669	3.703	2.45	1.27
PrS + FS	0.7114	3.209	2.27	1.14

Hφ and Sφ were obtained by heating the H and S fractions at 100°C for 10 min, the protein precipitate was centrifuged off at 10,000 rev/min for 10 min and the clear supernatant used as source of factor. PrH and PrS FH and FS were prepared by gel filtration, as described in legends to Figs 2 & 3. Protein eluted between 35 and 65 ml was employed as PrH and PrS, and the protein free retarded column fractions eluted between 85 and 115 ml used as FH and FS. In all reaction mixtures 1 ml of enzyme preparation was assayed for activity either alone or plus 1 ml of factor solution (Hφ, Sφ, FH or FS). Other experimental conditions were as indicated in Materials and Methods. Activity is here expressed both as nmol of porphyrins formed/mg and μg of substrate consumed/mg. (*) Relative activity is calculated taking the amount of porphyrins formed/mg of protein of the corresponding control as 1. (**) One unit of factor is defined as the amount of factor which entrances 2-fold the activity of the corresponding control.

The activating properties were, however, destroyed by treating FH, FS, or Hφ, Sφ with equal volumes of varying concentrations (ranging from 6 to 1 N) of NaOH and HCl, during 1 and 2 min at room temperature. After neutralization, their action on the activity of H, S, PrH and PrS was examined and in all cases, it was found that the stimulating action was destroyed by base or acid treatment.

Samples of PrH, PrS, FH and FS were stored at 4–6°C for a week and their activities measured daily. As can be seen in Fig. 4, as expected, spontaneous activation of enzymic activity was prevented by gel filtration; also, both the enzyme fractions PrH and PrS and the factor FH and FS are rather stable when stored at low temperature; therefore upon storage the stimulating effect produced by the factor was not modified.

Effect of folic acid and gluthation (GSH) on enzymic activity

Taking into account previous reports, indicating that the stimulatory action of a factor isolated from rat liver cytosol could be antagonized by folic acid (Tephly, 1975; Piper & van Lier, 1977), and those about the protecting and activating effect of gluthation on the bovine liver enzymes (Sancovich *et al.*, 1969), and in order to determine whether or not there might be any relation between the liver cytosol factor and our factor, we decided to comparatively investigate the effect of FS, folic acid and gluthation on enzymic activity.

Folic acid concentrations ranging from 10^{-3} to 10^{-10} M were used; in Fig. 5 we have only shown results obtained with those exhibiting maximum effect

Table 2. Heat stability of the factor

Incubation mixture	Activity porphyrins formed nmol/mg	Activity PBG consumed μg/mg	Relative activity	Units of factor
H	0.0341	0.4825	–	-
S	0.0387	0.7582	–	
PrH	0.3237	1.9452	9.5	
PrH + FH	0.8490	3.5631	2.6	1.30
PrH + FH 10′	0.8904	3.6018	2.7	1.35
PrH + FH 20′	0.7882	3.5766	2.4	1.20
PrH + Hφ	0.3238	1.9556	1.0	—
PrS	0.3975	3.6811	10.2	
PrS + FS	0.8528	3.8346	2.4	1.20
PrS + FS 10′	0.9660	3.7368	2.4	1.20
PrS + FS 20′	0.8128	3.9320	2.1	1.05
PrS + Sφ	0.3401	1.9890	1.05	

FH 10′, FH 20′, FS 10′, FS 20′ were obtained by heating FH and FS, 10 and 20 min at 100°C in a water bath, with shaking, and immediately cooling in ice. Other experimental conditions are those described or referred to in legend to Table 1. Relative activity and units of factors have also been defined in legend to Table 1.

and of the same order as that produced by 2–4 units of factor, depending on the enzyme fraction they acted on.

It was evident that folic acid produced a significant activation of the enzyme (up to 400%) at a concentration as low as 0.1 μM; therefore the stimulatory effect of the FS, can be replaced by folic acid. These findings are in agreement with those of Piper & van Lier (1977); however, the effect of both our factor and that of folic acid on the *E. gracilis* enzymes is several

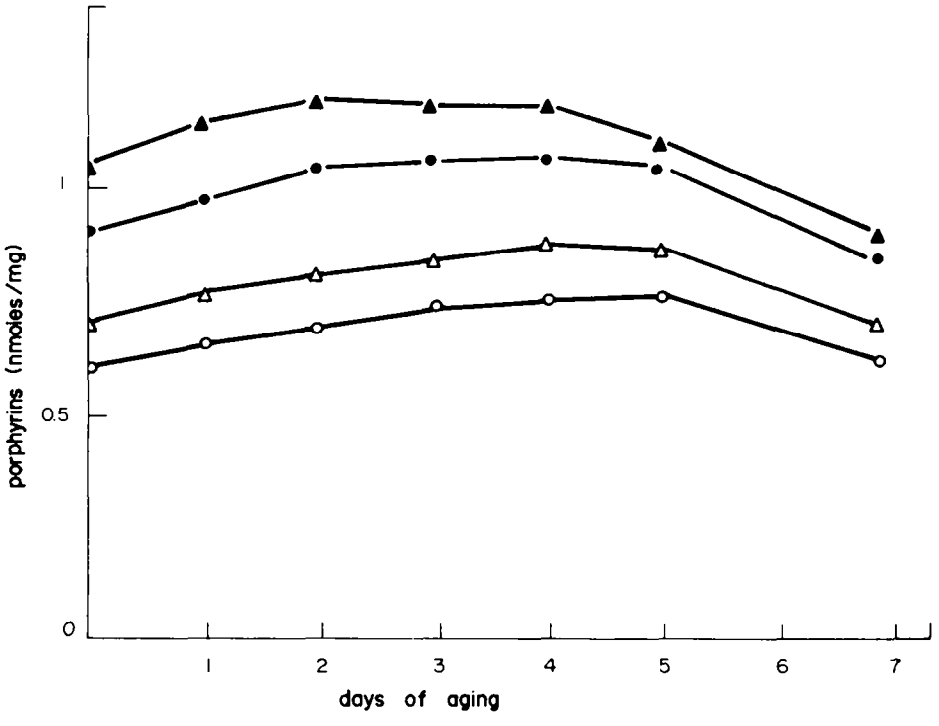

Fig. 4. Effect of aging solutions of PrH (○), PrS (△), FH and FS, in 0.05 M phosphate buffer pH 7.4 at 4–6°C. Samples were removed daily and enzymic activity was measured following the experimental conditions indicated in Materials and Methods, except that in the mixtures PrH + FH (●) and PrS + FS (▲) the ratio of enzyme to factor solution was 1:0.5 (ml:ml).

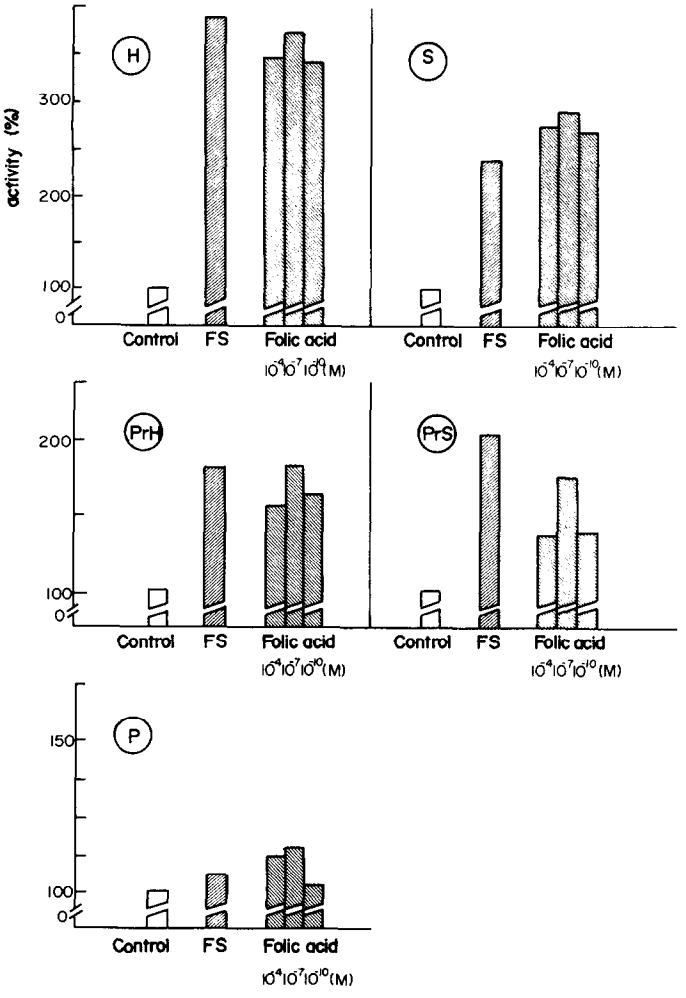

Fig. 5. Effect of factor FS (■) and Folic acid (▩) on the enzyme activity of H, S, PrH, PrS and P preparations obtained from *E. gracilis*. Activities are expressed on the basis of taking the activity of the control as 100%. Reaction mixtures contained 1 ml of enzyme fraction; 1 ml of enzyme fraction and 1 ml of FS and 1 ml of enzyme and Folic acid at the final concentration indicated. Other experimental conditions were as described in Materials and Methods.

times greater than that observed in rat liver, where maximum stimulation of folic acid was 20–25% at a concentration of 100 μM.

Next, to examine whether sulphydryl groups might be involved in either the chemical structure of the factor or as a protein functional residue in the site of interaction between the enzyme and the factor, or both; the effect of varying concentrations of GSH on the activity of different enzyme preparations, was studied (Fig. 6). Once again, the stimulating action of FS, was antagonized by gluthation at concentrations 10^{-2} or 10^{-3} M in H, S, PrH and PrS but only slightly (15%) in P.

From these results we can not yet dilucidate which is the actual role of the –SH groups, in the activating mechanism; however, we can advance that they should be of some importance in controlling enzymic activity and would also suggest that the factor might be a thiol derivate.

Effect of FH, FS, folic acid and GSH on the particulate enzyme

It was striking that this stimulating factor had no action on the pellet-bound enzyme (Table 1); therefore, in order to obtain some evidence to let us explain the reasons for this differential behaviour, we have released the particulate PBGase using 0.5 M sodium thiocyanate and then tested the effect of the factor, Folid acid and GSH on the solubilized and particulate bound enzyme.

As can be seen in Table 3, the activity of the solubilized PBGase was 31 times higher than that of the membrane bound enzyme; neither the factor nor folic acid or GSH were found to stimulate further the activity of the released PBGase, suggesting that this highly active enzyme, either has no sites for interating with the factor or it has already adopted the structural configuration corresponding to its maximal ac-

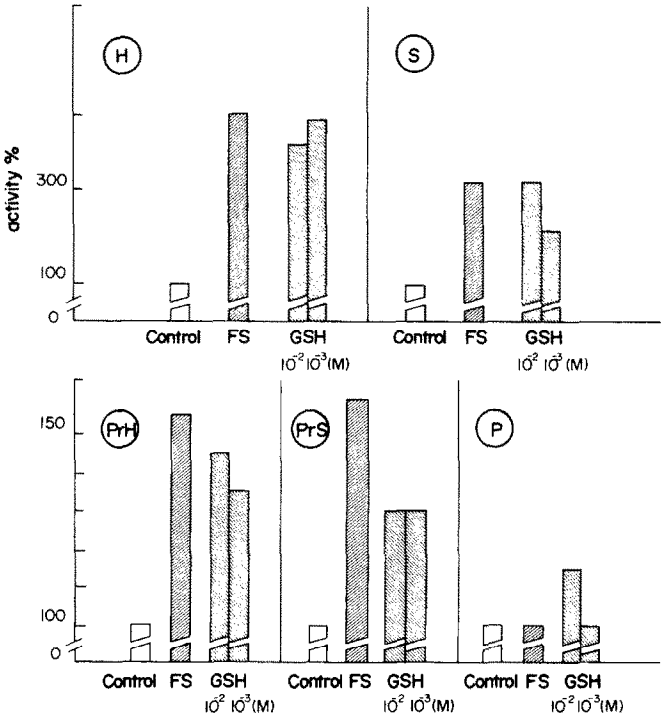

Fig. 6. Effect of factor FS (▩) and GSH (▩) on the enzyme activity of H, S, PrH, PrS and P preparations obtained from *E. gracilis*. Activities are expressed on the basis of taking the activity of the control as 100%. Reactions mixtures were as described in Fig. 4. Concentrations of gluthation shown are the final concentration in the mixture. Other experimental conditions were as indicated in Materials and Methods.

tivity. It is interesting to note that the more active the enzyme was, the less the activating effect of the factor; so there seem to be some correlation between the specific activity of the enzyme preparation and the possibility of the factor to further stimulate its activity.

The potential use of this factor in the treatment of acute porphyrias

It is accepted that a 50% reduction in the activity of deaminase and also PBGase is the primary genetic abnormality in acute intermittent porphyria (AIP)

Table 3. Effect of the factor, folic acid and GSH on the particulate enzyme

Incubation mixture	Activity porphyrins formed nmol/mg	PBG consumed μg/mg	Relative activity
P	0.083	0.5192	—
P + FH	0.086	0.7639	1.03
P + FS	0.087	0.8120	1.04
P + folic acid 10^{-7} M	0.093	0.8220	1.12
P + GSH 10^{-2} M	0.092	0.8310	1.10
P_{SCN}	2.599	4.0828	31.00
P_{SCN} + FH	2.234	5.2329	0.86
P_{SCN} + FS	2.305	5.6456	0.89
P_{SCN} + folic acid 10^{-7} M	2.212	3.7600	0.85
P_{SCN} + GSH 10^{-2} M	2.622	3.7709	1.01

The enzyme was released from the membrane by using 0.5 M sodium thiocyanate as described by Rossetti *et al.* (1980) and designated as SCN. Other experimental conditions were those described or referred to in legends to Table 1 and Figs 5 & 6.

(Heilmeyer & Clotten, 1969; Strand *et al.*, 1970; Batlle *et al.*, 1978). Therefore because of the role of deaminase in AIP, Tephly (1975) has already suggested that his rat liver activating factor could be of some importance in such porphyria. Both the American team results (Tephly, 1975; Piper & van Lier, 1977) and ours, prompted us to treat AIP patients in early or acute attack with folic acid (Wider de Xifra *et al.*, 1979, 1980).

A 32 yr old woman (Case 1) showed all the clinical symptoms characterizing AIP. δ-aminolevulinic acid (ALA), PBG and total free porphyrins levels in urine were 20 mg/24 hr, 150 mg/24 hr and 1200 μg/24 hr respectively. PGBase activity was 11.8 units/ml RBC (normal, 22.5 ± 5) and deaminase 27.5 units/ml RBC (normal: 55 ± 10); these data confirmed the diagnosis of AIP. Although she responded well to high carbohydrate therapy the first time she was admitted to hospital, when she came to us, 6 months later, beginning to feel the signs of one of her acute attacks, we decided to try folic acid therapy. Folic acid was administered orally at a dose of 10 mg every 8 hr (30 mg daily). On the following day her clinical condition was improved and remission was complete within 3–5 days. There was also a decline in urinary ALA, PBG and porphyrins (Fig. 7); however PBGase and deaminase activities were not significantly modified at any time. The therapy resulted in both symptomatic and biochemical improvement. After 10 days folic acid intake was suspended. Case 2 was a 19 years old woman who came in acute attack. ALA, PBG and porphyrins levels in urine were 40 mg/24 hr, 200 mg/24 hr and 1850 μg/24 hr respectively. PBGase and deaminase activities were reduced to 11 units/ml RBC and 24.5 units/ml RBC. Folic acid therapy was immediately started as described for Case 1. Rapid remission of the acute symptoms was followed along with a decrease in urinary levels of precursors and porphyrins (Fig. 7). After 10 days folid acid intake was suspended. Therefore, oral administration of folic acid seems to produce both clinical and biochemical improvement. Results to date, applied in more than 15 AIP patients along the last 3 yr, indicate that this simple and short-term therapy appears to be beneficial to control and suppress the acute attack and also to be an effective agent in preventing early attacks.

Tephly (1975) and Piper & van Lier (1977) have also demonstrated that the rat liver factor protected against inhibition of deaminase by lead or other metals and may be of potential consideration in lead poisoning. Studies on the purification properties and the effect of the *E. gracilis* factor on the enzymic synthesis of porphyrinogens both *in vivo* and *in vitro*, in tissue cultures and cases of lead and cadmium intoxication as well as AIP and induced experimental porphyrias, will shortly be published. Although some of the properties of the *E. gracilis* factor resemble those of the rat liver activator, there seem to be some differences between them. Nevertheless undoubtedly this factor should play an important role in the regulation of tetrapyrrole biosynthesis and might perhaps be involved in the metabolic derangements leading to the acute porphyria.

Postulated scheme for the release and action of the factor regulating the activity of E. gracilis *PBGase*

It is clear that further work is necessary to gain additional evidence as to be able to elucidate the structure of this factor, the nature of its interaction with the enzyme and its mode of action. However, although rather speculative, we postulate the following scheme (Fig. 8), which would be in accordance to the experimental data so far obtained.

In *E. gracilis* we find PBGase activity in both the particulate and soluble fractions and we assume that factor F would normally be associated to the latter,

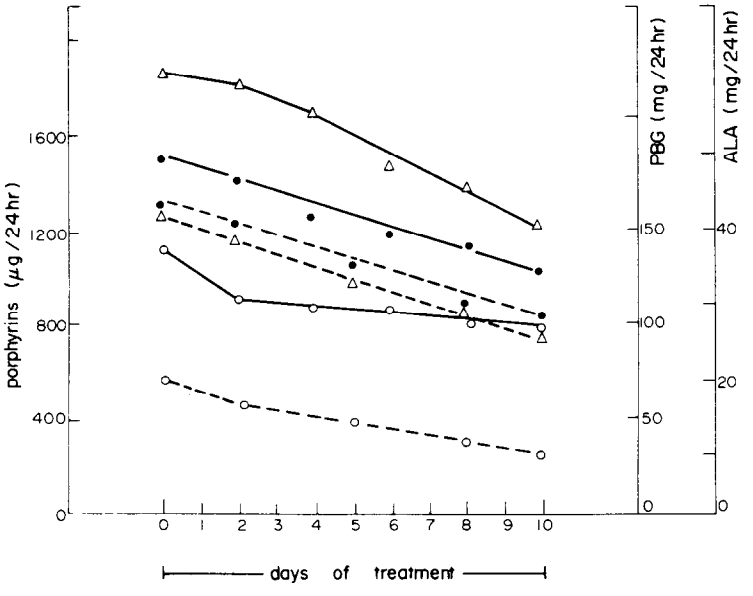

Fig. 7. Effect of folic acid therapy on 2 patients in early (Case 1 - - -) and acute attack (Case 2 ——). Daily urinary excretion of ALA (○), PBG (●) and total porphyrins (△) before and during treatment.

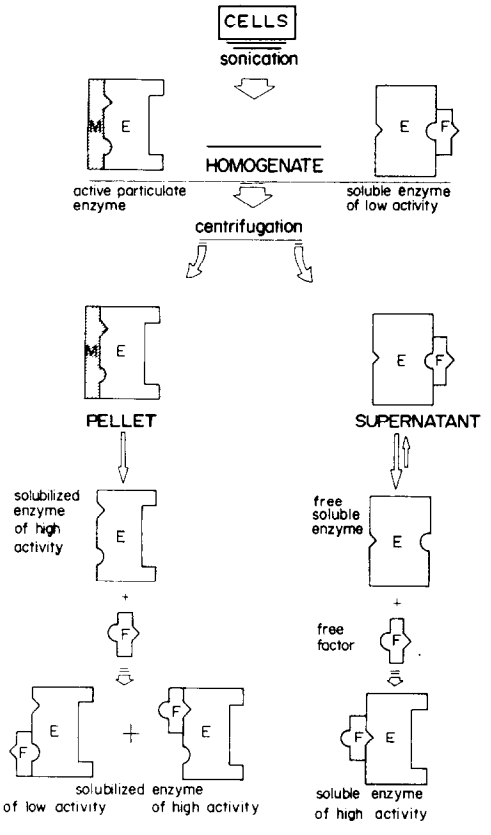

Fig. 8. Postulated scheme for the release and activation of a low molecular weight factor, regulating the enzymic synthesis of porphyrinogens, in *E. gracilis*. See the text for explanation. M, membrane; E, enzyme; F, factor.

which would be a low activity form of the enzyme, having regulatory properties. So, depending on certain physiological conditions *in vivo* or, after disrupting the cell *in vitro*, this factor might be released and allowed to act. We also suppose that there should exist an equilibrium between the enzyme bound to the factor and the free enzyme and its modifier ($E\text{–}F \rightleftharpoons E + F$), the displacement of this equilibrium to one side or the other would be dependent on the state of the cell. Once free, this factor would be able to express its stimulating properties, but apparently only on the soluble PBGase.

Although we can not explain yet, which is the mechanism of releasing and making this factor an activating compound, we assume that it could naturally be non-covalently bound to the enzyme, in such a way that the E–F complex might be a constrained molecular structure of low activity; then, as a consequence of either cellular requirements or cell disruption, this factor can be easily released, the functional groups in both the protein and the factor involved in the association would therefore be exposed, and now this low molecular weight substance would be free to interact again with the enzyme and bind it, although very likely at a different site, inducing perhaps some conformational changes in the protein which in turn will result in a high activity enzyme. It is also possible that this factor could protect the enzyme against inhibition by certain compounds by interfering with their binding to the protein.

The fact that the factor can not act on the particulate enzyme, made us to think that the structural configuration of the pellet PBGase can be slightly different and very likely the functional groups involved in the binding to the factor are embedded into the membrane, so this modifier finds no site on the particulate PBGase to interact with. After releasing the enzyme from the membrane, it is also probable that it already exists in a structural molecular arrangement exhibiting maximal activity, therefore the factor can not stimulate it further; alternatively, the factor might bind the protein at any of its receptor sites; producing a mixture of highest and low activity species, and consequently no measurable changes in the resulting activity could be detected; however this last possibility is considered the less attractive.

We have also found evidence on the existance of a similar regulator compound in *Rh. palustris* (Juknat de Geralnik *et al.*, 1980) and searches for its presence in other organisms and tissues are now under progress, as well as studies to dilucidate its role, if any, in the control of porphyrin biosynthesis.

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