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High-performance liquid chromatographic detection of pitfalls in porphobilinogen deaminase determination

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ABSTRACT

Potential pitfalls in the determination of porphobilinogen deaminase activity, as well as ways of eliminating these sources of error and determining the activity accurately, are discussed. In addition to measurement of the accurate activity, the described method (a combination of incubation of homogenate with porphobilinogen and high-performance liquid chromatographic separation) can also be used to detect enzymic defects in the haem biosynthetic pathway, according to the pattern of accumulation of the various porphyrins.

INTRODUCTION

Porphobilinogen deaminase (PBGD) (EC 4.3.1.8) functions in the biosynthetic pathway that produces haem; it transforms four porphobilinogen (PBG) molecules into hydroxymethylbilane. The latter cyclized spontaneously, and in the presence of uroporphyrinogen III cosynthease (EC 4.1.1.37) uroporphyrin III is produced. This is followed by a series of enzymic and non-enzymic reactions, which transform one porphyrin intermediate into another and culminate in the production of protoporphyrin. Finally, protoporphyrin combines with iron to form haem. This reaction is catalysed by the enzyme ferrochelatase (FC) (EC 4.99.1.1) and may also occur spontaneously.

In patients with acute intermittent porphyria (AIP), and in their relatives with latent AIP, the activity of PBGD in various tissues is generally about half the normal activity [1,2]. In addition, it has lately been observed that the measurement of PBGD activity may be of importance in the diagnosis of malignant lymphoproliferative diseases [3,4]. Moreover, PBGD is thought by some investigators to be the rate-limiting enzyme in the pathway of haem formation in erythropoietic tissues [5]. The accurate determination of its relatively low activity in nucleated cells is of importance in investigations concerning the haem biosynthetic pathway and its regulation in different tissues.

During our investigations of PBGD activity in peripheral mononuclear cells (PMNC) of patients with various diseases [4] by the method of Magnussen *et al.*

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[6], we observed that the measured activity in PMNC of healthy donors was higher in those separated from blood donated to the blood bank than in those obtained from heparinized blood. This indicates either that heparin depresses PBGD activity or that some factor in the blood bank samples enhances it. Thus it became apparent that the measurement of PBGD activity can be affected by one or more factors not taken into account in the generally utilized assay procedures, none of which takes into account that haem formation during incubation of homogenate of nucleated cells with PBG might reduce the amount of porphyrins determined and thus lead to underestimation of the activity of PBGD. The employment of a haem synthesis inhibitor might, therefore, be necessary for determining the true activity of PBGD.

This work was carried out in order to investigate this assumption, as well as to document other potential pitfalls in the determination of PBGD activity and to suggest ways for eliminating these sources of error.

EXPERIMENTAL

Separation of PMNC

PMNC of normal subjects were separated by the method of Boyum [7] from defibrinated blood [8], or from blood to which either heparin or other anticoagulants had been added.

Activity of PBGD

The activity of PBGD was determined basically according to Magnussen *et al.* [6]. The assay mixture consisted of 0.5 ml of sonicated sample, 0.95 ml of 0.05 *M* Tris–HCl (pH 8.2) and 0.05 ml of 3 m*M* PBG. The mixture was incubated at 37°C for 60 min. The reaction was stopped by the addition of 1.5 ml of 25% (w/v) trichloroacetic acid (TCA). After centrifugation the porphyrins in the supernatants were determined fluorimetrically (Shimadzu RF-540 spectrofluorophotometer with R-928-08 photomultiplier) at 404 nm excitation and 595 nm emission, and their concentration was calculated according to a standard curve of uroporphyrin in 12.5% TCA.

Protein was measured by the method of Lowry *et al.* [9], with bovine serum albumin as a standard.

High-performance liquid chromatography (HPLC) apparatus

An HP 1090 L solvent-delivery system (Hewlett-Packard, Avondale, PA, USA), equipped with a Rheodyne 7010 injector (Rheodyne, Cotati, CA, USA) and a 100-μl external loop, was used. A type 73XX inlet filter was installed between the sample injector and the column. An HP reversed-phase column was used (100 mm × 4 mm I.D., HP Hypersil ODS, 5 μm), and the fluorescence was measured by a programmable fluorescence detector, HP 1046. The excitation wavelength was 404 nm and the emission wavelength 615 or 620 nm. Quantification was performed by an HP-3393, a computing integrator.

Sample and standard preparation

To prepare the porphyrin standard mixture, a lyophilysed porphyrin acids marker kit (Porphyrin Products, Logan, UT, USA) containing 10 nmol each of eight, seven, six, five, four and two (2–8) carboxylic porphyrins was dissolved in 2 ml of 3 *M* HCl. The final porphyrin concentration was 5 μM. This stock solution was kept for up to two weeks at 4°C. Before injection into the column, the standard mixture was diluted 1:20, 1:50 or 1:100 with the solution in which the corresponding samples were prepared and was centrifuged for 10 min at 3000 *g* at 4°C. A 100-μl aliquot of the supernatant was injected directly into the column without any pretreatment. Protoporphyrin and uroporphyrin III were prepared and diluted as above.

Separation methods

Three methods for separating porphyrins were used.

Method 1: 4 min separation (Fig. 1A). We developed this method for the rapid separation of protoporphyrin. The elution is isocratic by 90% of 10% (v/v) aceto-

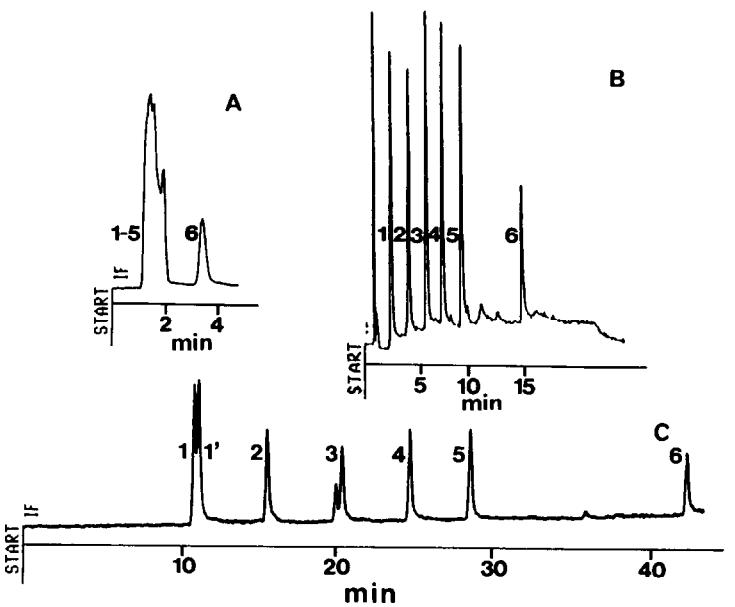


Fig. 1. Chromatograms of free acids of 2–8-carboxylic porphyrins separated by different methods. Three separation methods (1, 2 and 3, see Experimental) were employed. A mixture of 2–8-carboxylic porphyrins (I isomer) was prepared in 3 *M* HCl as described in Experimental. The final concentration of each of the porphyrins, after further dilution in 1 *M* acetic acid, was 250 pmol/ml; 100 μl were injected into the column in methods 1 and 2. For method 3, uroporphyrin III was added to the mixture of the 2–8-carboxylic porphyrins. The final concentration of each porphyrin was 125 pmol/ml. Peaks: 1 = uroporphyrin I; 1′ = uroporphyrin III; 2 = heptacarboxylic porphyrin; 3 = hexacarboxylic porphyrin; 4 = pentacarboxylic porphyrin; 5 = coproporphyrin; 6 = 2-carboxylic porphyrin.

nitrile in 1 *M* ammonium acetate (pH 5.0) and 10% (v/v) acetonitrile in methanol. Two peaks are observed: one large peak composed of all the porphyrins containing four to eight carboxyl groups (*ca.* 1.5 min retention time) and one peak of 2-carboxylic porphyrins (protoporphyrin or mesoporphyrin) with *ca.* 3.0 min retention time.

Method 2: 30 min separation of 2–8-carboxylic porphyrins (Fig. 1B). This is a modification of the method described by Bonkovsky *et al.* [10] Eluents: methanol (A); methanol–0.1 *M* ammonium phosphate buffer (pH 3.5) (44:56, v/v) (B). Elution: 15 min linear gradient from 70% B to 0% B, followed by 5 min isocratic conditions of 0% B, and returning to 70% B during the following 5 min.

Method 3: 60 min separation of I and III isomers of 4–8-carboxylic porphyrins and of 2-carboxylic porphyrins (Fig. 1C). The method of Lim and Peters [11] was modified for our system. Eluents: 10% (v/v) acetonitrile in methanol (A); 10% (v/v) acetonitrile in 1 *M* ammonium acetate (pH 5.1) (B). Elution: 30 min linear gradient from 100% B to 35% B, followed by 12 min linear gradient form 35% B to 10% B; after 4 min of isocratic elution, returning to 100% B during the following 14 min.

The flow-rate in all the procedures was maintained at 1.0 ml/min. For fluorimetric detection the excitation wavelength was 404 nm in all the methods; the emission wavelength was 620 nm in method 1 and 615 nm in methods 2 and 3.

Materials

Histopaque, PBG and sodium citrate (C-8532) were obtained from Sigma (St. Louis, MO, USA) and solvents for HPLC from Merck (Darmstadt, Germany). Porphyrins were purchased from Porphyrin Products. All other chemicals were of the highest purity available.

Student's *t*-test and Pearson's product moment correlation test were employed for statistical evaluation.

RESULTS AND DISCUSSION

In seeking the cause for the higher activity of PBGD, observed in PMNC separated from blood obtained from the blood bank when compared with the activity measured in PMNC separated from heparinized blood, we first tested whether heparin has an inhibitory effect on PBGD activity. Blood was withdrawn from five healthy volunteers. Each sample was divided between two test-tubes, one containing glass beads and the other heparin. No change in PBGD activity in PMNC obtained from the latter and the former was observed, which indicated that heparin did not interfere with the determination of PBGD activity. Together with the finding that PBGD activity is higher in PMNC obtained from the transfusion pack of the blood bank, this result led us to the conclusion that one of the constituents of the anticoagulant solution used by the blood bank interferes with the determination of PBGD activity. Indeed, sodium citrate, which is present in

the solution at a concentration of 22 m*M*, was found to increase the PBGD activity by 80%. None of the other constituents affected the PBGD activity.

The activity of citrate as an iron chelator is well known. Since FC binds iron, the presence of citrate in the incubation medium during the determination of the PBGD activity in cell homogenates may, owing to the inhibition of either enzymic or non-enzymic haem formation, lead to increased accumulation of porphyrins, *i.e.* to an increase in the measured activity of PBGD. Although it is believed that haem synthesis in aerobic conditions is very low, its inhibition might be crucial during the determination of low activities of PBGD.

One might, therefore, suggest that measuring the true activity of PBGD requires the presence of an inhibitor of haem formation.

In order to test this hypothesis HPLC methods were employed, and a few pitfalls in the common method of determining PBGD activity were revealed.

Precipitation of porphyrins in the presence of proteins by TCA

In order to demonstrate which porphyrins are formed during incubation of rat liver homogenate with PBG, the HPLC method 2 (see Experimental) for separating 2–8-carboxylic porphyrins was employed. The homogenate was incubated with PBG as described in Experimental. The effect of including citrate and desferrioxamine, inhibitors of haem formation in the incubation medium, was also studied. The reaction was terminated by TCA (12.5% final concentration).

After centrifugation, 100 μl of the supernatant were injected into the column. As shown in Fig. 2a–c only uroporphyrin and heptacarboxylic porphyrin were obtained in the presence and absence of the various inhibitors. This surprising observation could indicate that only these porphyrins are formed during incubation. However, a different conclusion can be drawn from the pattern of separation of the standard porphyrins, under similar conditions. Fig. 2d shows that when the standard porphyrins are mixed with 12.5% TCA, centrifuged and separated, the chromatogram obtained is similar to that of a standard mixture in 1 *M* acetic acid (see Fig. 1C). On the other hand, if liver homogenate protein (1.25 mg/ml) is added to the standard mixture, followed by 12.5% TCA, only 50% of the coproporphyrin is observed and 2-carboxylic porphyrin is hardly visible (Fig. 2e). It should be noted that both 2-carboxylic porphyrins tested (mesoporphyrin, which is present in the standard mixture, and protoporphyrin, prepared separately), shared the same retention time and were similarly affected by TCA (not shown).

The above data might indicate that even if protoporphyrin were formed during incubation of homogenate with PBG, it would not be detected because it would probably be precipitated by TCA in the presence of homogenate protein. It was shown recently that, in the presence of 10% TCA, it is impossible to extract protoporphyrin from human plasma [12].

In order to further understand the effect of protein precipitation by TCA on porphyrin detection, further experiments were carried out.

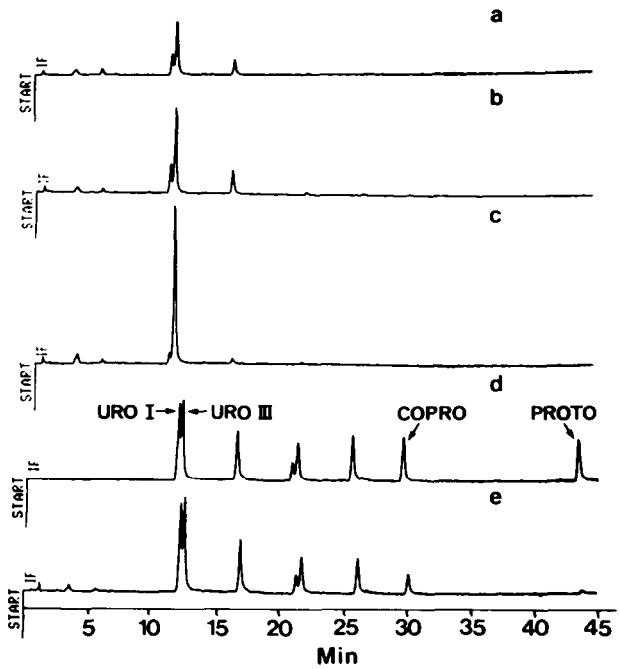


Fig. 2. Detection of formation of porphyrins by homogenate in the presence of protein and 12.5% TCA. Rat liver homogenate (1.25 mg protein per ml) was incubated with PBG (a) alone, or (b) with 1.5 m*M* desferrioxamine, or (c) with 20 m*M* citrate. The reaction was terminated by 12.5% TCA. After centrifugation the supernatant was brought to pH 2.4 and 100 μl were injected into the column and separated by HPLC method 3. (d), (e) Chromatograms of standards (250 pmol/ml), in the absence (d) or the presence (e) of 1.25 mg of homogenate protein. Peaks: URO I, III = uroporphyrin I, III; COPRO = coproporphyrin; PROTO = protoporphyrin.

Uroporphyrin, coproporphyrin and protoporphyrin were added separately (12 pmol/ml) to rat liver homogenate (1.25 mg protein per ml). TCA was added at various concentrations and, after centrifugation, the concentration of the porphyrins was determined in the supernatant and compared with the concentration in the absence of homogenate. As shown in Fig. 3, TCA did not change the concentration of uroporphyrin but reduced those of coproporphyrin and protoporphyrin in the presence of protein. The decrease was correlated positively and significantly with the concentration of TCA ($p < 0.001$) and inversely with the number of carboxylic groups of the porphyrins.

In order to prove that TCA causes precipitation of porphyrins in the presence of protein and does not simply interfere with their fluorescence, the precipitates were extracted with 0.5 *M* HCl and recentrifuged. The supernatants did indeed contain quantitatively the "missing" porphyrins (results not shown).

The precipitation of porphyrins was also positively and significantly correlated with the concentration of homogenate protein in the reaction medium, even at

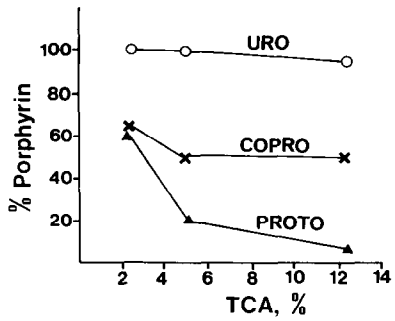


Fig. 3. Effect of protein precipitation by TCA on the detection of porphyrins. Uroporphyrin (URO), coproporphyrin (COPRO) or protoporphyrin (PROTO) were added at a final concentration of 12 pmol/ml to rat liver homogenate prepared in 50 m*M* Tris–HCl buffer (pH 8.2) (1.25 mg protein per ml). TCA at various concentrations was added and, after centrifugation, the fluorescence of the supernatant at 404 nm excitation and 595 nm emission was determined. The fluorescence of the various porphyrins in TCA in the absence of homogenate was taken as 100%. The results are the means of three separate determinations.

low concentrations of TCA (Fig. 4). In the presence of 2.5% TCA and 1.25 mg protein per ml only 30% of the protoporphyrin precipitated, but we decided not to use this concentration of TCA because it is too low to precipitate proteins adequately. The concentration of 5% TCA was chosen for further experiments.

Dimethyl sulphoxide (DMSO) as preventor of precipitation of porphyrins caused by TCA

DMSO was shown by Guo *et al.* [13] to increase the fluorescence of protoporphyrin when homogenate proteins were precipitated by 5% TCA. It was suggested that DMSO releases protoporphyrin from the protein precipitated by

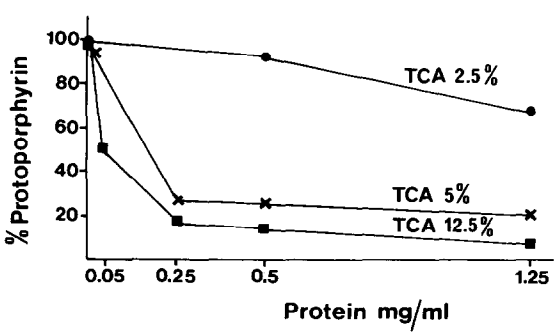


Fig. 4. Correlation between the concentrations of protein and of TCA on the precipitation of protoporphyrin. Standard protoporphyrin at a final concentration of 12 pmol/ml was added to rat liver homogenate prepared in Tris–HCl buffer and containing 0.05–1.25 mg protein per ml. Triplicates were prepared for each dilution of the homogenate, and TCA was added to each of them at different final concentrations, (2.5, 5 and 12.5%). After centrifugation the fluorescence of the supernatants was determined (see Fig. 4). Each point is the average of two determinations in two separate experiments.

TCA. In preliminary experiments we found that once the protoporphyrin has been precipitated by TCA it cannot be extracted by DMSO. However, if DMSO (25% final concentration) is added to the mixture of protein and protoporphyrin before addition of TCA, it prevents the binding of *ca.* 55% of the protoporphyrin to proteins (results not shown).

DMSO (25%) also partially prevents the precipitation of coproporphyrin, which occurs in the presence of TCA and protein, as shown in Fig. 5. Therefore, the concentration of DMSO chosen for further experiments was 25%.

In order to determine the optimal concentration of TCA in the presence of 25% DMSO, the experiment shown in Fig. 6 was carried out. In the presence of 12.5% or 5% TCA, the addition of 25% DMSO resulted in recovery of 30% or 60% of the protoporphyrin and coproporphyrin, respectively, in the supernatant. Therefore, the concentrations of 5% TCA and 25% DMSO were chosen for further experiments. As to the concentration of protein, although at a lower concentration, 0.25 mg of protein per ml, *ca.* 90% of the protoporphyrin was found in the supernatant, we continued our experiments with 1.25 mg of protein per ml. Only at that concentration were enough porphyrins for HPLC detection formed by rat liver homogenate incubated with PBG.

Optimal assay and detection conditions for determination of accurate PBGD

Homogenate was incubated in the presence of the various inhibitors of haem synthesis, and the reaction was terminated by the simultaneous addition of 5% TCA and 25% DMSO. The porphyrins in the supernatant were separated and quantitated by HPLC using the 4-min method (method 1), which separates 4–8-carboxylic porphyrins from protoporphyrin. As shown in Fig. 7, protoporphyrin

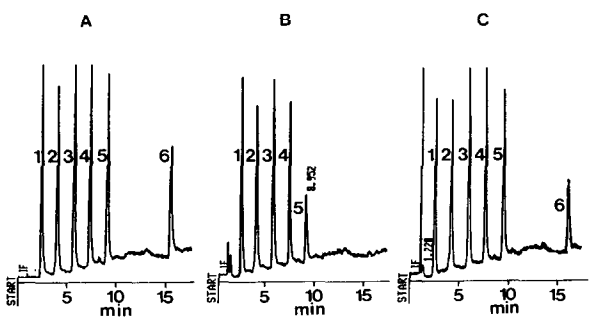


Fig. 5. HPLC separation of standard mixtures of porphyrins in 5% TCA in the presence and absence of protein and DMSO. The standards of 2–8-carboxylic porphyrins were prepared as described in Experimental. The final concentration of each of the six porphyrins was 5 nmol/ml in 3 *M* HCl, and was further diluted 1:20 (v/v) in the following solutions: (A) 5% TCA; (B) homogenate (1.25 mg protein per ml) and 5% TCA; (C) homogenate (1.25 mg protein per ml), 25% DMSO and 5% TCA. After centrifugation the supernatant was brought to pH 2.4, and 100 μl were injected into the column. HPLC method 2 was used. Peaks: 1 = uroporphyrin; 2 = heptacarboxylic porphyrin; 3 = hexacarboxylic porphyrin; 4 = pentacarboxylic porphyrin; 5 = coproporphyrin; 6 = 2-carboxylic prophyrin.

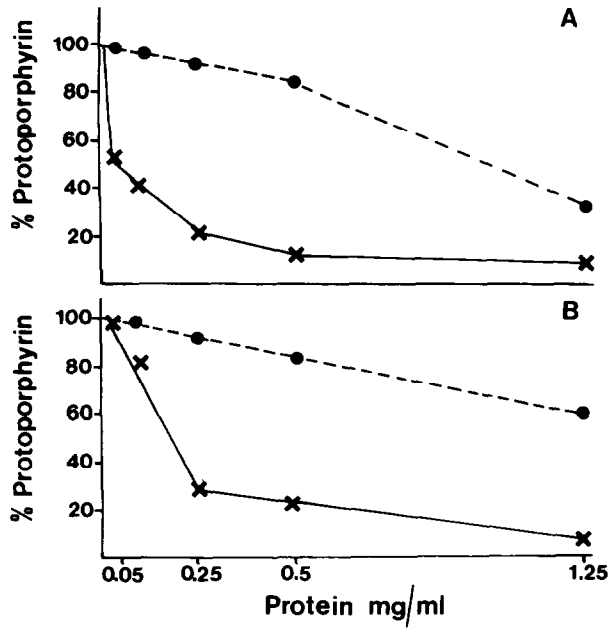
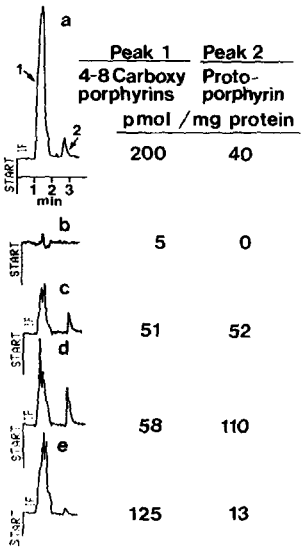


Fig. 6. Effect of DMSO on protoporphyrin precipitation at different concentrations of protein and TCA: 12.5% TCA (A) or 5% TCA (B) was added to homogenates in various dilutions in buffer, containing standard protoporphyrin (12 pmol/ml) in the absence (×) or presence (●) of 25% DMSO (v/v). After centrifugation the concentration of protoporphyrin present in the supernatant was determined fluorimetrically. Its concentration in the absence of protein was considered to be 100%. The values shown are the averages of determinations obtained in two separate experiments.

accumulated during the incubation of homogenate with PBG even without an inhibitor of haem synthesis in the medium (Fig. 7c). Its concentration was 52 pmol/mg of protein, which was similar to the sum of the concentrations of the other porphyrins formed. In the presence of 1.5 m*M* desferrioxamine its concentration was doubled (Fig. 7d), indicating that for the accurate determination of PBGD, haem formation should be inhibited. Since, in the presence of citrate, 4–8-carboxylic porphyrins were increased and protoprophyrin was decreased (Fig. 7e), one may assume that citrate inhibits an enzyme proximal to FC in the haem biosynthetic pathway.

In order to examine the site of the assumed inhibition, the porphyrins formed during incubation of the homogenate with and without citrate were separated by HPLC method 3, which also partially separates isomers I and III of uroporphyrin, in addition to separation of the other porphyrins (see Fig. 1C). As demonstrated in Fig. 8A and B, in the presence of citrate uro-III is markedly increased and 7–2-carboxylic porphyrins are decreased, probably owing to inhibition of uroporphyrinogen decarboxylase (UROD) by citrate. It should be pointed out that a quantitatively similar increase was obtained in uro-I instead of uro-III



	Peak 1 4-8 Carboxy porphyrins	Peak 2 Proto-porphyrin
	pmol / mg protein	
a	200	40
b	5	0
c	51	52
d	58	110
e	125	13

Fig. 7. Porphyrins formed by rat liver homogenate under various conditions. Homogenate containing 1.25 mg protein per ml was incubated in the presence of PBG alone (c) or with added 1.5 m*M* desferrioxamine (d) or 20 m*M* citrate (e). (a) Standard mixture containing 2–8-carboxylic porphyrins was handled exactly as the samples. (b) The situation at time zero of the reaction. After the addition of DMSO–TCA, and pH adjustment, 100 μl were injected into the column. HPLC method 1 was used for separation.

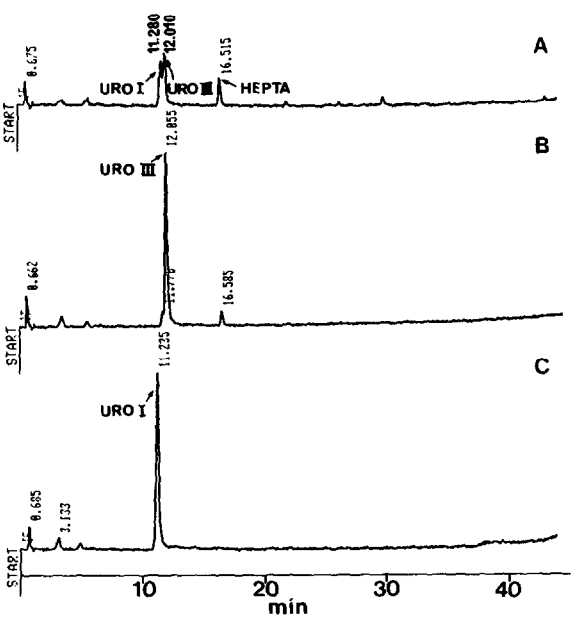


Fig. 8. Effect of citrate and heating on the formation of porphyrins by homogenate. Rat liver homogenate (1.25 mg protein per ml) was incubated with PBG (A) in the absence of (B) in the presence of 20 m*M* citrate. (C) Another sample of homogenate was heated for 1 h at 56°C prior to incubation. The reaction was terminated by addition of DMSO–TCA. After pH adjustment a 100-μl aliquot of the supernatant was injected into the column. HPLC method 3 was employed. Peaks: URO I, III = uroporphyrin I, III; HEPTA = heptacarboxylic porphyrin.

TABLE I

PBGD ACTIVITY OBTAINED USING VARIOUS ASSAY CONDITIONS

Rat liver homogenate (1.25 mg protein per ml) was incubated with PBG (1), with the addition of 20 m*M* citrate (2), 1.5 m*M* desferrioxamine (DES) (3) or both (4). One of the samples (5) was heated at 56°C for 1 h prior to the incubation. The reaction was terminated by 12.5% TCA, or 25% DMSO–5% TCA, or 1 *M* perchloric acid–methanol (1:1, v/v). After centrifugation, the fluorescence of 1 ml of the supernatant was determined at 404 nm excitation, 595 nm emission. The pH of the remaining supernatant was brought to 2.4, and 100 μl were injected into the column. HPLC method 2 was used to separate the various porphyrins formed during incubation. Quantification was performed by comparing the chromatograms obtained with chromatograms of standards handled exactly in the same way as the samples. We could not obtain a separation of the porphyrins by HPLC in perchloric acid–methanol, therefore the corresponding values are missing. The values are in pmol/mg protein/h, and are averages of three separate determinations, which differed by less than 10% from each other.

Treatment	Fluorimetry			HPLC	
	TCA	DMSO–TCA	Perchloric acid–methanol	TCA	DMSO–TCA
1. None	55	65	76	45	92
2. Citrate	110	72	78	125	138
3. DES	62	82	90	60	153
4. Citrate + DES	100	105	92	120	155
5. 56°C	123	110	–	138	140

when the homogenate was heated for 1 h at 56°C prior to incubation with PBG (Fig. 8c), owing to inhibition of uroporphyrinogen III cosynthase.

Because it was shown that factors such as the presence of inhibitors of haem synthesis or use of TCA affect the determination of PBGD, various conditions of the assay procedure were compared in order to reveal which method leads to the most accurate estimation of PBGD activity.

As shown in Table I, the highest activity of PBGD was obtained when the incubation was carried out in the presence of desferrioxamine, with or without citrate, stopping the reaction by addition of DMSO–TCA and injecting the supernatant into the HPLC column. A value of 153 pmol/mg protein/h was calculated, which is three times the value obtained in the absence of haem synthesis inhibitors, with fluorescence detection. However, if HPLC is not available, we recommend incubation with citrate, which, apparently, inhibits UROD, or heating of the sample before incubation at 56°C for 1 h, which inhibits uroporphyrinogen III cosynthase. Under these conditions (citrate or heating), only uroporphyrin and heptacarboxylic porphyrin are formed. Therefore it is possible to use 12.5% TCA for precipitation of protein and to determine the concentration of uroporphyrin by fluorimetery, leading to values close to those obtained with HPLC.

It is interesting to note that when porphyrins are extracted after incubation

into media, such as perchloric acid–methanol, which extract protoporphyrin as well as other porphyrins, and determined fluorimetrically without separation by HPLC, the calculated activity of PBGD is lower when compared with those obtained under similar assay conditions and with HPLC. This is due to the difference in uroporphyrin and protoporphyrin fluorescence spectra, both in DMSO–TCA and in perchloric acid–methanol (1:1, v/v), as shown in Fig. 9.

PBGD activity is determined fluorimetrically at 404 nm excitation and 595 nm emission, and uroporphyrin serves as a standard. At these wavelengths the fluorescence of protoporphyrin is only one third of that of a similar concentration of uroporphyrin. Therefore, it is inaccurate to calculate the concentration of a mixture of porphyrins containing approximately the same amount of uroporphyrin and protoporphyrin fluorimetrically, *i.e.* at one fixed wavelength.

We may therefore conclude that for accurate determination of the activity of PBGD one should use an inhibitor of haem synthesis during the incubation of the sample with PBG.

If desferrioxamine is used, protoporphyrin as well as other porphyrins are formed, and therefore TCA–DMSO should be used for precipitation of proteins. Since a few porphyrins with different spectra are involved, the use of HPLC is necessary for determining the exact concentration of each of them.

However, if 20 m*M* citrate is added to the incubation medium, or it the sample is heated for 1 h at 56°C (more time-consuming) only uroporphyrin and heptacar-

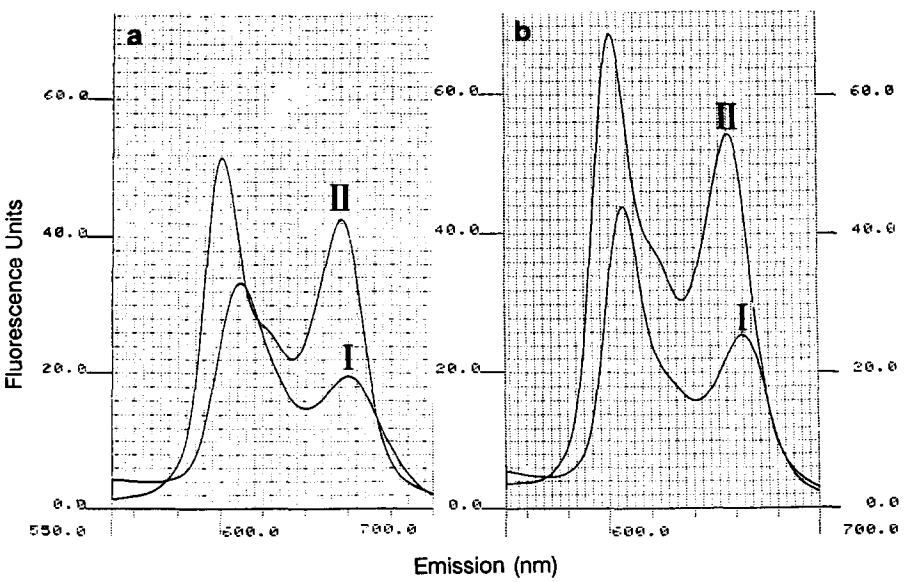


Fig. 9. Fluorescence spectra of protoporphyrin and uroporphyrin in perchloric acid–methanol and in DMSO–TCA. Protoporphyrin (I) and uroporphyrin (II) were prepared, each 1 μM, in either (a) 1 *M* perchloric acid–methanol (1:1, v/v) or (b) 5% TCA–25% DMSO. The spectra were measured at 404 nm excitation (fixed wavelength) and 550–700 nm emission (variable wavelength).

boxylic porphyrin are formed. Therefore, TCA alone, without DMSO, may be used in the absence of HPLC to precipitate porphyrins, and the determination can be carried out with a fluorimeter.

Based on the data reported, the detailed procedure recommended for determining PBGD activity is as follows. Incubation of 1 ml of homogenate in Tris–HCl buffer (pH 8.2) (3 mg protein per ml) with 1 ml of 20 m*M* sodium citrate dissolved in Tris–HCl buffer (pH 8.2) and 0.4 ml of 0.5 m*M* PBG. The reaction is terminated by the addition of 0.9 ml of DMSO followed by 0.36 ml of 50% TCA. After centrifugation for 10 min at 4000 *g*, 20 μl of 10 *M* NaOH are added to 1 ml of the supernatant. A 100-μl aliquot of the latter is then injected into the column. The supernatant obtained may be kept for up to a week at 4°C. The addition of NaOH should be carried out immediately prior to injection into the HPLC column.

The recovery of uroporphyrin (125 pmol/ml) and protoprophyrin (125 pmol/ml) added to the homogenate prior to the incubation was found to be 96.5 ± 3.2% and 48.2 ± 3.7%, respectively.

The within-assay coefficient of variation (C.V.) was 4.4% for PBGD activity of 156 ± 6.9 pmol porphyrin/mg protein/h (n = 7) and the between-assay C.V. was 6.9% for an activity of 162 ± 11 pmol porphyrin/mg protein/h.

None of the methods commonly used for measuring PBGD activity (14–16) takes into account that haem formation during the incubation of homogenate with PBG might reduce the measured PBGD activity. Therefore, the values of PBGD activity reported in many studies are probably underestimated and should be re-examined.

Many reports have been published concerning enzymic defects in the haem biosynthetic pathway in the various types of porphyria. In variegate porphyria (VP) all the investigators agree that the last steps of haem synthesis are inhibited, either by protoporphyrinogen oxidase (PO) or FC [17,18]. However, there are conflicting reports concerning PBGD activity [17–20]. Some investigators report decreased activity [17,18] and others unchanged activity [19,20]. These discrepancies may be due to different methods employed for the determination of PBGD. In VP, PO or FC are inhibited; thus, the true activity of PBGD should be measured and compared with normal in the presence of citrate or any other inhibitor of FC that does not affect PBGD activity, otherwise an increased PBGD activity would be obtained if its actual activity is normal, and a normal activity if its actual activity is reduced. Support is lent to this last assumption by our findings (not shown) that PBGD activity determined in the presence of citrate in lymphocytes of two VP patients is significantly reduced, compared with normal people.

Therefore, in order to eliminate any interfering effect of haem formation, and to obtain a better analytical approximation of PBGD activity, we recommend including 20 m*M* citrate (final concentration) in the incubation medium as a routine procedure in all PBGD determinations examined according to Magnus-

sen *et al.* [6]. This is of special importance when measuring PBGD activity in unknown systems or in experimental systems in which the effect on haem formation of various investigated factors is unclear. In addition to measurement of true activity of PBGD, one may also use the method described above, *i.e.* combination of incubation of homogenate with PBG and HPLC separation for detecting defects in the haem biosynthetic pathway, according to the pattern of accumulation of the various porphyrins.

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