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BIOSYNTHESIS OF UROPORPHYRINOGEN III IN BOVINE LIVER

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in partial fulfillment of the requirements
for the degree of Doctor of Philosophy,
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ABSTRACT

Bovine liver porphobilinogenase, an enzyme which converts porphobilinogen (PBG) to uroporphyrinogen III, has been studied.

Active site specificity for the leaving group was investigated by using PBG derivatives in which the amine group of the sidechain had been replaced by imidazole, imidazole derivatives (N-methylimidazole and 2-methylimidazole) and methylated amine. These derivatives were characterized by gel filtration, thin layer chromatography, and paper electrophoresis. PBG derivatives of imidazole, N-methylimidazole, 2-methylimidazole, and a mixture of methylamine, dimethylamine, and trimethylamine served as good substrates for bovine liver porphobilinogenase in the biosynthesis of uroporphyrinogen III. When these PBG derivatives were enzymatically consumed, they were completely converted to uroporphyrinogen III. Enzymatically-consumed PBG was only partially ($\sim$50%) converted to uroporphyrinogen III. When glucose and glucose oxidase were added to the PBG incubation

mixture to "scour" residual oxygen, PBG was completely converted to uroporphyrinogen III. Apparently, under our experimental conditions, PBG and oxygen are substrates for a PBG oxygenase whereas the derivatives of PBG do not serve as substrates for PBG oxygenase.

The effects of alkylation on the enzyme and of anaerobic and aerobic conditions on the enzymatic utilization of PBG were investigated. Porphobilinogenase alkylated with methyl iodide was partially deactivated. Anaerobiosis was much more effective than aerobiosis for the formation of uroporphyrinogen III from enzymatically-consumed PBG, although more PBG was enzymatically consumed under aerobic conditions. This again points to consumption of PBG by porphobilinogenase and by oxygenase in presence of oxygen.

Conditions were sought for accumulating and isolating intermediates. All attempts involving electrophoresis, thin layer chromatography, and spectrophotometric assays failed to detect or characterize any intermediates in the biosynthesis of uroporphyrinogen III.

Some speculations about the active site and the mechanism of action of bovine liver porphobilinogenase are made in the light of the enzymatic and non-enzymatic experimental results with PBG and its derivatives.

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To my husband Raymond and my parents,

I dedicate this thesis with love and appreciation

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to the following people who have made this thesis possible.

Foremost, I am deeply grateful to my mentor, Professor C.S. Russell, whose adroit guidance and relentless encouragement have given me an invaluable experience in research. Her personal involvement in this work has been most rewarding to me and I am indebted to her professionalism.

Also I would like to express my sincere appreciation to members of my committee——Dr. Lukton, Dr. Mazur, Dr. Schulz, and Dr. Meislich. Their suggestions and insights have supplemented in molding this thesis.

My thanks also go to my colleagues——Sy Garte and Angelo Turturro——who have made my stay both pleasant and rewarding.

Finally, I would like to express my deepest gratitude to my parents for their encouragement and their understanding and to my husband for his love and patience throughout the completion of this work.

INTRODUCTION

Uroporphyrinogen III is the biological precursor of hemes, chlorophylls, cytochromes, and vitamin B_{12}[1]. Its biosynthesis involves the tetramerization of porphobilinogen (PBG) and is catalyzed by porphobilinogenase, a combination of two enzymes: uroporphyrinogen I synthetase (deaminase) and uroporphyrinogen III cosynthetase (isomerase). In the presence of deaminase alone, four molecules of PBG are converted by head to tail condensation and ring closure to one molecule of uroporphyrinogen I (urogen I). However, deaminase and isomerase (porphobilinogenase) together catalyze the condensation of four molecules of PBG to form one molecule of uroporphyrinogen III (urogen III) where at least one step of the condensation must involve an isomerization. Isomerase alone has no catalytic effect on PBG or on urogen I and might be considered as a modifier protein (Fig. 1).

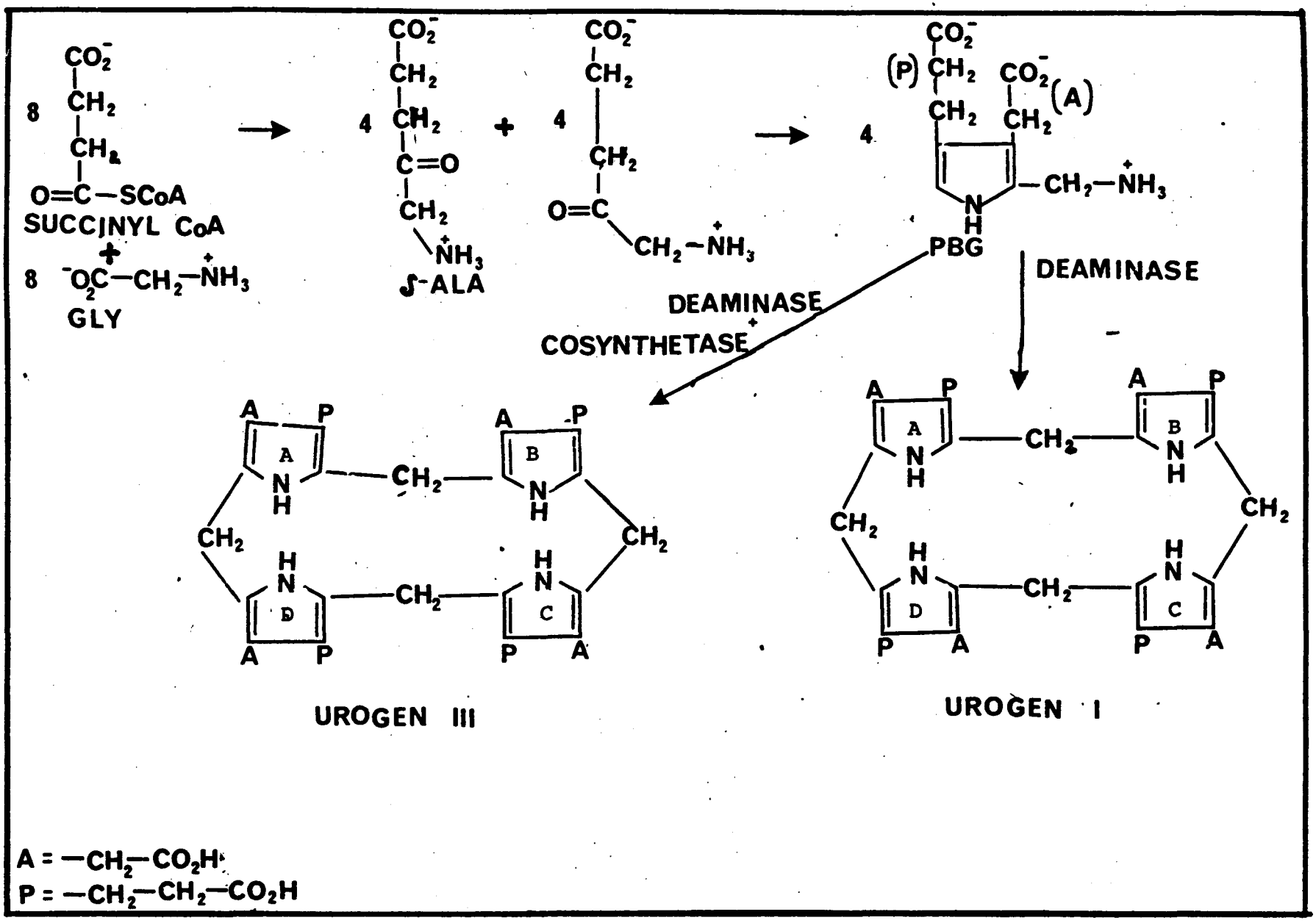
The mechanism for the catalytic condensation of PBG to urogen III is unclear and is an area of active investigation. Many recent experimental results, however, are beginning to unravel the condensation mechanism. Pluscec and Bogorad[2]

)

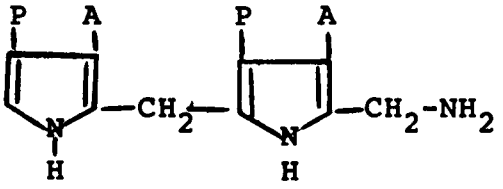
4

Fig. 1

Biosynthesis of Urogen I and Urogen III Catalyzed by Deaminase and by Deaminase + Cosynthetase (Porphobilinogenase) Respectively.


8
SUCCINYL CoA
8
GLY
4
δ-ALA
4
4
PBG
DEAMINASE
DEAMINASE
COSYNTHETASE
UROGEN III
UROGEN I
A = —CH2—CO2H
P = —CH2—CH2—CO2H

isolated a dipyrrylmethane (DPM) from the incubation of spinach deaminase with PBG in the presence of hydroxylamine.

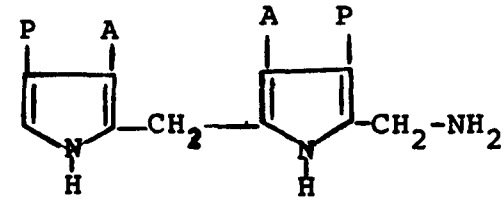


A = Acetic Acid
P = Propionic Acid

DPM

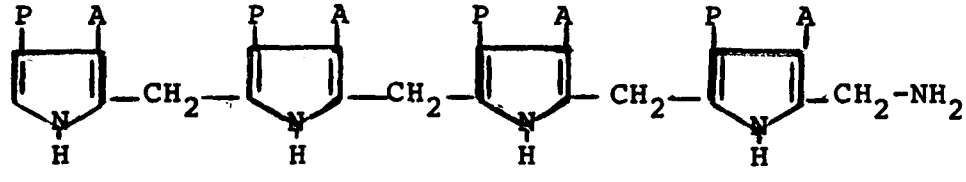
It was found that uroporphyrinogen I synthetase did not catalyze the condensation of two molecules of DPM to form urogen I; however, in the presence of ^{14}C-PBG, isotope dilution data showed that DPM is incorporated into urogen I suggesting that deaminase acts as a polymerase and that DPM is a poor substrate for the enzyme. The fact that DPM is incorporated into urogen I in the presence of PBG also suggests that DPM is like an intermediate in the biosynthetic pathway. Frydman and coworkers[3] incubated synthetic ^{14}C-DPM (identical with the DPM isolated by Bogorad) with wheat germ deaminase and the extent of enzymatic incorporation of DPM was much lower than that of Bogorad. It was found that DPM is neither a substrate nor an intermediate in the biosynthesis of urogen III. The experimental results indicated that DPM is not a free substrate but could resemble an enzyme-bound intermediate formed in the process because the enzymatic incorporation of ^{14}C-DPM into urogen I was quite low. Frydman and coworkers[4] incubated

the synthetic dipyrrylmethane (DPM-III) corresponding to a rearranged dimer of PBG with wheat germ porphobilinogenase.



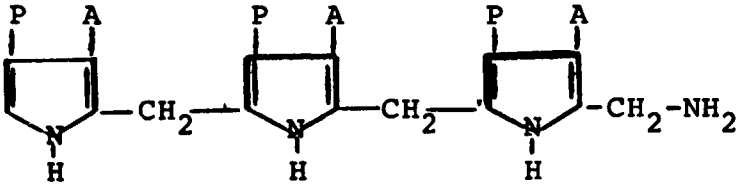
A = Acetic Acid
p = Propionic Acid DPM-III

in the presence of ^{14}C-PBG, a decrease in the specific activity of urogen III was evident, indicating that DPM-III was incorporated. No decrease was observed in the specific activity of urogen I. DPM-III was incorporated to a very small extent into urogen III and is considered to be a very poor substrate for the enzyme system. It may resemble an intermediate in the reaction and may get onto the active site to some extent. Radmer and Bogorad[5] reported the isolation from the spinach deaminase system in the presence of ammonium ion, of a tetrapyrrylmethane (TEPM) which was converted to urogen I enzymatically.



TEPM

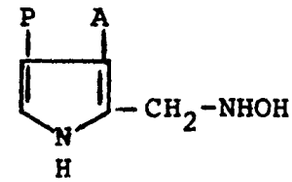
TEPM was neither a substrate nor an intermediate in the biosynthesis of urogen III but probably resembles the enzyme-bound tetrapyrrole which yields urogen I. TEPM was not converted to urogen III by urogen III cosynthetase. ^{14}C-TEPM was incubated with ^{12}C-PBG with spinach porphobilinogenase. No radioactivity was incorporated in urogen III; radioactivity was incorporated only into urogen I. Radmer and Bogorad also showed that the rate of cyclization of the isolated TEPM is not increased by uroporphyrinogen I synthetase indicating that TEPM probably does not exist free in solution as a precursor of urogen I in a normal, noninhibited enzymatic reaction. Frydman and coworkers[6] incubated a synthetic tripyrrylmethane (TRPM) with wheat germ prophobilinogenase.



TRPM

They showed that TRPM, in the presence of ^{14}C-PBG, was neither a substrate nor an intermediate for the uroporphyrinogen III cosynthetase. Addition of TRPM in the presence of ^{14}C-PBG to wheat germ porphobilinogenase had a marked influence on the

total porphyrin formation and the isomer distribution. The proportion of urogen I increased while a parallel decrease was observed in the amount of urogen III and of total porphyrin. A large excess of TRPM must be used in order to detect the small but significant (2% to 8%)[6] enzymatic incorporation of TRPM into urogen I. Davies and Neuberger[7] working with deaminase from R. spheroides in the presence of ammonia, hydroxylamine, or methoxyamine, observed the accumulation of polypyrroles. They reported that these inhibitory amines are incorporated into the polypyrroles. A monopyrrole compound different from PBG was observed in incubation mixtures when hydroxylamine was used. It was formed enzymically and the following structure is postulated.



Llambias and Batlle[8] obtained supernatant solutions (from soybean callus porphobilinogenase incubation mixtures) without using inhibitors which, together with PBG, were converted to urogen III in the presence of deaminase alone. In other words, they were able to obtain a free polypyrrolic intermediate which is used as a substrate for the deaminase to form urogen

III. These results have not been obtained with other systems. Battersby, Hunt, and McDonald[9], using ^{13}C-N.M.R. measurements on enzymatically produced protoporphyrin IX (which is directly derived from urogen III), showed that in avian blood and Euglena, ring D (Fig. 1) is derived from a PBG unit which has undergone intramolecular rearrangement and that PBG has been incorporated intact into rings A,B, and C. All of this evidence, with the exception of Llambias' and Batlle's work, suggest 1) that the entire enzymatic process in the biosynthesis of urogen I and urogen III takes place on the enzyme surface, with no liberation of free polypyrrylmethane intermediates at any stage of the biosynthetic process 2) that urogen I and urogen III must originate by different pathways from the start of the enzymatic polymerization[3,4].

In this thesis, experimental results are presented to probe further into the mode of biosynthesis of urogen III by bovine liver porphobilinogenase (urogen I synthetase and urogen III cosynthetase). The areas explored are as follows:

1) The effects of new substrates on porphobilinogenase: PBG derivatives with leaving groups other than $-\overset{+}{N}H_3$.

a) Non-enzymatic reactions of methyl iodide (CH_3I) or imidazole (Im) or N-methylimidazole (N-Me-Im) or 2-methyl-imidazole (2-Me-Im) with porphobilinogen (PBG).

b) The effect on substrate consumption and product formation of incubating PBG derivatives (PBG-Im, PBG-N-Me-Im, PBG-2-Me-Im, and methylated PBG) with porphobilinogenase.

2) Detection and characterization of intermediate polypyrroles during the lag period (after substrate (PBG) consumption and before product formation (urogen I&I)).

3) Enzymatic effects of anaerobiosis versus aerobiosis on substrate consumption and product formation during incubation.

4) Alkylation of bovine liver porphobilinogenase by methyl iodide.

Based on the results of experiments pursuing the goals described above, some conclusions and suggestions about the nature of bovine liver porphobilinogenase and its enzymatic reaction are presented.

MATERIALS AND METHODS

Chemicals

Porphobilinogen (PBG) was a generous gift from Professor David Shemin of Northwestern University. Earlier batches of PBG used were purchased from Sigma Chemical Co. (St. Louis, Mo.) and from Protex Research and Consulting (Montreal, Canada). Uroporphyrin I-octamethyl ester, uroporphyrin III-octamethyl ester (Waldenström Type), coproporphyrin I-octamethyl ester, coproporphyrin III-octamethyl ester, and glucose oxidase (purified, type II) were purchased from Sigma Chemical Co. Uroporphyrin I and III were prepared from the corresponding methyl esters by hydrolysis in concentrated HCl in the dark at room temperature for at least 24 hours. p-Dimethylaminobenzaldehyde for Ehrlich's reagent was purchased from J.T. Baker Chemical Co. (Phillipsburg, N.J.). Calcium phosphate gel was purchased from Bio Rad Laboratories (Richmond, Calif.). Sephadex G-15, G-25, and G-100 were purchased from Pharmacia. The modified Ehrlich's reagent in acetic acid-perchloric acid used to detect pyrroles quantitatively was

prepared according to the method of Mauzerall and Granick[10]. The Ehrlich's reagent used for detection in thin layer chromatography and paper electrophoresis was prepared according to the method of Sohler[11] and Irvine[12].

Substrates

Porphobilinogen was dissolved in .05M Tris buffer (pH7.4) and the pH adjusted to 7.4.

The derivatives of porphobilinogen were chemically synthesized in the following manner. Four stock solutions of PBG (1mg/5ml; 10^{-3}M), .1M imidazole (Im), .1M N-methyl-imidazole (N-Me-Im), and .1M 2-methylimidazole (2-Me-Im) were all made in .05M Tris buffer with a final pH of 7.4. A stock solution of 10^{-3} M PBG (1mg/5ml) and .1M 2-methylimidazole (2-Me-Im) were also made in .05M Tris buffer with a final pH of 8.5. Excess Im or N-Me-Im or 2-Me-Im solution at pH 7.4 or 2-Me-Im solution at pH 8.5 was mixed with PBG solution at 25^{o}C such that the molarity of the various imidazoles was at least $2x10^{-2}$M. A typical run consisted of 1 ml of PBG and 1.5 ml of the various imidazoles. The mixtures were allowed to react at 25^{o}C for at least 30 minutes or at 37^{o}C for at least 15 minutes. Aliquots of the PBG derivatives were then kept frozen for all other assays. Polygram thin layer chromatography was used to

gauge time required for completion of the reaction and it was shown that 30 minutes were sufficient for relatively small amount of PBG to react completely with a large excess of Im or N-Me-Im or 2-Me-Im. At pH 7.4 PBG did not react appreciably with 2-Me-Im (pK_a 8.1) because at pH 7.4 the nitrogens on 2-Me-Im are protonated. When 10^{-3}M PBG (dissolved in .05M Tris buffer, pH 8.5) was allowed to react with 2-Me-Im at pH 8.5, the reaction took place.

Polymethylated PBG (attempts were not made to determine the extent of methylation) was made in the following manner. Approximately .5 ml to 1 ml of CH_3I was used to react with 10^{-3}M PBG at pH 7.4 and at pH 9.5 in a test tube with constant stirring for 30 minutes. It was found that methylation occurs much more effectively if the PBG solution is at pH 9.5 (pK_a of the ammonium group on PBG is 10.1)[13].

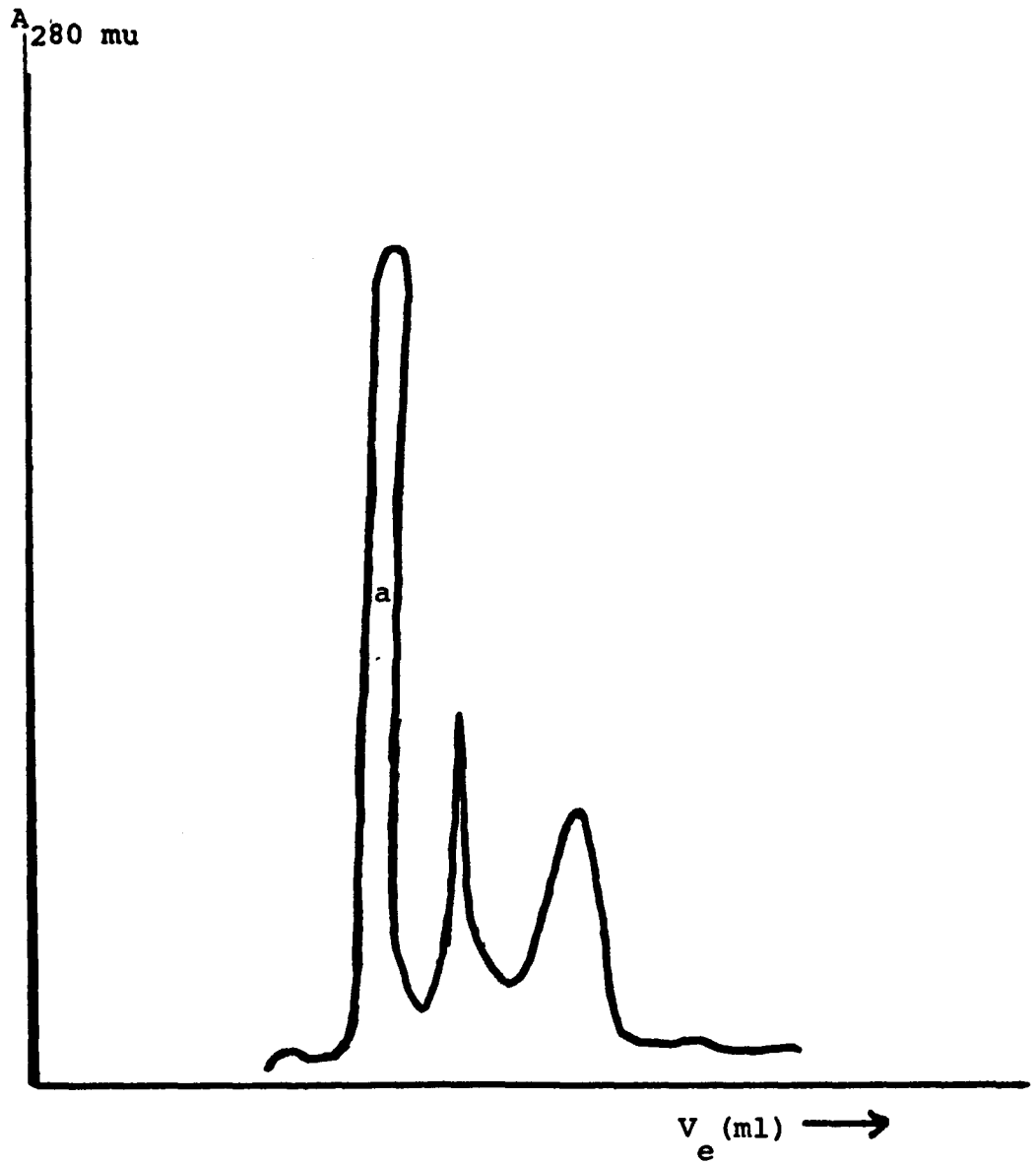
Isolation and Purification of Enzymes

Bovine liver porphobilinogenase was isolated and purified from fresh frozen beef liver (Pel-Freeze of Arkansas) according to the method of Grinstein et al.[14]. A typical isolation and purification procedure of bovine liver porphobilinogenase is described. All operations were carried out in the cold room at 4°C. Homogenate (10% w/v) of bovine liver (80 gms) was prepared in .25M sucrose and the homogenate thus obtained was

centrifuged at 11,000g for 10 min. and the sediment was discarded. Glacial acetic acid was added dropwise to the supernatant to adjust it to pH 5.0; and after 20 min., the material was centrifuged for 10 min. at 11,000g and again the sediment was discarded. The supernatant solution was then fractionated with solid $(NH_4)_2SO_4$ to 34% saturation and stirred for 1 hr. The material was centrifuged for 15 min. at 11,000g and the sediment was discarded. The supernatant solution was again fractionated with solid $(NH_4)_2SO_4$ to 50% saturation and stirred for 3 hrs. The material was centrifuged for 15 min. at 11,000g and the pellet was kept frozen at $-15^{o}C$ overnight. The $(NH_4)_2SO_4$ pellet was dissolved in a small volume of .05M Tris buffer (pH 7.4) and passed through a Sephadex G-25 column (2 x 30 cm). The eluant from the first protein peak which contained enzyme activity (Fig. 2) was treated with calcium phosphate gel (1 mg protein: 5 mg gel). After being stirred for 10 min. the mixture was centrifuged at 11,000g for 5 min. and the sediment was discarded. Solid $(NH_4)_2SO_4$ was added to the supernatant to 50% saturation and the protein precipitate was dissolved in a small volume of Tris buffer. This enzyme preparation was then applied to the Sephadex G-100 column (3 x 35 cm) and fractions of 4 ml were collected. The eluting buffers for Sephadex G-25 and Sephadex G-100 were .05M Tris buffer (pH 7.4) and .05M Tris buffer-.1M NaCl (pH 7.4)

Fig. 2

Elution Profile of 50% Ammonium Sulfate Precipitate of Bovine Liver Porphobilinogenase on Sephadex G-25 Column (2 x 30 cm) as Monitored by LKB Uvicord II. Peak a Used in Further Purification.


A
280 mu
a
V_e (ml) ⟶

respectively.

Bovine liver porphobilinogenase is easily inactivated by trace quantities of ammonium ions. Therefore both Sephades G-25 and Sephadex G-100 columns were always carefully checked with Nessler's Reagent for the absence of NH_4^+. When Sephadex G-25 column was monitored by the LKB Uvicord, the first major protein peak contained the enzyme and was collected. A higher ratio of calcium phosphate gel to protein was used (5 mg gel to 1 mg protein instead of 2 mg gel to 1 mg protein) than Grinstein et al. Earlier enzymatic runs involved the use of calcium phosphate gel freshly prepared according to the method of Keilin and Hartree[15]. More recent enzymatic incubations involved the use of commercial calcium phosphate gel (Bio Rad Laboratory, Richmond, Calif.). The commercial calcium phosphate gel proved to be as effective as the freshly prepared calcium phosphate gel. The ammonium sulfate precipitates (before purification on G-25 and G-100) were stable in the freezer (-15°C) for three months. Bovine liver porphobilinogenase in .05M Tris buffer-.1M NaCl (pH 7.4) after being eluted off Sephadex G-100 was stable for a few weeks at 4°C. Storing the enzyme solution at -15°C for a few days seemed to deactivate the enzyme. Assays of the enzymes were done on all fractions off Sephadex G-100 between the two large protein peaks (Fig. 26). The active fractions

were pooled (16 ml) and used immediately.

Methylation of porphobilinogenase was carried out in the following manner. 0.5 ml to 1 ml of CH_3I was allowed to react with porphobilinogenase at pH 7.4 with occasional shaking at $4^{\circ}C$ for 10 min. and the excess CH_3I was blown out with pre-purified nitrogen. The methylated porphobilinogenase was then used immediately for incubation.

Equipment

Polygram cel 300, plastic sheets, pre-coated with cellulose, 20 x 20 cm (Brinkman, Westbury, N.Y.) were used for thin layer chromatography. Paper electrophoresis was done on Whatman #1 paper (10 x 30 cm) cut to (10 x 25 cm) in a Shandon electrophoresis apparatus (Model U77) with a Shandon VoKam power supply (BAE 2781). Gel chromatography was performed at $4^{o}C$ with a Pharmacia column (1.8 x 82 cm). Spectrophotometers used include Cary 15 (Varian Associates Instrument Division), Zeiss M4QIII (8742)(Germany), Beckman DU (Model 2400), and Hitachi fluorescence spectrophotometer (Perkin Elmer MPF-2A). Protein fractions and PBG derivatives were automatically collected with an LKB collector (Stockholm). A BLE (Black Light Eastern) viewing cabinet with excitation sources at 366 mu and 308 mu was used to detect fluorescence of porphyrins. Mass spectra were obtained with the Varian CH-5 mass spectrometer.

Determinations

a) Porphobilinogenase Assay

Porphobilinogenase has two measurable activities: the consumption of porphbilinogen or derivatives of porphobilinogen and the formation of uroporphyrinogens. The standard incubation system, unless stated otherwise, contained the following: substrate (100 nmoles to 600 nmoles) in .05M Tris buffer; .5 mM in EDTA (pH 7.4); .15 mmoles of NaCl; and .03 mmoles of $MgCl_2$; ($2x10^{-3}$ mmoles of cysteine in the earlier incubations); porphobilinogenase (.2 ml to . ml) made up to a final volume of 5 ml with Tris buffer(pH7.4). In one of the enzymatic runs, glucose (20 mg) and glucose oxidase (2 mg) were added in amounts specified by Delwiche et al.[16] to "scour" for residual oxygen in the PBG incubation mixture. It is known that glucose oxidase utilizes glucose and oxygen as its substrates. In later enzymatic runs, cysteine was omitted because it interfered with PBG assay with Ehrlich's reagent[17]. The enzymatic results did not seem to be different whether or not cysteine was present as long as the environment was kept anaerobic. Incubations were carried out anaerobically in the dark in Thunberg tubes with repetitive flushing and evacuation with pre-purified

nitrogen or aerobically in open test tubes in the dark with mechanical shaking at 37°C for 6 hrs. Blanks were generally run with all components except for the enzyme. At certain times, blanks were run with all components except for the substrate. Aliquots of incubation mixtures were taken out for analyses at t = 0, t = 3 hrs., t = 6 hrs.

i) Porphobilinogen: Spectrophotometric assays of PBG and its derivatives were made with modified Ehrlich's reagent[10]. A sample of incubation mixture containing PBG or PBG derivative was made up to .6 ml and was reacted with an equal volume of modified Ehrlich's reagent (.2 ml incubation mixture + .4 ml of .05M Tris + .6 ml of modified Ehrlich's reagent). The full magenta color at 553 mu was reached and measured after 15 minutes of development. The difference in absorbance for test and control samples from zero time measured the enzymatic consumption of PBG. A value of $\varepsilon = 5.77 \times 10^4$ l-mol^{-1} lcm^{-1} at 553 mu[10] was used in all experiments. PBG blanks were run in each case and used to correct for non-enzymic PBG loss.

ii) Uroporphyrins: Aliquots (.5 ml to 1 ml) of incubation mixtures containing uroporphyrinogens were oxidized to uroporphyrins in the dark according to the method of Jordan and Shemin[18]. In the earlier experiments, oxidation of the uroporphyrinogens by light was done according to the method

of Grinstein et al.[14] and Rimington[19]. Oxidation by I_2 in 2% HCl in the dark and addition of thiosulfate to reduce unreacted I_2 as described by Jordan and Shemin proved to be most satisfactory because oxidation was complete without producing a nonporphyrin band at 500 mu and was complete within a short period of time. After oxidation, the sample was centrifuged to remove denatured protein and scanned on the Perkin-Elmer 402 uv-visible spectrophotometer from 350 mu to 670 mu to obtain the overall absorption profile of uroporphyrins. The sample was then read on the Zeiss spectrophotometer M4QIII (8742)(Germany) at 405 mu for the exact absorbance. A value of $\epsilon = 5.41 \times 10^5 \text{l-mol}^{-1}\text{-cm}^{-1}$ at 405 mu in 2% Hcl[20] was used in all experiments. Test values were corrected for non-enzymatic formation of porphyrins by subtracting control results. The non-enzymic formation of uroporphyrin in 6 hours at 37°C was negligible.

b) Analysis of Uroporphyrin Isomers:

Uroporphyrins were esterified according to the method of Rimington[21] in 5% H_2SO_4-methanol for 24 hours. Paper chromatography of the uroporphyrin methyl esters followed the method of Falk and Benson[22]. The method involved two solvent systems. The first solvent system: $CHCl_3$ in 1% absolute

C_2H_5OH—kerosene (6:4) was used to move porphyrin esters away from the origin, leaving behind impurities and unesterified porphyrins. The paper chromatogram was then cut off about .5 cm below the porphyrin ester spots and was subjected to the second developing system: kerosene—dioxane (4:1.5)[23]. In our case, Whatman no. 3 paper was used instead of Whatman no. 1 because it was stiffer and could stand conveniently in the developing tank. No difference in resolution was observed between Whatman #1 and Whatman #3 paper.

This method has many drawbacks. Molecular complexes of methyl esters of uro I and III are known to be formed[24]. Entrainment of methyl ester of uro I by uro III and vice-versa is known to occur[25]. A more reliable method involves the decarboxylation of uroporphyrins to coproporphyrins and the analyses of the coproporphyrins I and III. The uroporphyrins I and III were decarboxylated to the corresponding coproporphyrins I and III according to the method of Edmondson and Schwartz[26]. Uroporphyrin in 2% HCl was diluted with distilled water until the concentration of HCl was 1%. The sample was placed in Carius tubes, frozen, evacuated for 10 min., and then sealed under vacuum. The Carius tube containing uroporphyrin was allowed to warm up to room temperature before placing in the oven for decarboxylation at 180°C for 3 hrs.

The tubes were opened. The method for the analysis of the corresponding coproporphyrin I and III isomers is described in detail in the Appendix. The uroporphyrin isomers or the resulting coproporphyrin isomers were cut out of the chromatograms and eluted with chloroform. The emission spectra of the porphyrins were measured on the Perkin Elmer fluorescence spectrophotometer MPF-2A, excitation λ 415 mu and fluorescence emission maximum λ 621 mu. The relative ratios of the fluorescence emission maxima at 621 mu was taken as a measure of uroporphyrin isomer distribution.

c) Non-enzymic Reactions of PBG and its Derivatives

i) Purification of PBG and its derivatives on Sephadex G-18 in .033M phosphate buffer, pH 8.0: The column (1.8 x 82 cm) had a void volume of 46.2 ml determined by using Blue Dextran (Pharmacia). PBG and its derivatives were determined quantitatively by mixing equal volumes of eluate and modified Ehrlich's reagent and reading the optical density after 15 min. on the Beckman Du (Model 2400) spectrophotometer. The elution profile of PBG was found to be different from the elution profiles of PBG derivatives (Fig. 3). Phenylalanine and tetra-alanine were used as rough standards for calibration of molecular weights. The elution profile of phenylalanine was determined by plotting absorbance of eluate at 280 mu and the elution profile of tetra-alanine was determined by treating eluate with ninhydrin (2mg/100ml .033M phosphate

buffer). A blue color developed after 10 min. of heating at 100°C and the absorbance was read at 570 mu. Graphs of elution volume versus log of molecular weights and K_{av} versus log of molecular weights were plotted (Fig. 4,5).

ii) Paper electrophoresis of PBG and its derivatives: Paper electrophoresis on Whatman #1 paper (10 x 25 cm) was run in .05M sodium diethylbarbiturate buffer, pH 9.2, for 2.5 hrs. at 4°C. The electrophoretograms were run at a constant voltage of 350 V (14V/cm) and at a minimum current of 16 milliamps. Uroporphyrin III was used as a standard marker by which to gauge electrophoretic mobility. After 2.5 hrs., the paper was dried. Uroporphyrins were detected by observing fluorescence by excitation at 366 mu. Porphobilinogen and its derivatives were visualized by the pink spots developed by spraying with Ehrlich's reagent[11,12].

iii) Cellulose thin layer chromatography on polygrams of PBG and its derivatives: Cellulose thin layer chromatography on polygrams was carried out at 25°C in two different solvent systems: n-butanol-acetic acid-water (63:11:26)[23] and n-propanol-ammonia-water (60:30:10)[27]. The solvents were allowed to equilibrate in the tanks for at least 1 hr. Chromatography on thin layer cellulose polygrams (20 x 10 cm) were run until the solvent fronts had moved more than 2/3 of the way from the origin. Uropor-

phyrin III-octamethyl ester which moves with the solvent front was applied in chloroform solution as an internal standard to each sample spot. Uroporphyrin esters and porphobilinogen and its derivatives were located as above.

iv) Visible spectra of PBG and its derivatives with Ehrlich's reagent: 8 to 12 nmoles of PBG were mixed with excess .1M Im or .1M N-Me-Im or .1M 2-Me-Im. One minute after mixing with the Ehrlich's reagent, the sample was scanned on the Cary 15 spectrophotometer at 553 mu. The maximum optical density was used to calculate the concentration of PBG using $\varepsilon = 5.77 \times 10^4$ liter-mol^{-1}-cm^{-1} [28]. Similar spectra were obtained with purified PBG and derivatives from Sephadex G-15.

v) Ultraviolet scans of PBG and its derivatives: The ultraviolet spectrum of porphobilinogen alone and in the presence of imidazole or N-methylimidazole was studied in split cells on the Cary 15. PBG in .05M Tris buffer (pH 7.4) was placed in one of the compartments of the split cell and excess imidazole or N-methylimidazole was placed in the remaining compartment. The reference cell contained the imidazole compound in one compartment and .05M Tris buffer in the remaining compartment. The above sample cell was scanned against the reference cell. The contents of the two compartments of the split cells were then mixed and after thirty minutes at 25°C, the sample cell was again scanned. The two ultraviolet spectra were different.

vi) Mass spectral studies of PBG and its imidazole derivative: Mass spectral studies were done on PBG in the acetate form and on PBG-Im and PBG-N-Me-Im after they have been desalted with deionized water on Sephadex G-15 and lyophilized. The amperage was 100 microamps and the temperature ranged from ambient to 300^{o}C. A blank was run at 300^{o}C and 100 microamps.

RESULTS

PBG Derivatives

In an attempt to "shake loose" intermediate pyrroles in the biosynthesis of urogen III, a group of nucleophilic compounds (imidazoles) were included in incubation mixtures of PBG and porphobilinogenase. It had been shown by Bogorad[2,5] and Grinstein[1,4] that ammonia and hydroxylamine inactivate co-synthetase and the only intermediates isolated were converted to urogen I only. Imidazole (Im) was chosen because it is an excellent nucleophile at pH 7.4 (pK_a 7.1) and the experimental results with an excess of imidazole added to the incubation mixture at pH 7.4 (Table 1) led to a series of experiments. In the presence of excess imidazole (2×10^{-2}M), it appeared that less PBG was consumed enzymatically but conversion to urogen was very efficient, whereas in the absence of imidazole, more PBG was consumed enzymatically but the yield of urogen was only about 50%. N-methylimidazole effected the same results as imidazole, but 2-methylimidazole had no effect on PBG utilization or porphyrin yield (Table 2). In order to determine whether the effects observed by these imidazoles were caused by changes wrought on substrate, enzyme, or both, the non-enzymatic reactions between PBG and the

Explanations and Abbreviations for Tables 1,2,3,4,5,6,7,8,9,10

RUN = Enzymatic runs done with blanks taken into account

COMPOUND = Substrates used for enzymatic runs

1) S = substrate

2) Superscript no. = number of hours incubated, e.g., 6 = 6 hr-incubation

3) Subscript refers to substrate used
   PBG = porphobilinogen
   PBG-Im = imidazole derivative of porphobilinogen
   PBG-N-Me-Im = N-methylimidazole derivative of porphobilinogen
   PBG-2-Me-Im = 2-methylimidazole derivative of porphobilinogen
   PBG + 2-Me-Im = unreacted PBG with 2-methylimidazole at pH 7.4.

nMoles PBG Enz Cons = nano-moles of PBG or PBG derivatives enzymatically consumed.

nMoles Porph Enz Formed = nano-moles of uroporphyrins enzymatically formed.

% PBG Used = nano-moles of PBG enzymatically consumed/ total nano-moles PBG x 100%

% Porph Formed = nano-moles of uroporphyrins formed/ nano-moles of PBG enzymatically used/4 x 100%.

% I, III = Percentage of urogen I and urogen III in total porphyrins formed.

Table 1. Enzymatic Effects of Exogenous Imidazole on Bovine Liver Porphobilinogenase in .05M Tris Buffer (PH 7.4)

RUN	COMPOUND	nMOLES PBG ENZ CONSUMED	nMOLES PORPH ENZ FORMED	% PBG USED	% PORPH FORMED
1	S^6_{PBG}	91.0	12.3	28.3	54.1
1	S^6_{PBG-Im}	61.0	11.0	18.3	72.1
2	S^6_{PBG}	45.0	7.3	40.0	64.9
2	S^6_{PBG-Im}	33.5	8.5	28.6	101.5

Table 2. Enzymatic Effects of Exogenous Im, N-Me-Im, 2-Me-Im on Bovine Liver Porphobilinogenase in .05M Tris Buffer (PH 7.4)

RUN	COMPOUND	nMOLES PBG ENZ CONSUMED	nMOLES PORPH ENZ FORMED	% PBG USED	% PORPH FORMED	% I, III
1	S^3_{PBG}	24.0	5.1	20.9	85.0	------
1	S^3_{PBG-Im}	15.0	5.0	12.8	100.0	------
1	$S^3_{PBG-N-Me-Im}$	10.0	3.2	8.8	94.3	------
1	$S^3_{PBG+2-Me-Im}$	20.0	5.9	17.4	91.5	------
1	S^6_{PBG}	45.0	7.3	40.0	64.9	------
1	S^6_{PBG-Im}	33.5	8.5	28.6	100	------
1	$S^6_{PBG-N-Me-Im}$	24.1	5.1	21.0	96.6	------
1	$S^6_{PBG+2-Me-Im}$	58.7	9.4	51.0	64.3	------
2	S^3_{PBG}	111	16.9	18.5	60.8	------
2	S^3_{PBG-Im}	88	15.4	14.9	65.2	------
2	$S^3_{PBG-N-Me-Im}$	59	16.9	10.1	100	------
2	$S^3_{PBG+2-Me-Im}$	112	16.3	19.9	58.0	------
2	S^6_{PBG}	256	29.0	42.7	45.3	0, 100
2	S^6_{PBG-Im}	165	25.4	27.9	61.6	4.7, 93.3
2	$S^6_{PBG-N-Me-Im}$	127	29.4	21.7	92.6	8.3, 91.7
2	$S^6_{PBG+2-Me-Im}$	250	25.4	44.4	40.6	0, 100

various imidazoles (Im, N-Me-Im, 2-Me-Im) were investigated.

In order to detect the formation of PBG derivatives with Im or N-Me-Im or 2-Me-Im, the following sets of experiments were done.

i) Purification of PBG and its derivatives on Sephadex G-15: To make sure that free imidazole, N-methylimidazole, and 2-methylimidazole did not interfere with porphobilinogenase in the enzymatic incubation and to isolate the effects of imidazoles on substrate and enzyme, PBG was reacted with excess Im (pK_a 7.1), N-Me-Im (pK_a 7.3), and 2-Me-Im (pK_a 8.1) at pH 7.4 and with 2-Me-Im(pK_a 8.1) at pH 8.5 and the reaction mixtures were placed on a Sephadex G-15 column at pH 8.0. This procedure should remove excess imidazoles and allow isolation of samples of PBG derivatives and permit a molecular weight estimation as well (Fig. 3). It appeared that PBG-2-Me-Im was formed at pH 8.5 whereas there was hardly any reaction between PBG and 2-Me-Im at pH 7.4. PBG and 2-Me-Im mixed in both .05M Tris buffer (pH 7.4) and .05M Tris buffer (pH 8.5) were used in the ensuing experiments. The mixture of PBG and 2-Me-Im in pH 7.4 buffer was indistinguishable from PBG alone. The rough standards used to calibrate the molecular weight were phenylalanine, tetra-alanine, and PBG. Elution volume V_e (Fig. 4) and K_{av} (Fig. 5) were plotted against the log of the molecular weights (Table 3). The derivatives being different from PBG and having higher molecular weights, were eluted before PBG. The various imidazoles were eluted much

Table 3. Parameters of PBG and PBG Derivatives Before and After Purification on Sephadex G-15.

COMPOUND	$R_{f(B:A:W:)}$	$R_{f(P:A:W)}$	$R_{m(pH\ 9.2)}$	A (mu)	K_{av} $\frac{V_e-V_o}{V_t-V_o}$
PBG(P)	.55	.45	.55		
PBG-Im(P)	.51	.50			
PBG-N-Me-Im(P)	.52	.51			
PBG-2-Me-Im(P) (pH 8.5)	.53	.48	.53		.163
CH_3-PBG(P) (pH 9.5)	.52	.45	.51		
PBG(UP)	.55	.45	.52		.244
PBG-Im(UP)	.51	.50	.54	228	.183
PBG-N-Me-Im(UP)	.52	.51	.49	223	.163
PBG + 2-Me-Im(UP) (pH 7.4)	.55	.47	.51		.244
PBG + 2-Me-Im(P) (pH 7.4)					
Phenylalanine	----	----	----	---	.305
Tetra-alanine	----	----	----	---	.132

(P) = purified on G-15
(UP) = unpurified on G-15

Fig. 3

Elution Profile of PBG and its Derivatives and Two Other Standards (Tetra-alanine and Phenylalanine) on Sephadex G-15 Column (1.8 x 82 cm).

a = Tetra-alanine
b = PBG-N-methylimidazole
c = PBG-Imidazole
d = PBG
e = PBG + 2-Methylimidazole (pH 7.4)
f = Phenylalanine
g = PBG-2-Methylimidazole (pH 8.5)

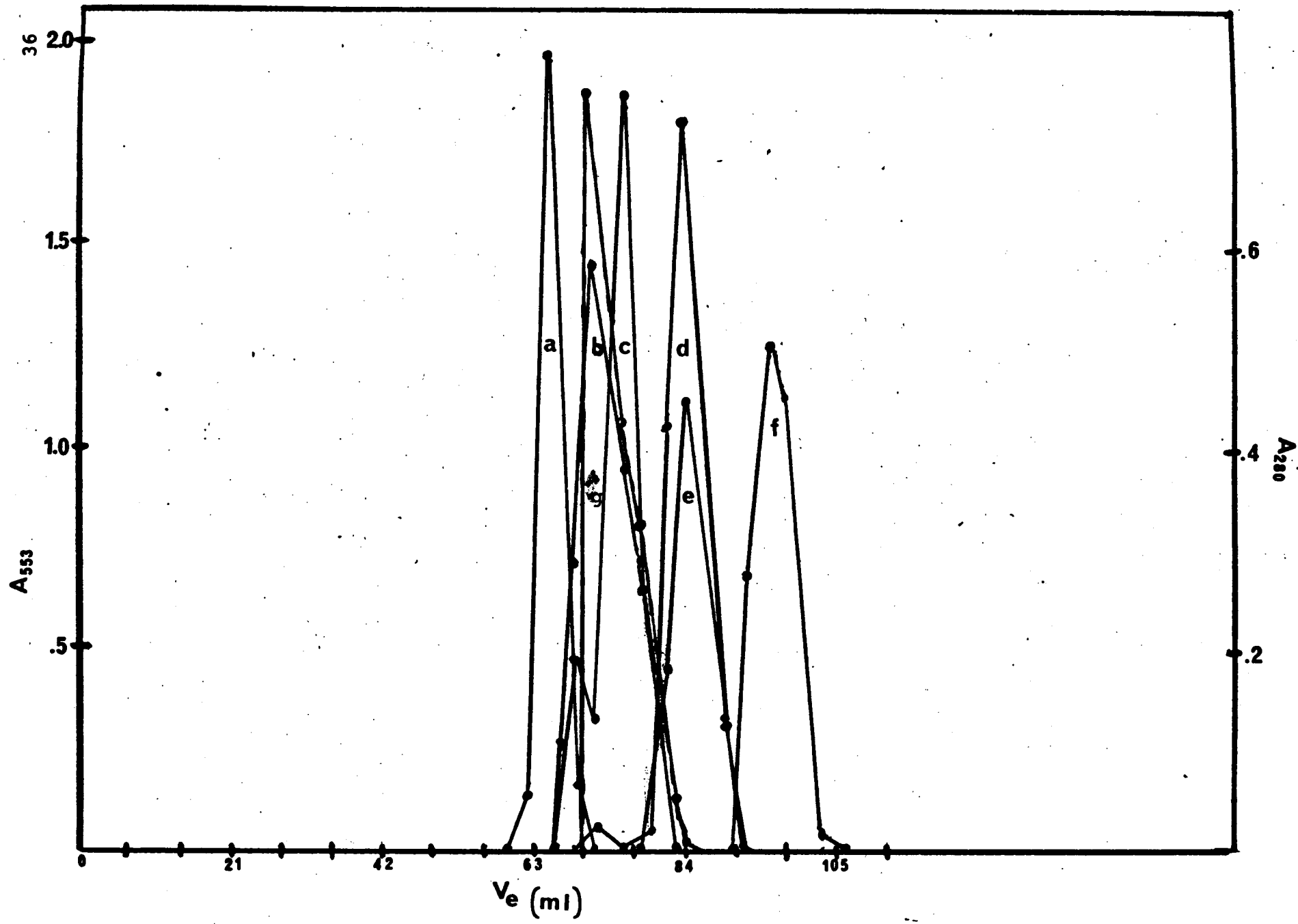

36
2.0
1.5
1.0
.5
A_{553}
.6
.4
.2
A_{280}
a
b
c
d
e
f
g
0
21
42
63
84
105
V_e (ml)

Fig. 4

Elution Volume vs. Log MW of PBG and its Derivatives and Two Other Standards (Tetra-alanine and Phenylalanine).

a = Tetra-alanine
b = PBG-N-methylimidazole
c = PBG-Imidazole
d = PBG
e = PBG + 2-Methylimidazole (pH 7.4)
f = Phenylalanine
g = PBG-2-Methylimidazole (pH 8.5)

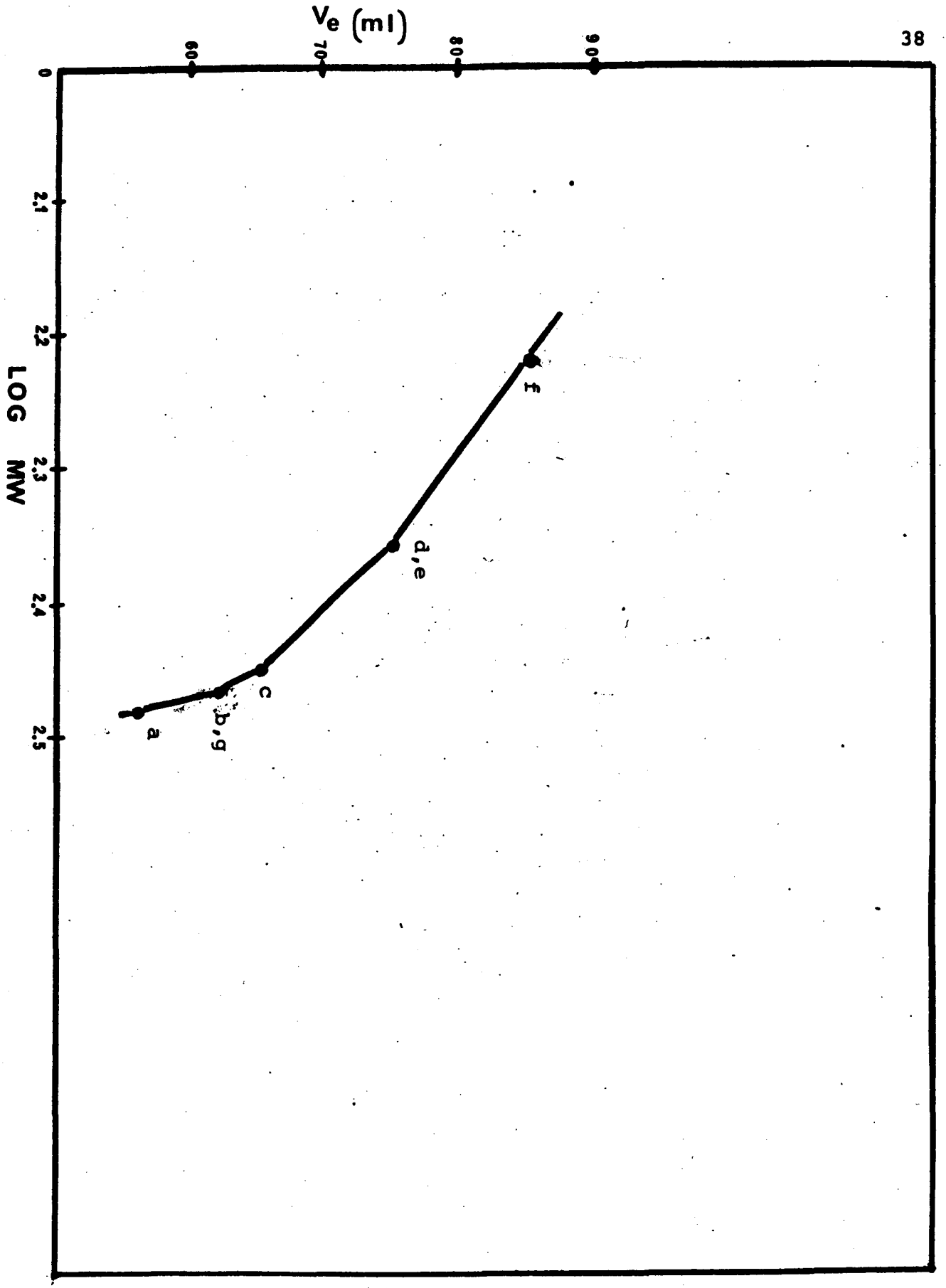
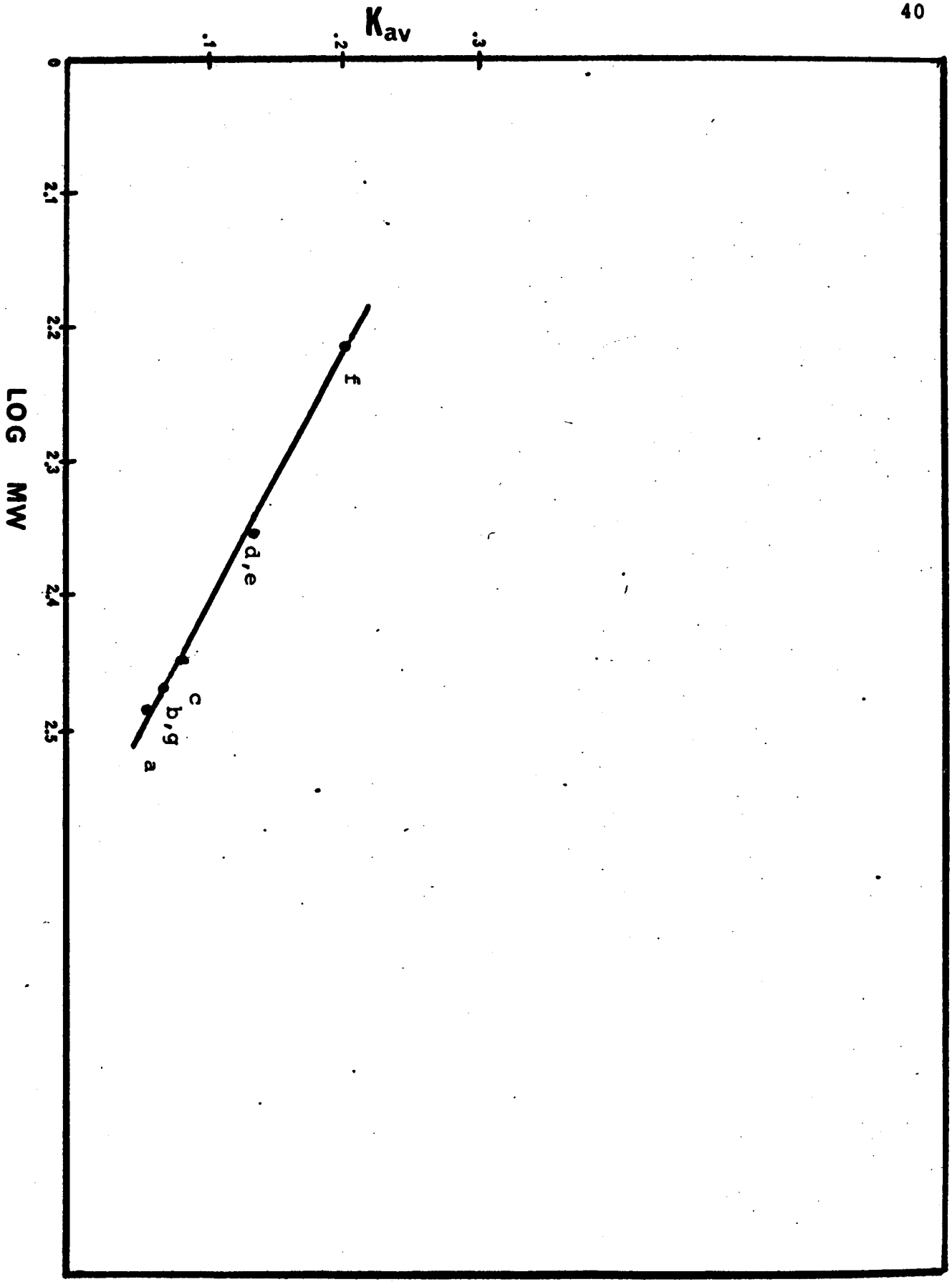

V_e (ml)
60
70
80
90
0
2.1
2.2
2.3
2.4
2.5
LOG MW
a
b,g
c
d,e
f

Fig. 5

K_{av} vs. Log MW of PBG and its Derivatives and Two Other Standards (Tetra-alanine and Phenylalanine)

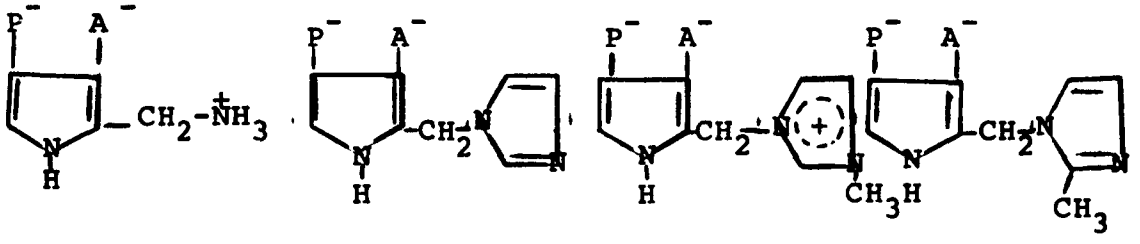
a = Tetra-alanine
b = PBG-N-methylimidazole
c = PBG-Imidazole
d = PBG
e = PBG + 2-Methylimidazole (pH 7.4)
f = Phenylalanine
g = PBG-2-Methylimidazole (pH 8.5)

Assumption was made that MW for b = 292, c = 277, g = 292.


K_{av}
0
.1
.2
.3
LOG MW
2.1
2.2
2.3
2.4
2.5
f
d,e
c
b,g
a

after PBG. From these results, it appeared that PBG derivatives of Im and N-Me-Im at pH 7.4 and 2-Me-Im at pH 8.5 were indeed formed and that the molecular weights of these derivatives fell in the approximate range on the calibration curve. The elution volume results indicated that PBG reacted with 2-Me-Im at pH 8.5 but not appreciably at pH 7.4.

ii) Paper electrophoresis of PBG and its derivatives: In paper elctrophoresis at pH9.2, mobility was calculated by the ratio distance in cm travelled by a sample spot and that travelled by uroporphyrin III (Table 3). At this pH, one would expect PBG and its derivatives to exist in the following states:



	PBG	PBG-Im	PBG-N-Me-Im	PBG-2-Me-Im
Net Charge:	- 1	-2	-1	-2

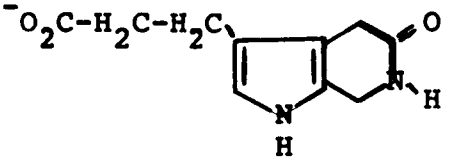
$A = CH_2\text{-}CO_2^-$

$P = CH_2\text{-}CH_2\text{-}CO_2^-$

Based only on net charges, one would expect the elctrophoretic mobility of the above four compounds at pH 9.2 to be in the following order: PBG-Im ≅ PBG-2-Me-Im > PBG ≅ PBG-N-Me-Im. Since the molecular weights of PBG, PBG-Im, PBG-N-Me-Im, and

PBG-2-Me-Im are 226, 277, 292, and 292 respectively, the larger molecule may be hindered in the electrophoretic mobility. The experimental values for the electrophoretic runs were .516, .540, .487, and .535 for PBG, PBG-Im, PBG-N-Me-Im, and PBG-2-Me-Im respectively (Table 3). The electrophoretic mobility for PBG + 2-Me-Im reacted at pH 7.4 and purified on Sephadex G-15 at pH 8.0 was .509 which was indicative of unreacted PBG (E_m .516) with 2-Me-Im at pH 7.4 (Fig. 6, 7, 8, 9).

iii) Cellulose thin layer chromatography of PBG and its derivatives: Thin layer chromatographic results in two different solvent systems showed that PBG behaved differently from its derivatives with imidazole, N-methylimidazole, and 2-methylimidazole. The purified PBG-Im and PBG-N-Me-Im (after Sephadex G-15) had the same R_f values as PBG-Im and PBG-N-Me-Im before gel filtration (Table 3). The chromatograms developed in the n-butanol-acetic acid-water (pH 3.5) are shown in Fig. 10, 11, 12, 13 and the chromatograms developed in the n-propanol-ammonia-water (pH 12) are shown in Fig. 14,15,16, 17. The possibility that PBG derivatives may exist in the lactam form



under our experimental conditions can be ruled out by the R_f value and Sephadex G-15 molecular weight estimates. Jackson and MacDonald[29] reported R_f values of .78 and .50 for porphobilinogen lactam and porphobilinogen respectively in their n-butanol-acetic acid-water system (60:40:10). The R_f value for PBG in our n-butanol-acetic acid-water system (63:11:26) is .55 and the derivatives had R_f's which were even lower than .55. In addition, derivatives of PBG were eluted off Sephadex G-15 before PBG indicating that the molecular weights of these derivatives are larger than that of PBG or PBG lactam.

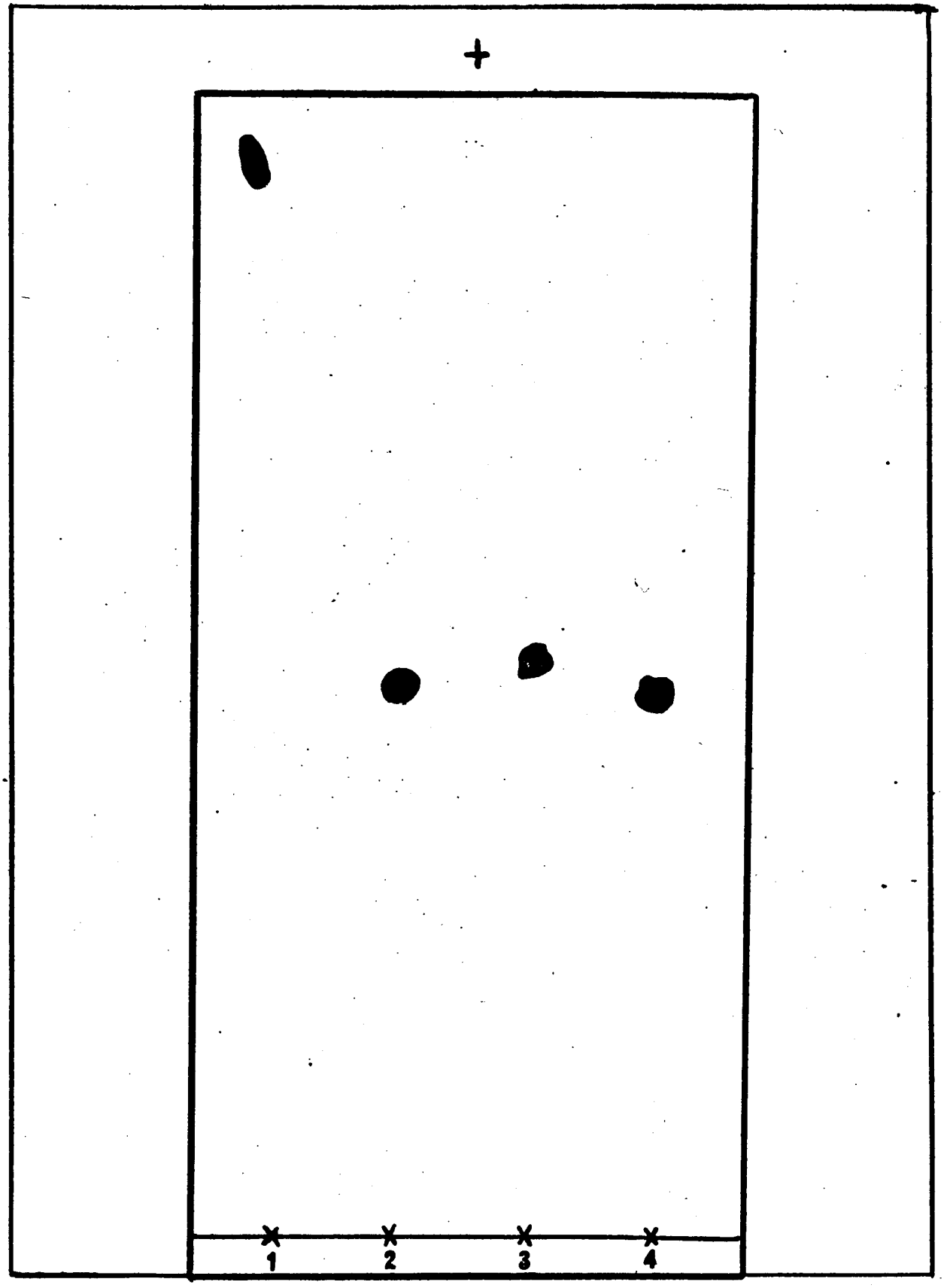
iv) The effect of various imidazoles on the Ehrlich reaction with PBG: PBG and modified Ehrlich's reagent gave characteristic visible spectra with a maximum absorption at 553 mu and a shoulder at 525 mu ($A_{525\ mu}/ A_{553mu} = .82$)[28] after fifteen minutes of reaction (Fig. 18). PBG in the presence of excess Im or N-Me-Im or 2-Me-Im with modified Ehrlich' reagent gave the same characteristic visible spectra (Fig 19). However, the rates of color development in the reaction of modified Ehrlich's reagent with PBG was dramatically decreased by the presence of imidazole, N-methylimidazole, and 2-methylimidazole although the final maximum optical density for all was the same. The rate of

color development at 553 mu in the presence of the various imidazoles was inversely dependent on the concentration of various imidazoles added (Im Fig. 20)(N-Me-Im Fig. 21)(2-Me-Im Fig. 22). When PBG derivatives were purified on Sephadex G-15, i.e., PBG derivatives devoid of various free imidazoles were used to react with the modified Ehrlich's reagent, the delayed rate of color development at 553 mu was far less marked (Fig. 23). There was a slight decrease in the initial rate of color development with PBG-Im and with PBG-N-Me-Im.

Fig. 6

Paper Electrophoresis on Whatman #1 Paper (10 x 25 cm) of Uroporphyrin III, PBG, and PBG Derivatives in .05M Sodium Diethylbarbiturate Buffer (pH 9.2) for 2.5 Hrs. at 4°C. PBG Derivatives not Purified on Sephadex G-15.

1 = Uro III
2 = PBG
3 = PBG-Imidazole
4 = PBG + 2-Methylimidazole (pH 7.4)

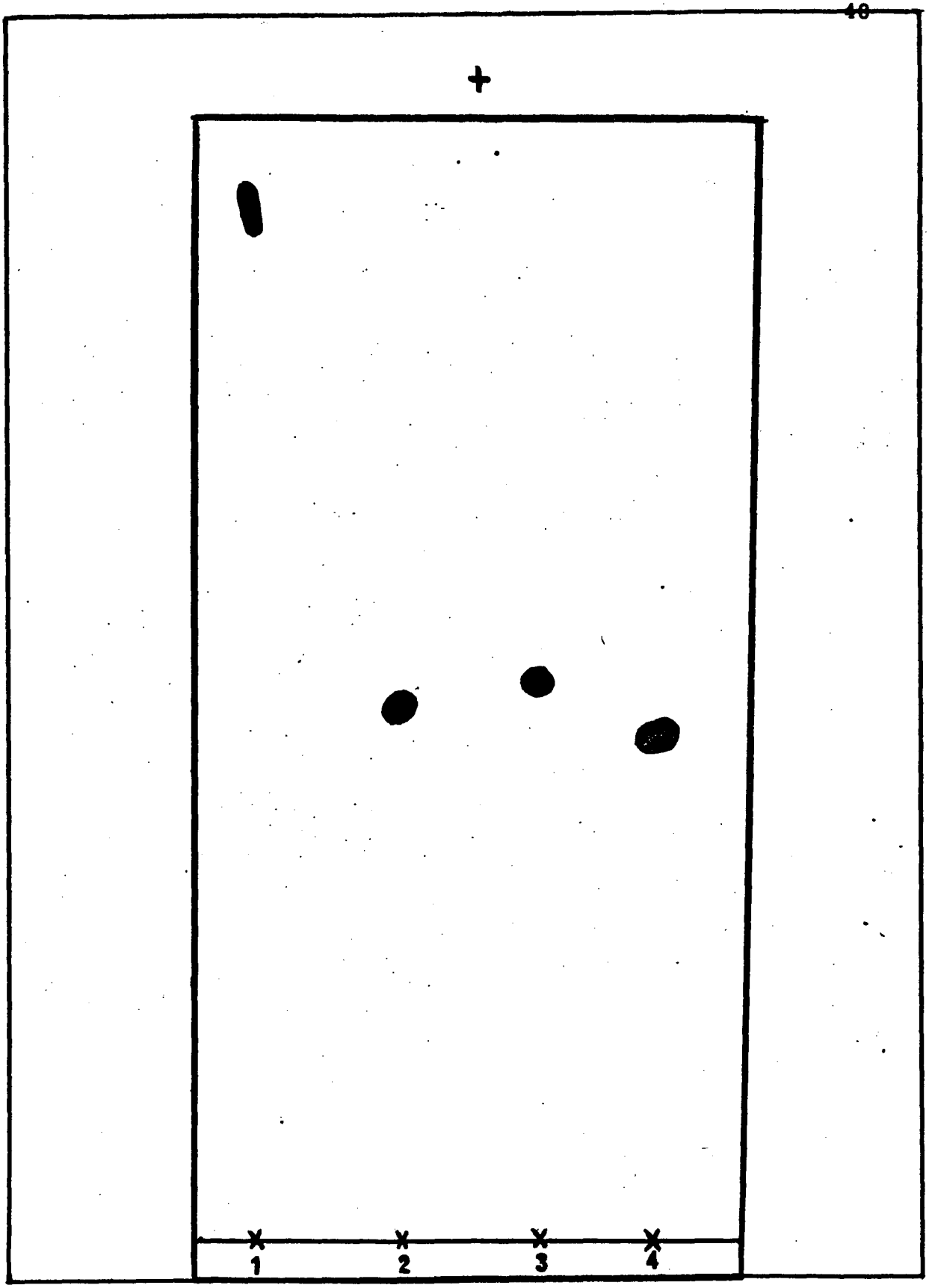

+
1
2
3
4

47

Fig. 7

Paper Electrophoresis on Whatman #1 Paper (10 x 25 cm) of Uroporphyrin III, PBG, and PBG Derivatives in .05M Sodium Diethylbarbiturate Buffer (pH 9.2) for 2.5 Hrs. at $4^{\circ}C$. PBG Derivatives not Purified on Sephadex G-15.

1 = Uro III
2 = PBG
3 = PBG-Imidazole
4 = PBG-N-methylimidazole


+
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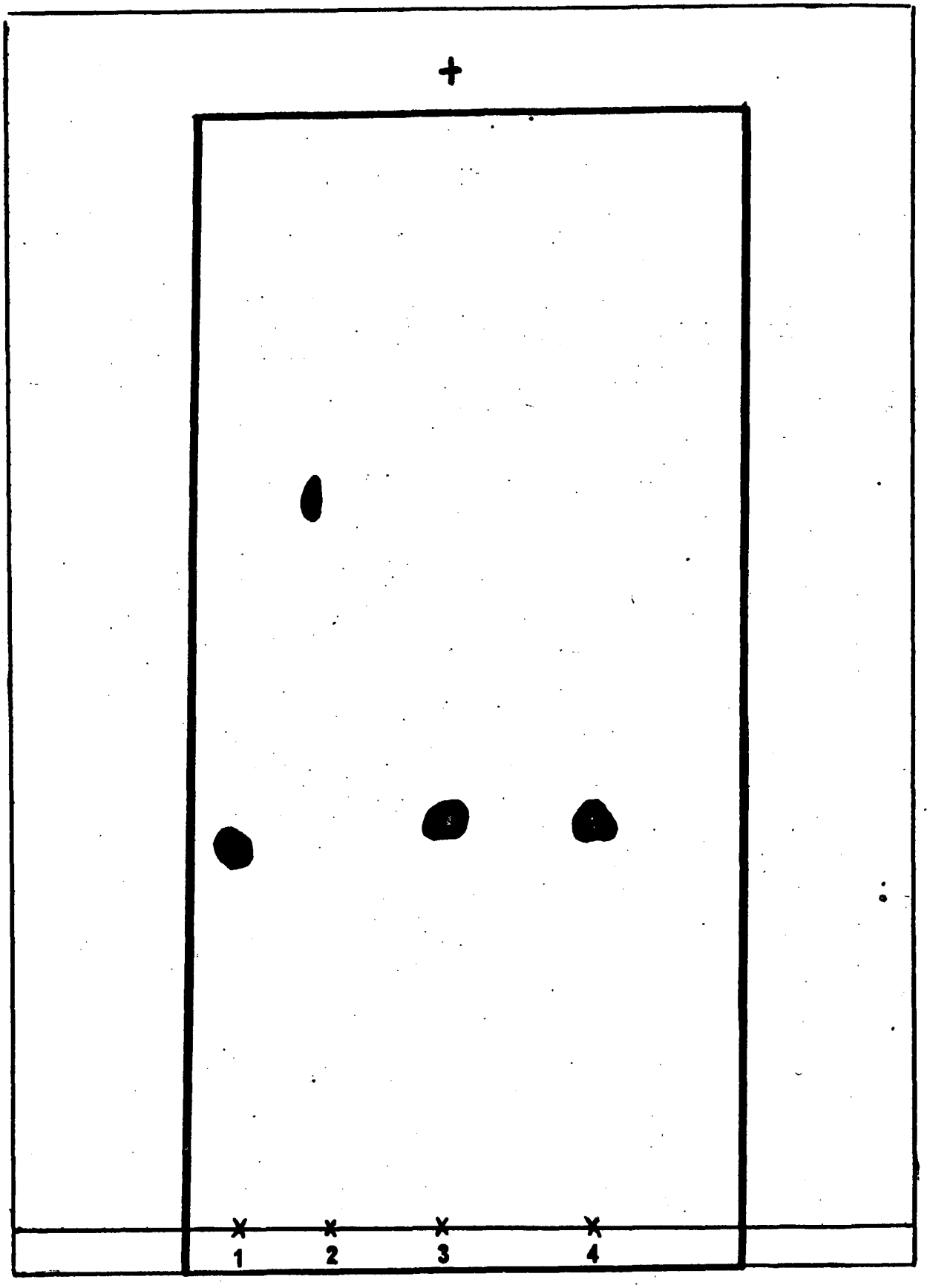
2
3
4

Fig. 8

Paper Electrophoresis on Whatman #1 Paper (10 x 25 cm) of Uroporphyrin III, PBG, PBG-Imidazole (purified), and PBG-Imidazole (unpurified) in .05M Sodium Diethyl-barbiturate Buffer (pH 9.2) for 2.5 Hrs. at $4^{o}C$.

1 = PBG
2 = Uro III
3 = PBG-Imidazole (unpurified)
4 = PBG-Imidazole (purified)


+
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2
3
4

Fig. 9

Paper Electrophoresis on Whatman #1 Paper (10 x 25 cm) of Uroporphyrin III, PBG, PBG-2-Methylimidazole (purified, synthesized at pH 8.5), and CH_3-PBG (purified, methylated with CH_3I at pH 9.5) in .05M Sodium Diethylbarbiturate Buffer (pH 9.2) for 2.5 Hrs. at 4°C.

1 = PBG
2 = Uro III
3 = CH_3-PBG
4 = PBG-2-Methylimidazole (pH 8.5)

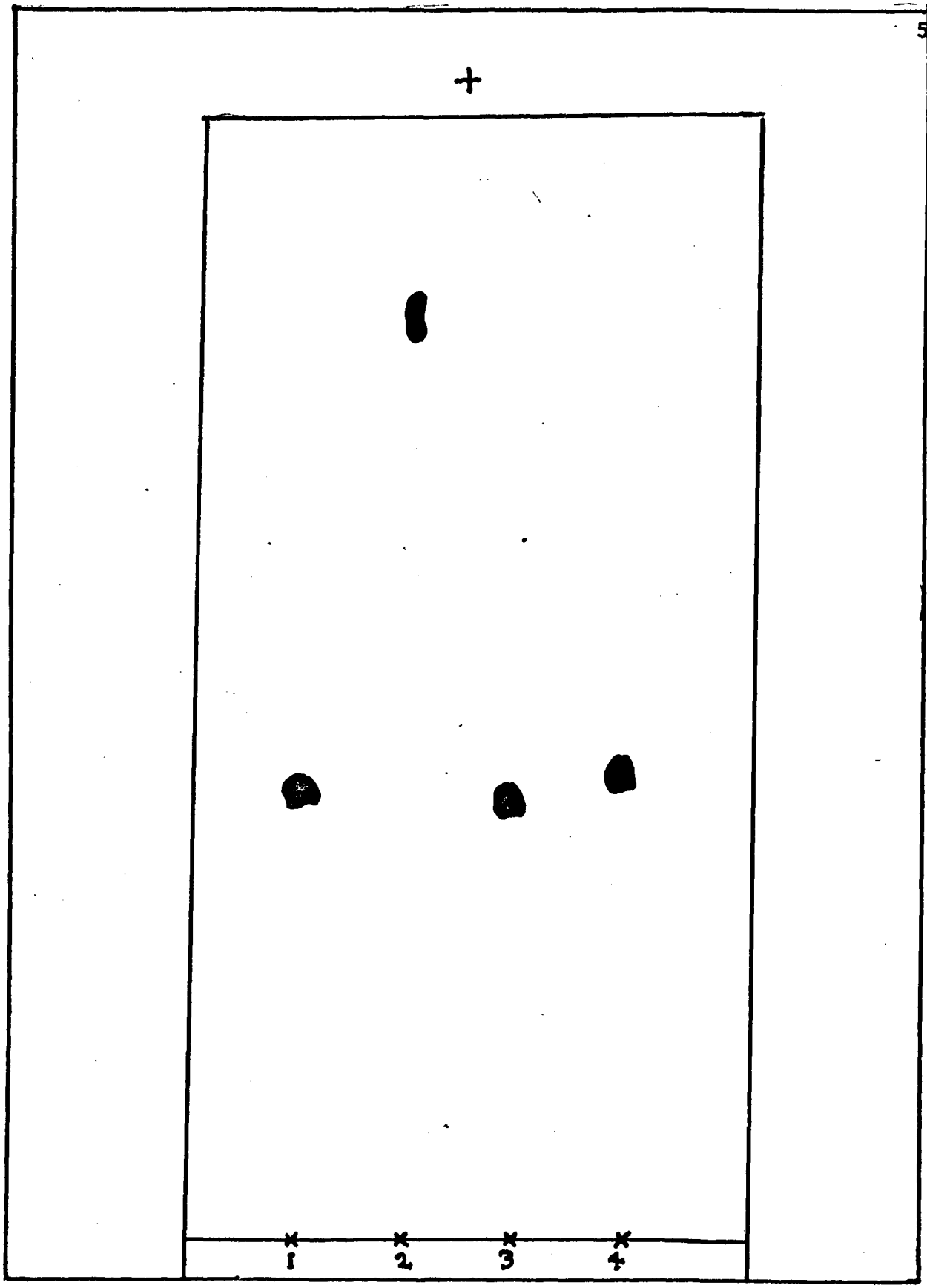

+
1
2
3
4

Fig. 10

Thin Layer Chromatography (TLC) of PBG, PBG-Imidazole, PBG-N-methylimidazole, and PBG + 2-Methylimidazole on Cellulose Polygram in n-Butanol-Acetic Acid-Water (63:11:26). Uroporphyrin III Present with Every Sample Spot as Marker. PBG and Derivatives not Purified on Sephadex G-15.

1 = PBG
2 = PBG-Imidazole
3 = PBG-N-methylimidazole
4 = PBG + 2-Methylimidazole (pH 7.4)

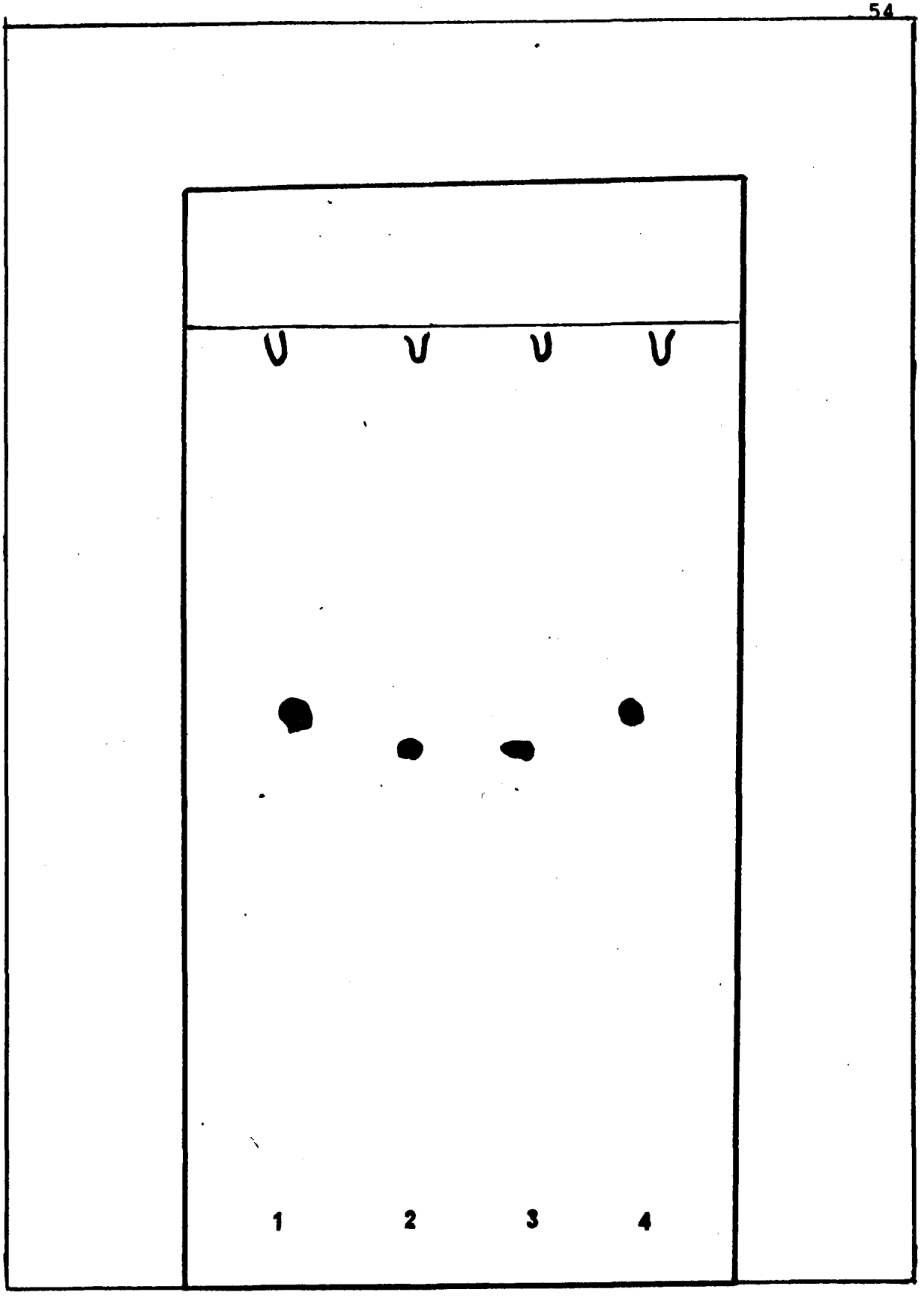

1
2
3
4

Fig. 11

Thin Layer Chromatography (TLC) of PBG, PBG-Imidazole, PBG-Imidazole (purified on G-15), and PBG + 2-Methyl-imidazole on Cellulose Polygram in n-Butanol-Acetic Acid-Water (63:11:26). Uroporphyrin III Present with Every Sample Spot as Marker.

1 = PBG
2 = PBG-Imidazole (unpurified)
3 = PBG-Imidazole (purified on G-15)
4 = PBG + 2-Methylimidazole (pH 7.4)

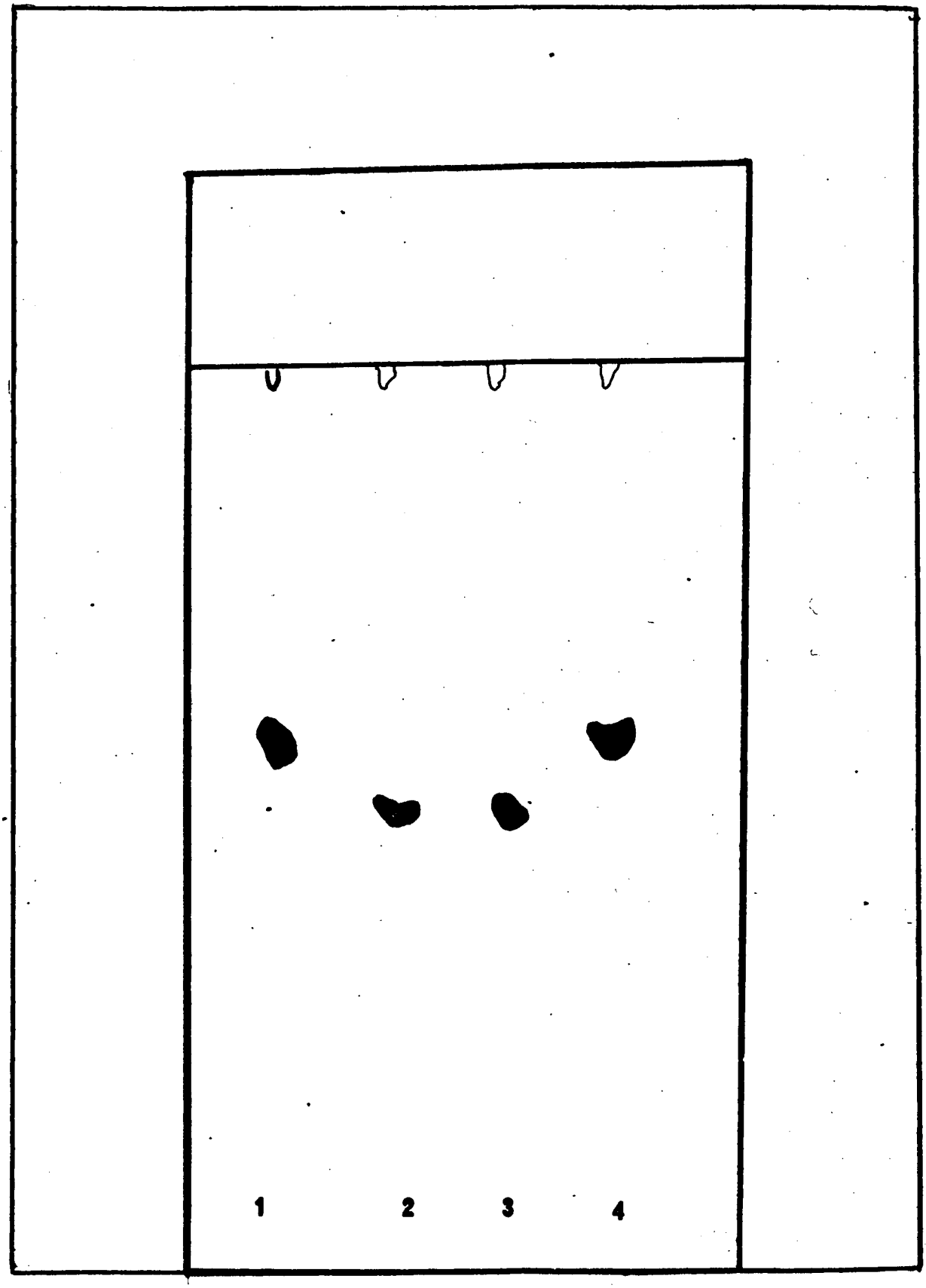

1
2
3
4

Fig. 12

Thin Layer Chromatography (TLC) of PBG + 2-Methylimidazole (unpurified at pH 7.4), PBG + 2-Methylimidazole (purified at pH 7.4), PBG-N-methylimidazole (unpurified), and PBG-N-methylimidazole (purified on G-15) on Cellulose Polygram in n-Butanol-Acetic Acid-Water (63:11:26). Uroporphyrin III Present with Every Sample Spot as Marker.

1 = PBG + 2-Methylimidazole (pH 7.4 and unprified)
2 = PBG + 2-Methylimidazole (pH 7.4 and purified)
3 = PBG-N-methylimidazole (unpurified)
4 = PBG-N-methylimidazole (purified)

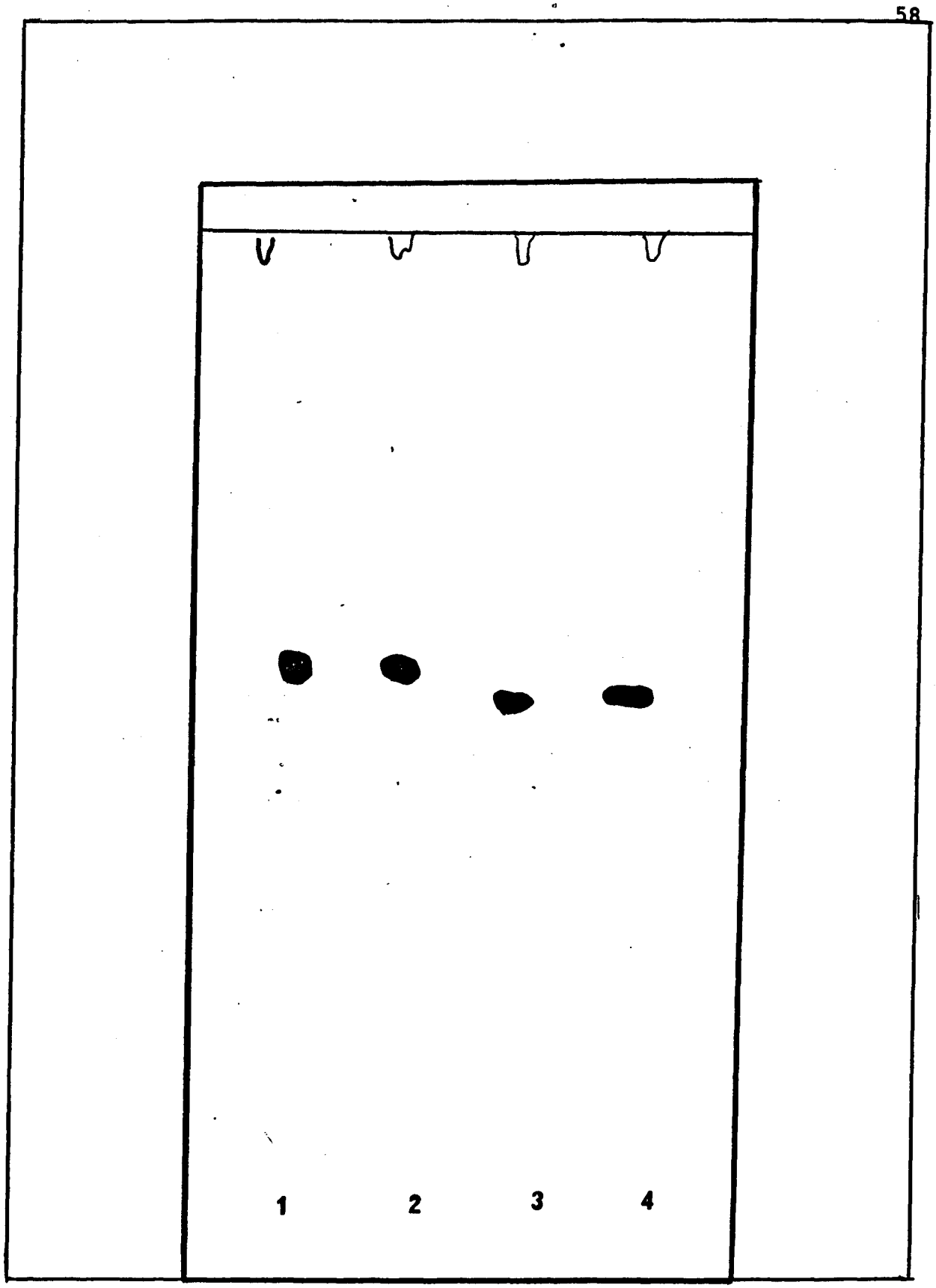

1
2
3
4

Fig. 13

Thin Layer Chromatography (TLC) of PBG, PBG-2-Methylimidazole (purified and synthesized at pH 8.5), CH_3-PBG (Methylated at pH 9.5 with CH_3I and purified on G-15) on Cellulose Polygram in n-Butanol-Acetic Acid-Water (63:11:26). Uroporphyrin Present with Every Sample Spot as Marker.

1 = PBG
2 = PBG-2-Methylimidazole
3 = CH_3-PBG
4 = PBG

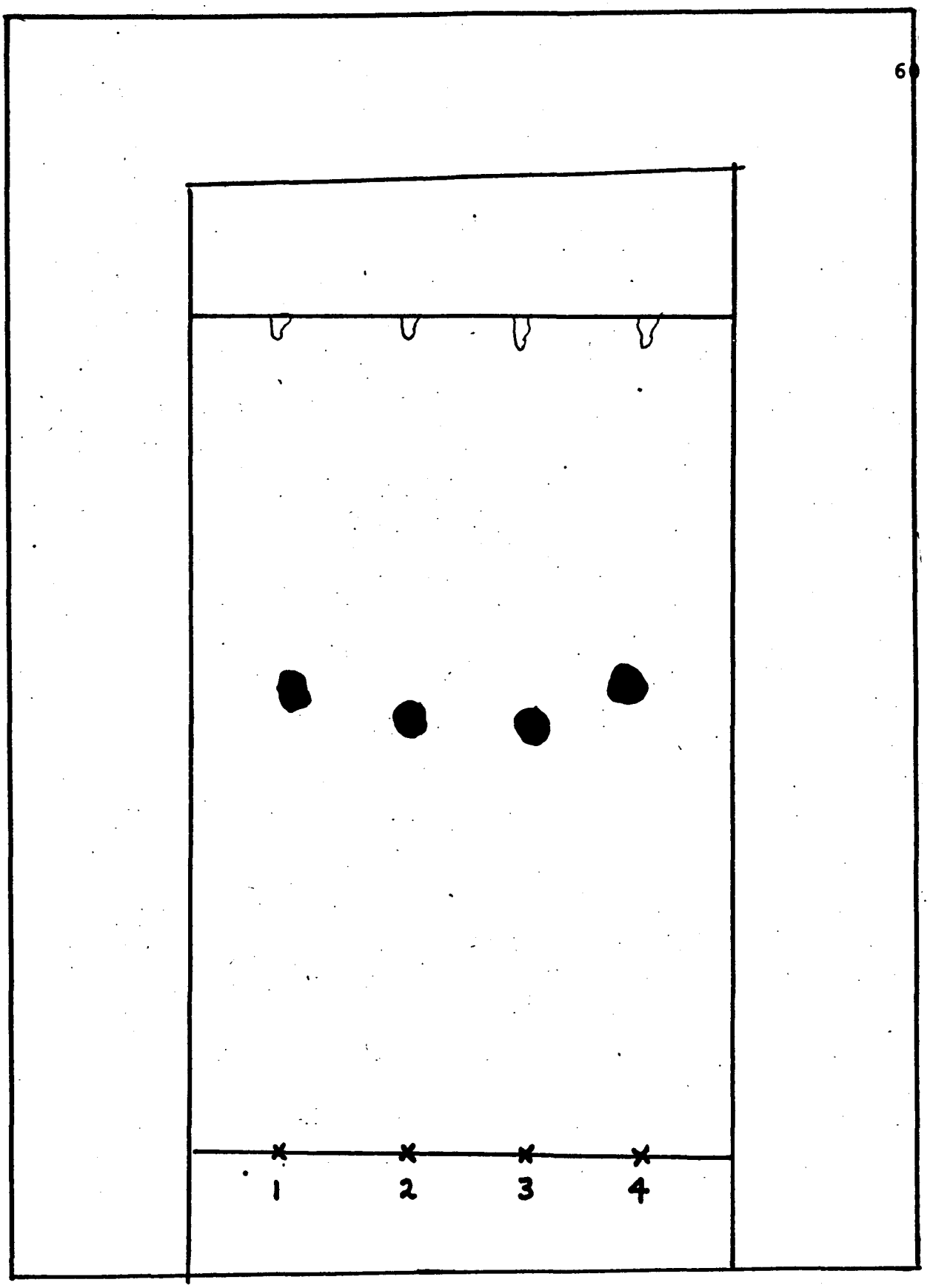

1
2
3
4

Fig. 14

Thin Layer Chromatography (TLC) of PBG, PBG-Imidazole, PBG-N-methylimidazole, and PBG + 2-Methylimidazole on Cellulose Polygram in n-Propanol-Ammonia-Water (60:30:10). Uroporphyrin III Present with Every Sample Spot as Marker. PBG and Derivatives not Purified on Sephadex G-15.

1 = PBG
2 = PBG + 2-Methylimidazole (pH 7.4)
3 = PBG-Imidazole
4 = PBG-N-methylimidazole

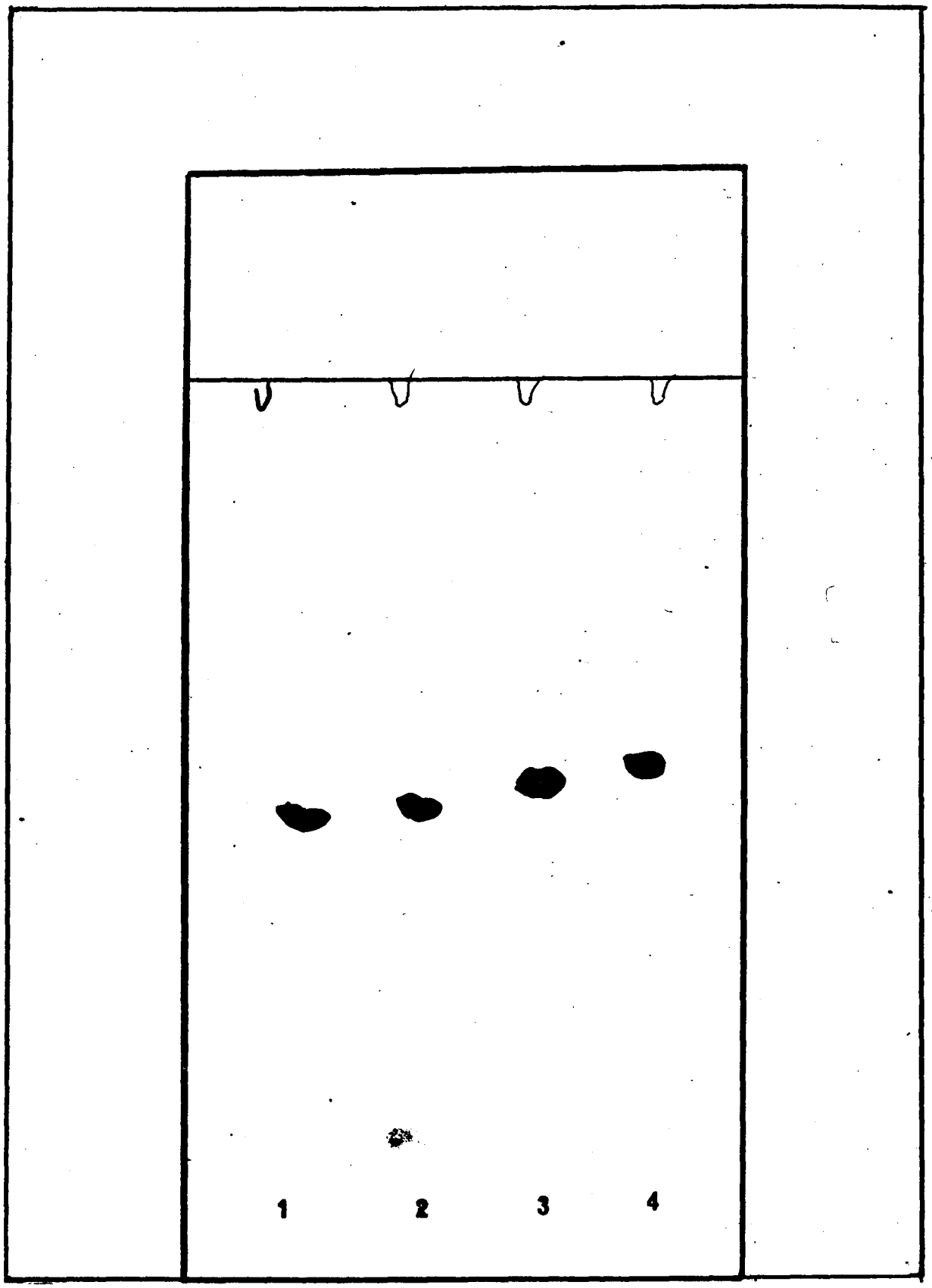

1
2
3
4

Fig. 15

Thin Layer Chromatography (TLC) of PBG (purified), PBG (unpurified), PBG-Imidazole (purified), and PBG-Imidazole (unpurified) on Cellulose Polygram in n-Propanol-Ammonia-Water (60:30:10). Uroporphyrin III Present with Every Sample Spot as Marker.

1 = PBG (unpurified on G-15)
2 = PBG (purified on G-15)
3 = PBG-Imidazole (unpurified on G-15)
4 = PBG-Imidazole (purified on G-15)

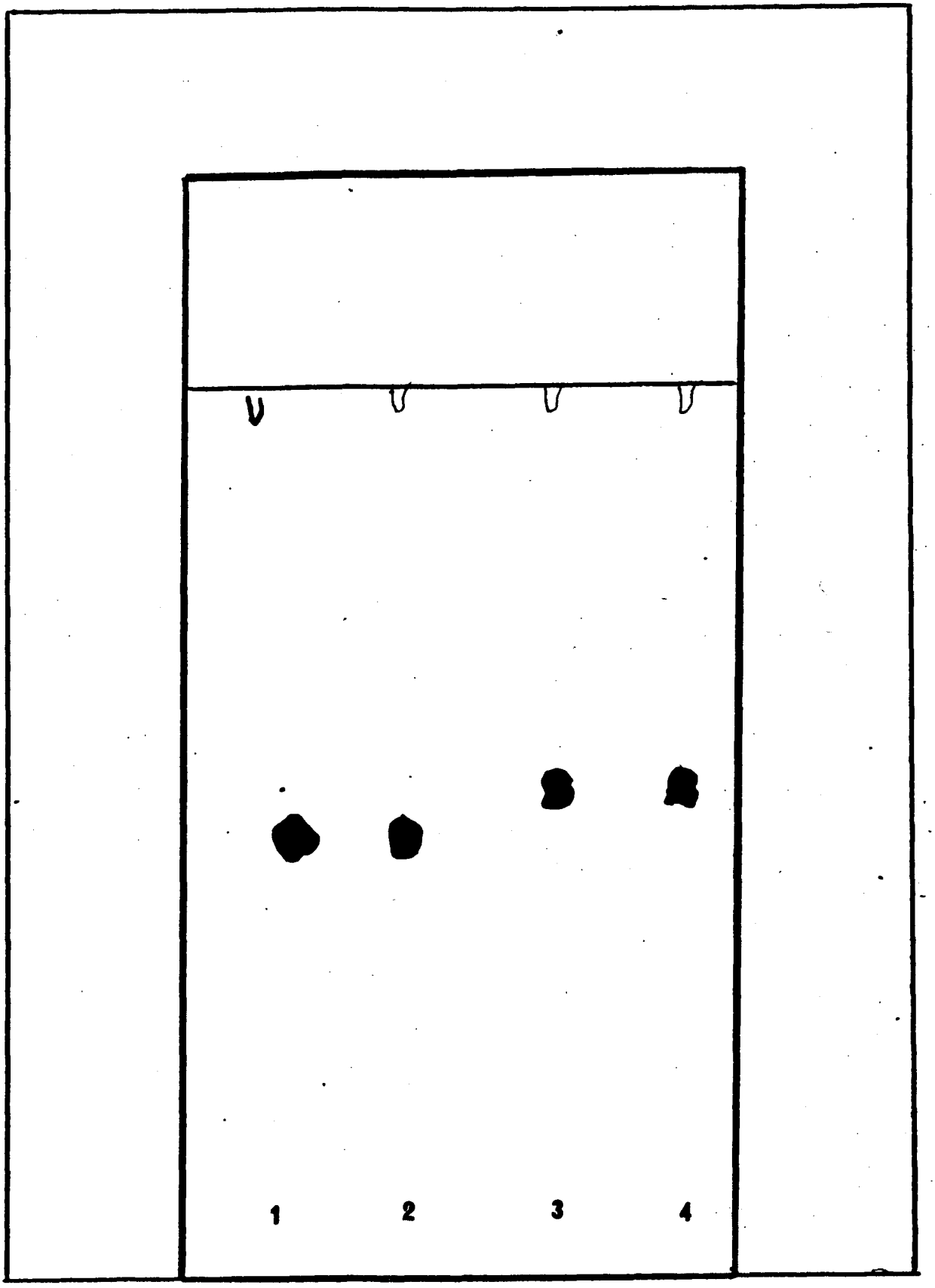

1
2
3
4

Fig. 16

Thin Layer Chromatography (TLC) of PBG-N-methylimidazole (purified), PBG-N-methylimidazole (unpurified), PBG + 2-Methylimidazole (purified and reacted at pH 7.4), and PBG + 2-Methylimidazole (unpurified and reacted at pH 7.4) on Cellulose Polygram in n-Propanol-Ammonia-Water (60:30:10). Uroporphyrin III Present with Every Sample Spot as Marker.

1 = PBG + 2-Methylimidazole (unpurified)
2 = PBG-N-methylimidazole (unpurified)
3 = PBG-N-methylimidazole (purified)
4 = PBG-N-methylimidazole (purified)

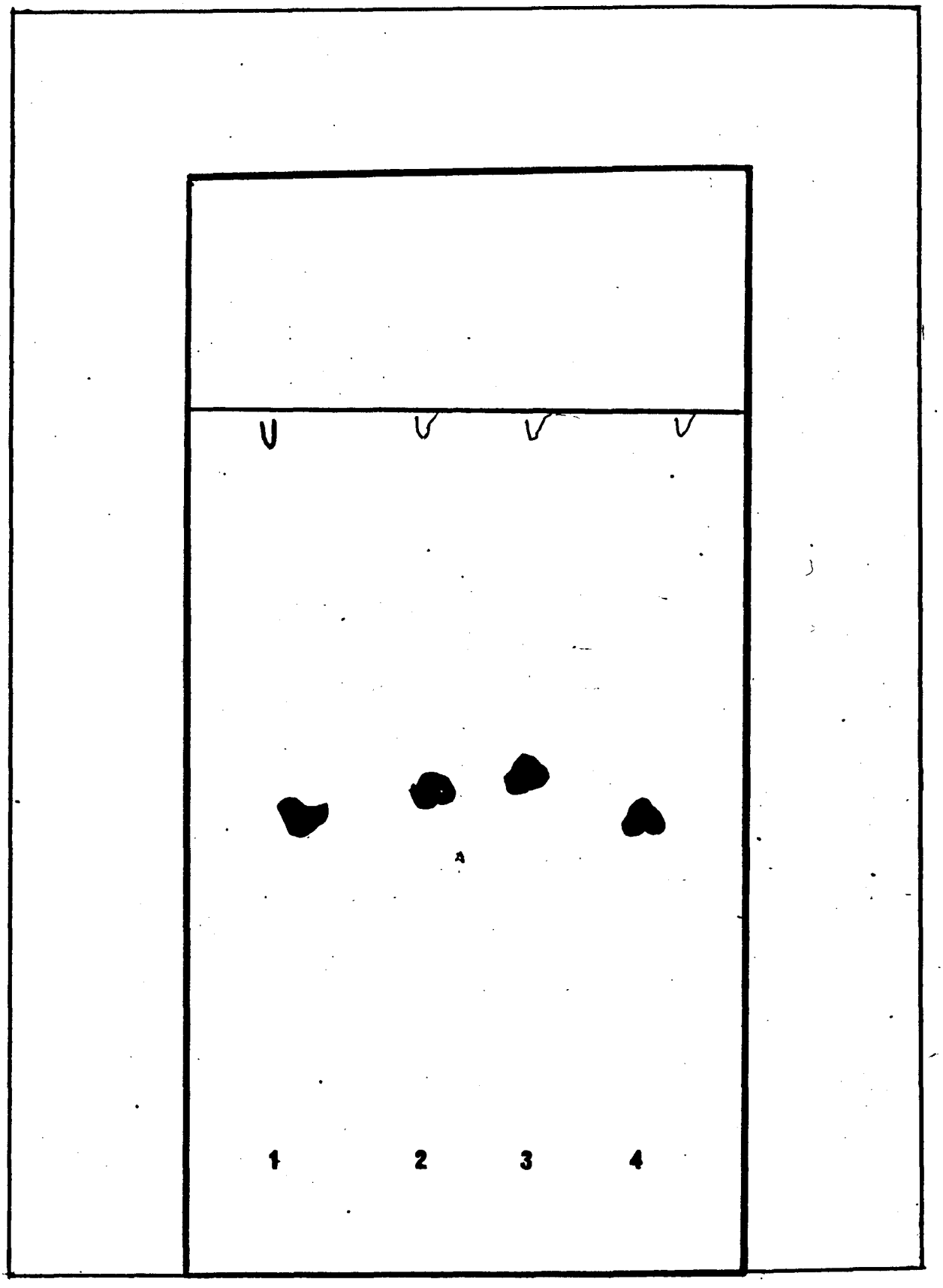

1
2
3
4

Fig. 17

Thin Layer Chromatography (TLC) of PBG, PBG-2-Methylimidazole (purified and synthesized at pH 8.5), CH_3-PBG (Methylated at pH 9.5 with CH_3I and purified on G-15) on Cellulose Polygram in n-Propanol-Ammonia-Water (60:30:10). Uroporphyrin III Present in Every Sample Spot as Marker.

1 = PBG
2 = PBG-2-Methylimidazole
3 = CH_3-PBG
4 = PBG

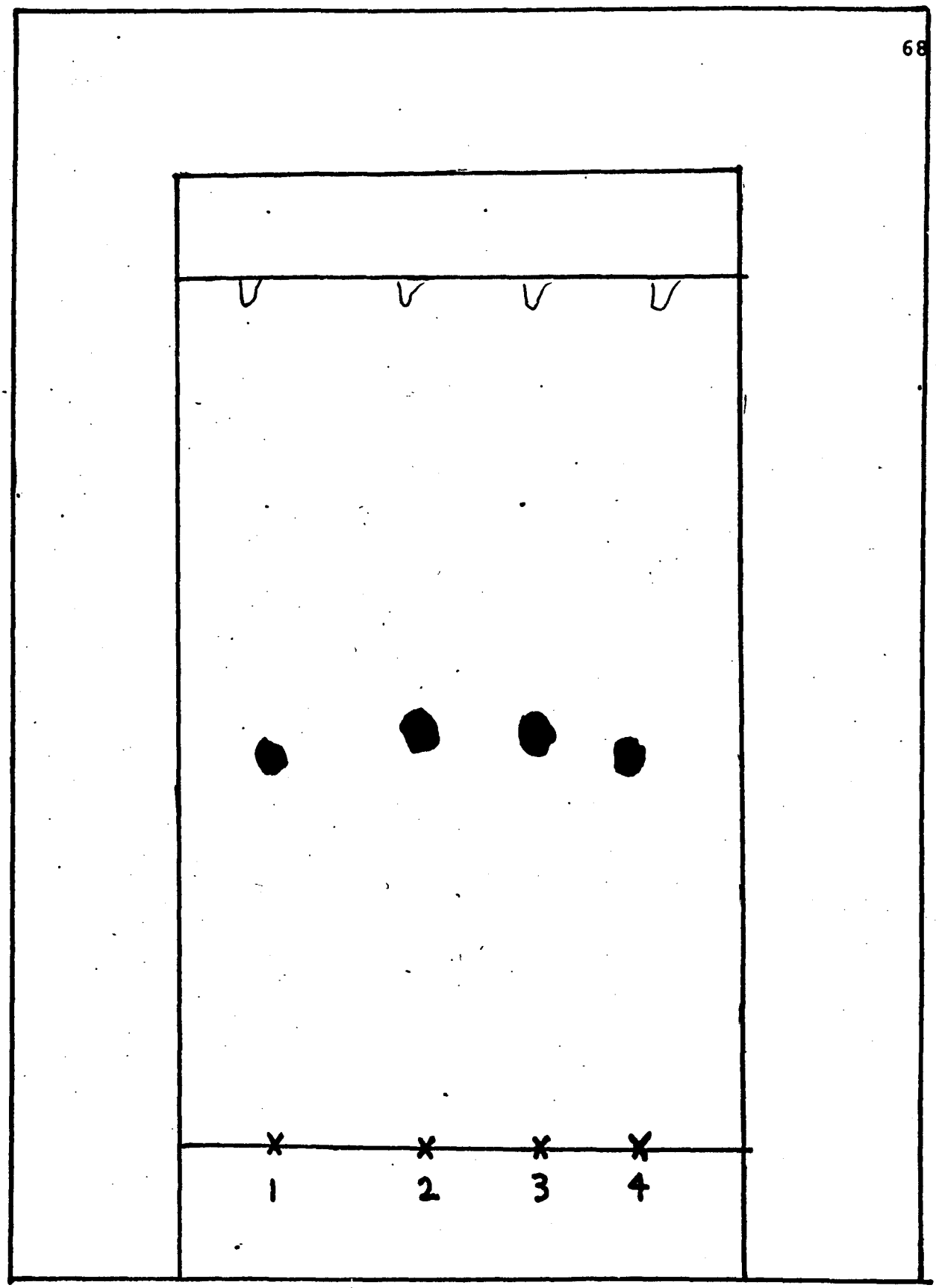

1
2
3
4

Fig. 18

Time Study of Ehrlich Reaction of PBG with Modified Ehrlich's Reagent (p-dimethyl-amino benzaldehyde).

a = 30 secs. after mixing PBG with Modified Ehrlich's Reagent. Concentration of PBG = 11.7 nmoles/1 ml.

X = Homium Oxide Standard

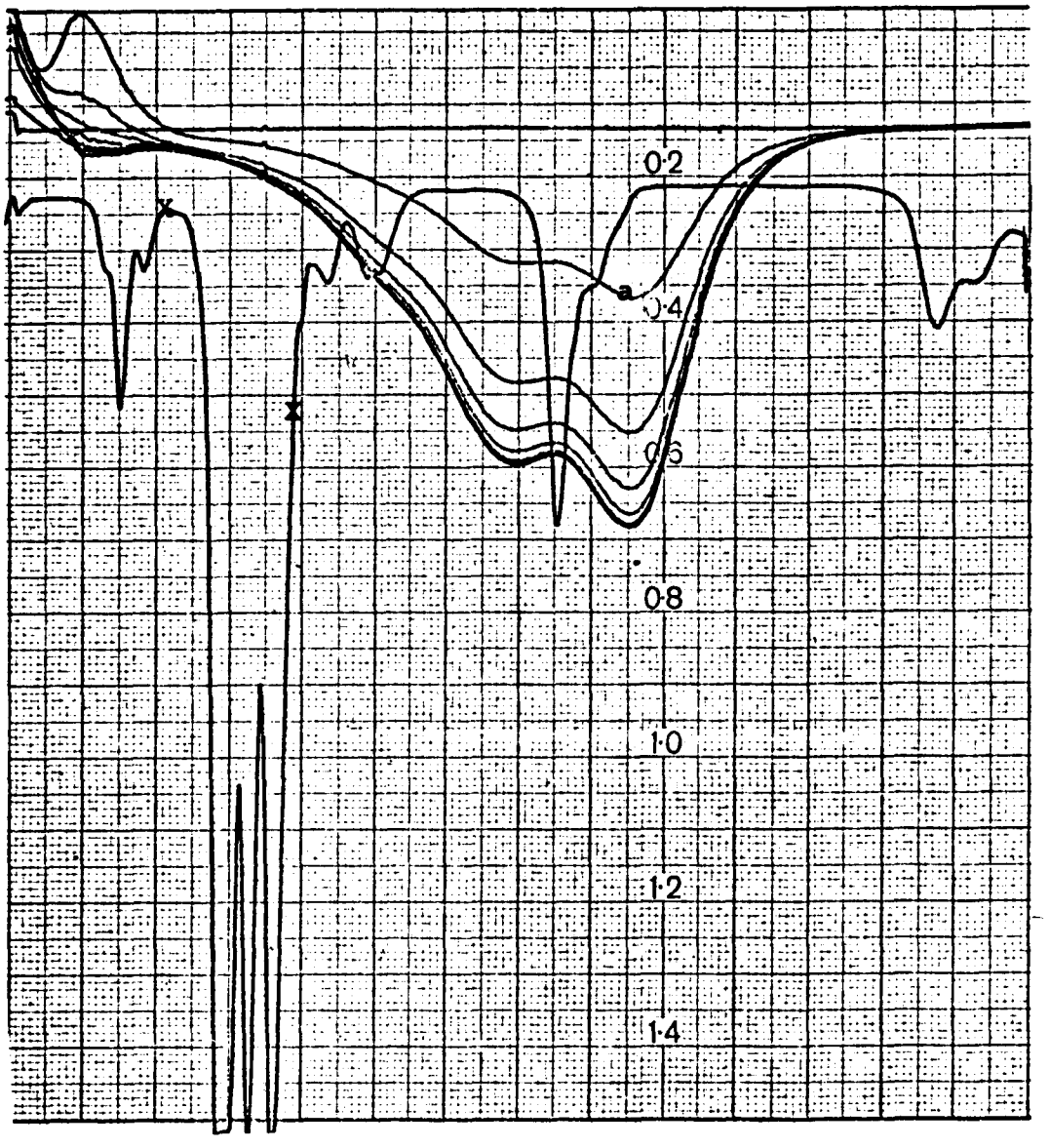

0·2
0·4
0·6
0·8
1·0
1·2
1·4

Fig. 19

Time Study of Ehrlich Reaction of PBG + N-methylimidazole with Modified Ehrlich's Reagent (p-dimethyl-amino benzaldehyde).

a = 30 secs. after mixing PBG + N-methylimidazole with Modified Ehrlich's Reagent.

Concentration of PBG = 11.7 nmoles/1 ml
Concentration of N-methylimidazole = 700 nmoles/1 ml

b = 30 secs. after mixing PBG + N-methylimidazole with Modified Ehrlich's Reagent.

Concentration of PBG = 11.7 nmoles/1 ml
Concentration of N-methylimidazole = 1400 nmoles/1 ml

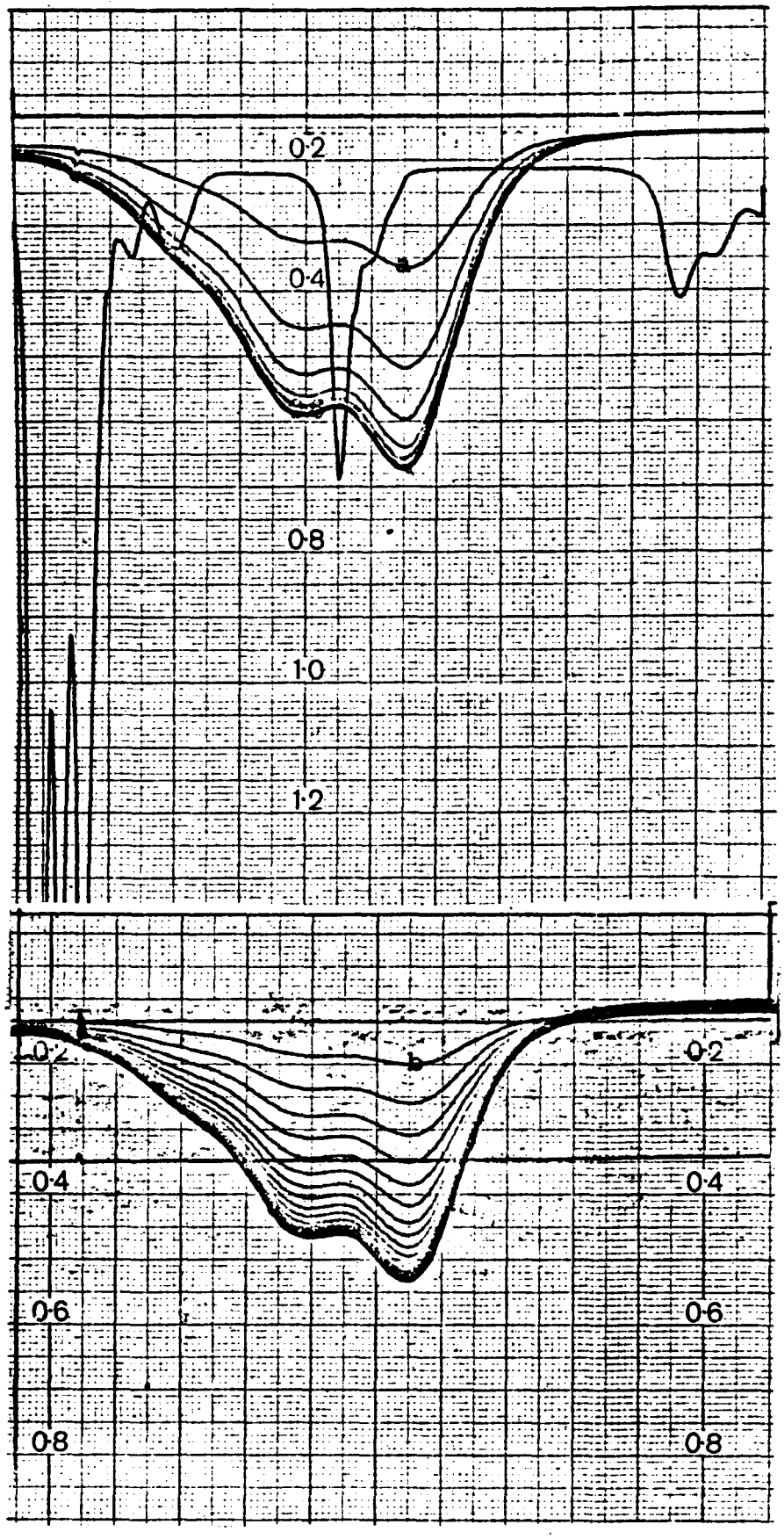

0·2
0·4
0·8
1·0
1·2
0·2
0·4
0·6
0·8
0·2
0·4
0·6
0·8

Fig. 20

Nonenzymatic Effect of Imidazole on the Ehrlich's Reaction with PBG.

1 = 12.4 nmoles PBG
2 = 12.4 nmoles PBG + 4000 nmoles Im
3 = 12.4 nmoles PBG + 7000 nmoles Im
4 = 12.4 nmoles PBG + 9000 nmoles Im

■ = a break in time after 3 hrs.

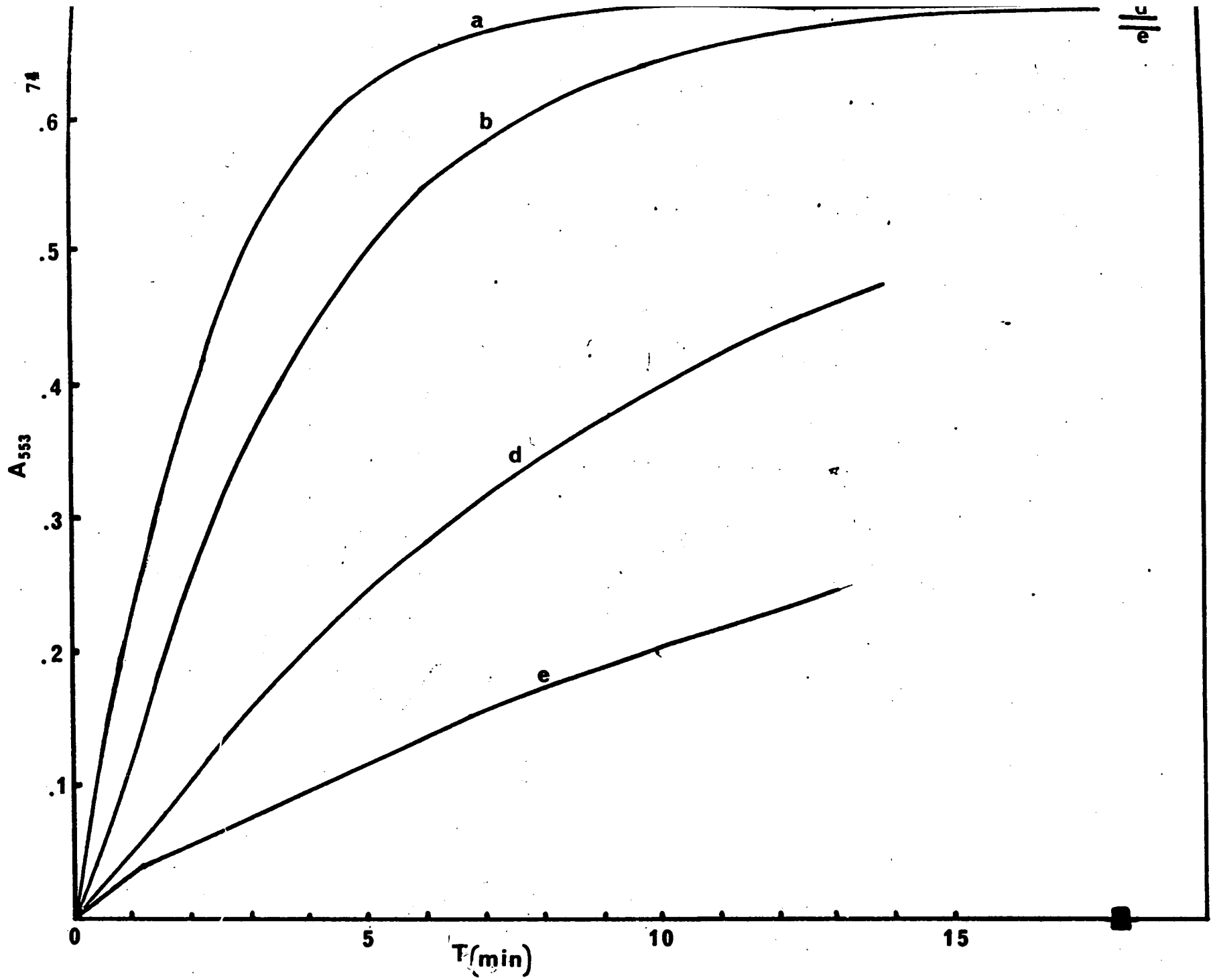

a
b
d
e
u
e
.6
.5
.4
.3
.2
.1
A_{553}
0
5
10
15
T(min)

Fig. 21

Nonenzymatic Effect of N-methylimidazole on the Ehrlich's Reaction with PBG.

a = 7.8 nmoles PBG
b = 7.8 nmoles PBG + 1400 nmoles N-Me-Im
c = 7.8 nmoles PBG + 4000 nmoles N-Me-Im
d = 7.8 nmoles PBG + 9400 nmoles N-Me-Im

■ = a break in time after 1 hr.
■ = a break in time after 3 hrs.

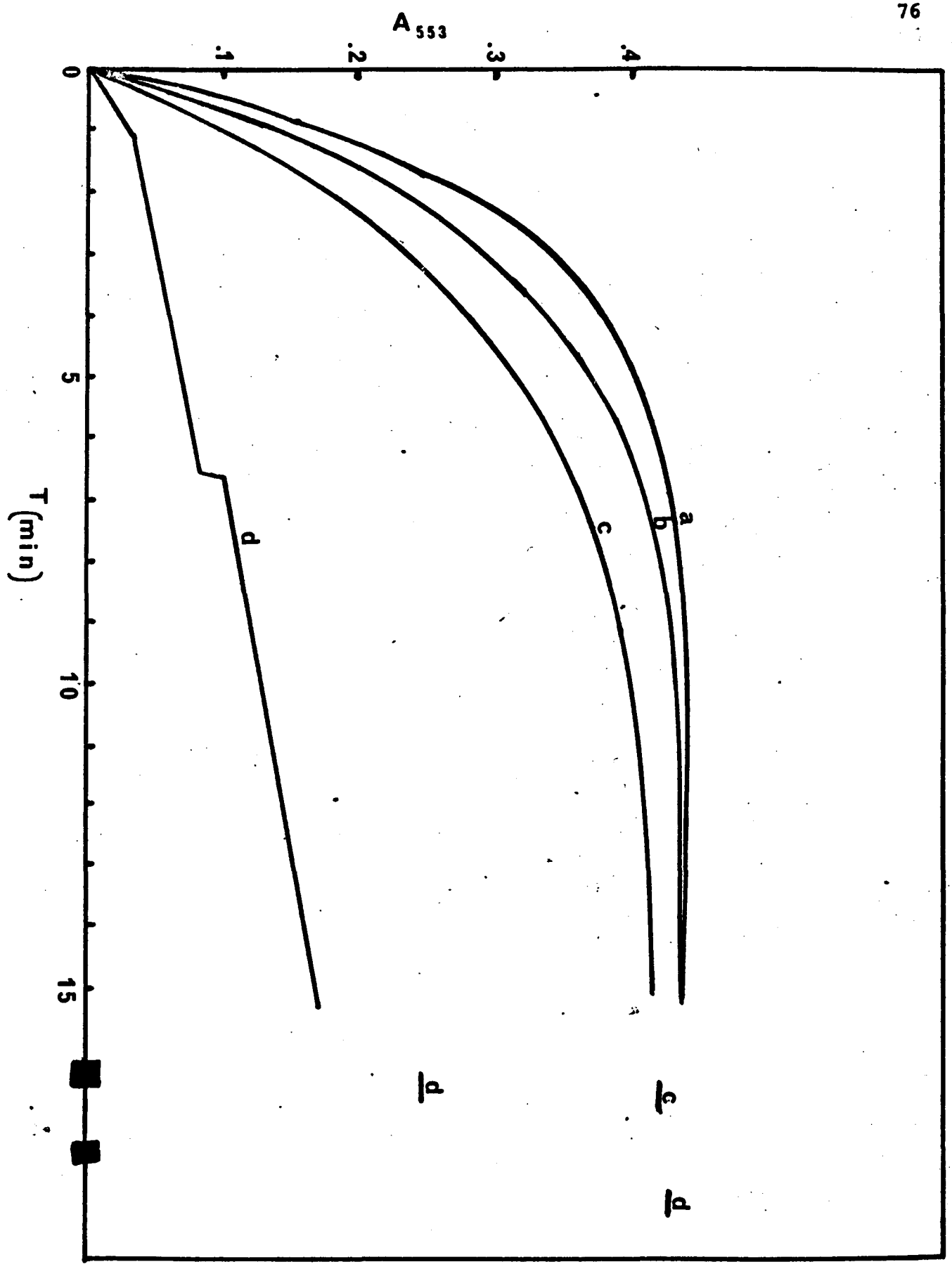

A 553
.1
.2
.3
.4
0
5
10
15
T (min)
a
b
c
d
d
c
d

Fig. 22

Nonenzymatic Effect of 2-Methylimidazole on the Ehrlich's Reaction with PBG.

a = 10.5 nmoles PBG
b = 10.5 nmoles PBG + 4000 nmoles 2-Me-Im
c = 10.5 nmoles PBG + 7000 nmoles 2-Me-Im

■ = a break in time after 1 hr.

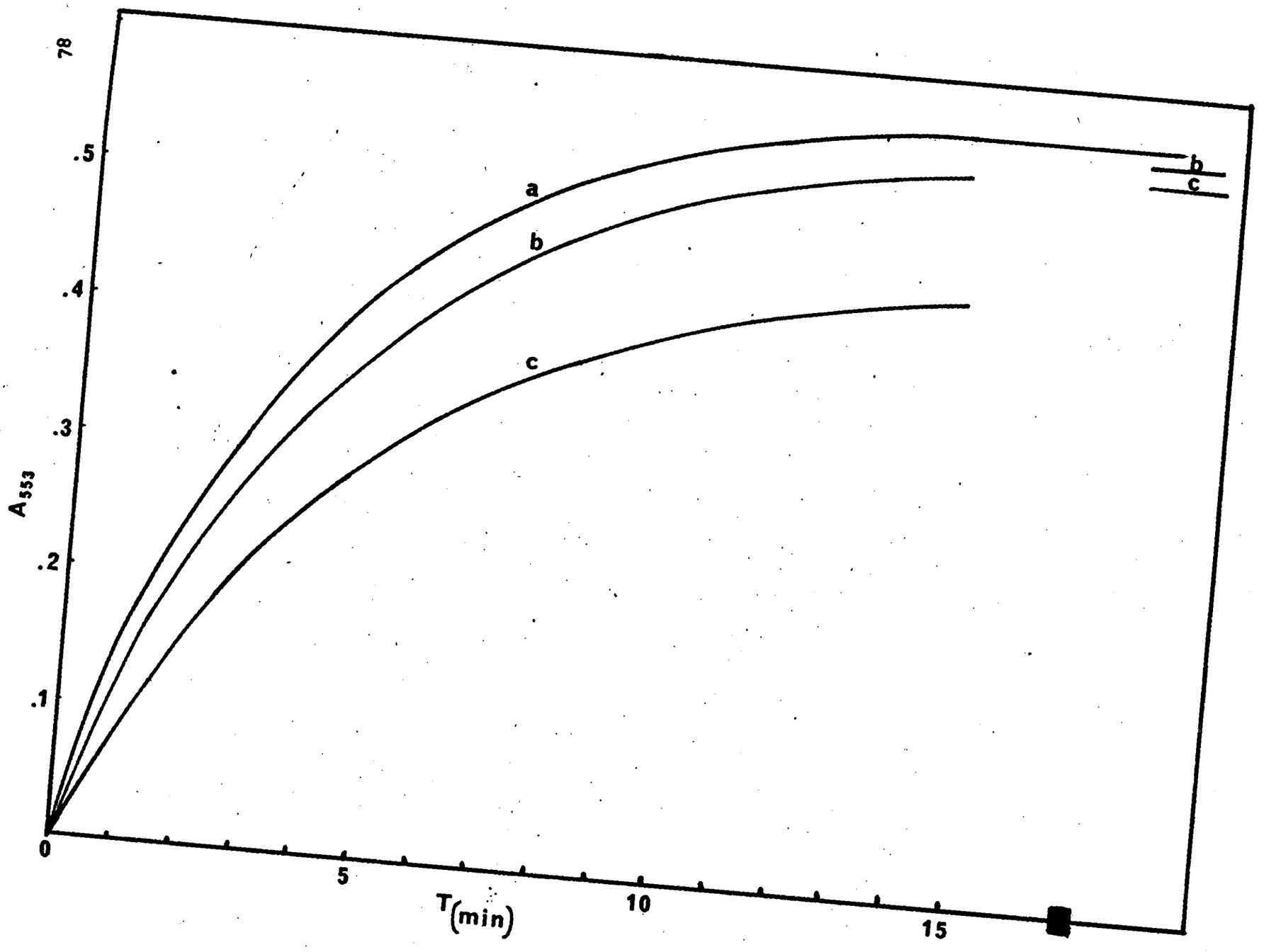
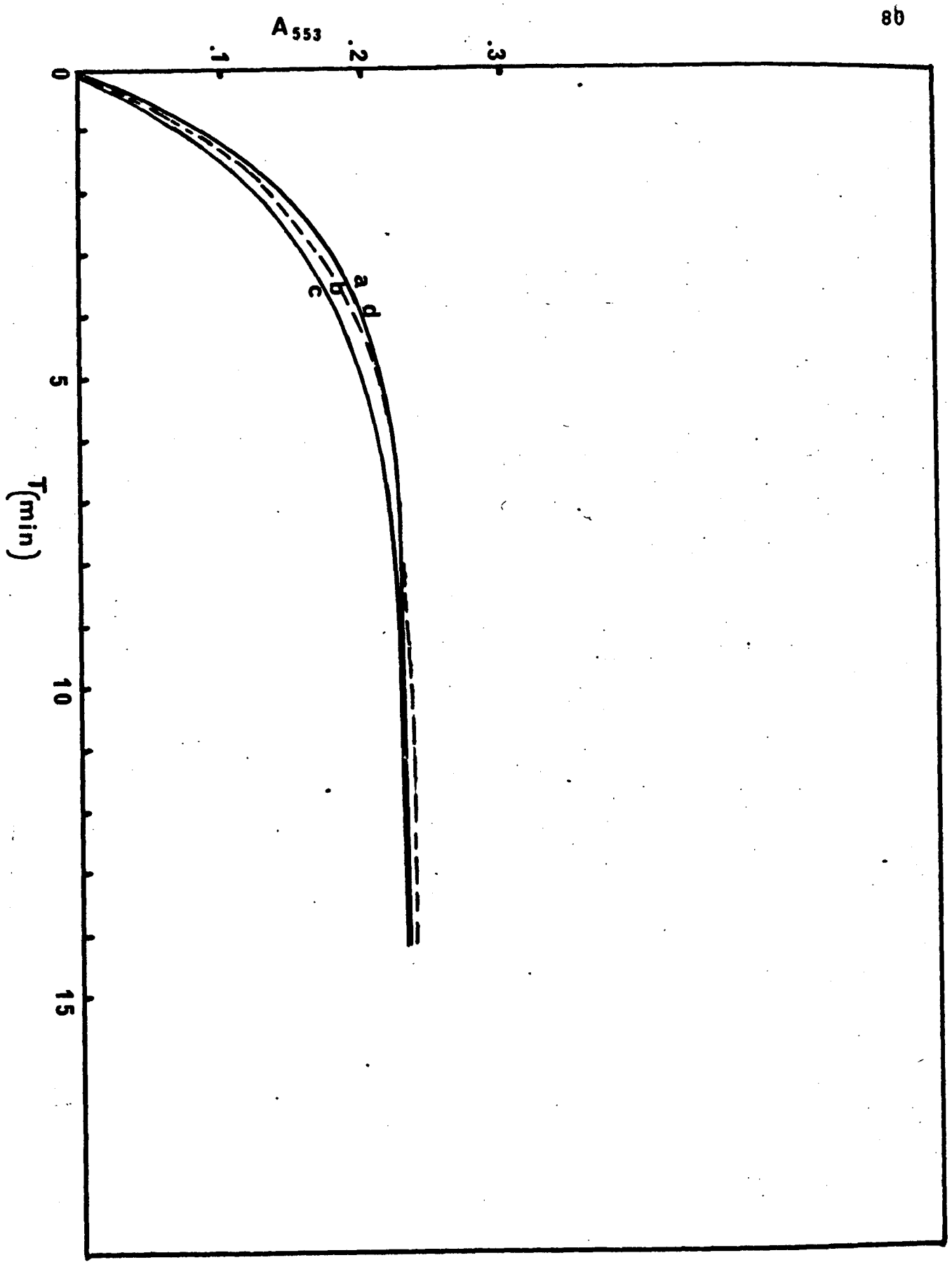

78
.5
.4
.3
.2
.1
A_{553}
a
b
c
b
c
0
5
10
15
T(min)

Fig. 23

Nonenzymatic Effect of Purified PBG Derivatives of Imidazole, N-methylimidazole, and 2-Methylimidazole (after Sephadex G-15) on the Ehrlich Reaction with PBG.

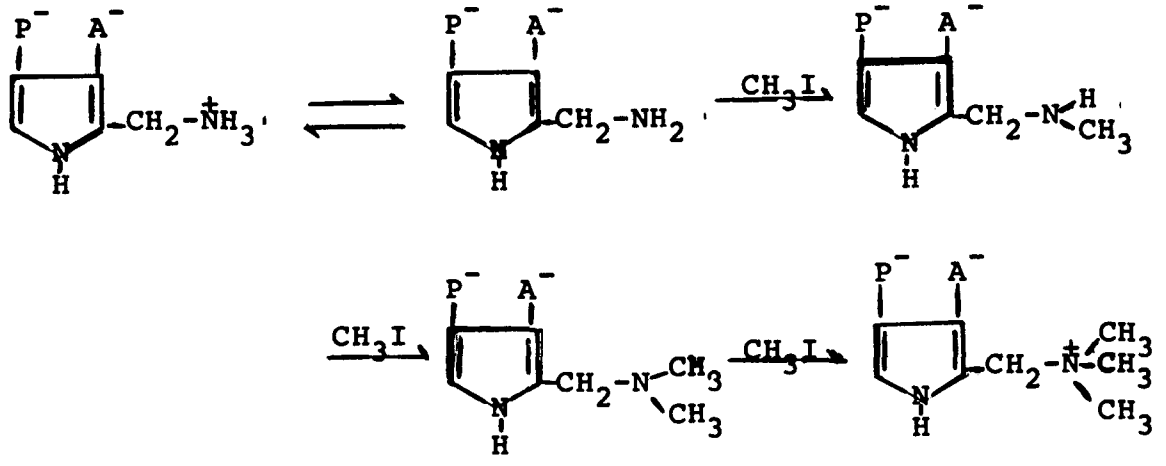
a = 4.2 nmoles PBG
b = 4.4 nmoles PBG-Imidazole
c = 3.9 nmoles PBG-N-methylimidazole
d = 4.3 nmoles PBG-2-Methylimidazole


A_{553}
.1
.2
.3
0
5
10
15
T(min)
a
b
c
d

v) Ultraviolet absorption studies of PBG and mixtures of PBG and imidazole and N-methylimidazole: The uv absorption was different for PBG than its derivatives. PBG-Im and PBG-N-Me-Im gave a uv absorption maximum at 228 mu and 223 mu respectively (Fig. 24,25). The results indicated that there might be chemical interactions between PBG and Im and between PBG and N-Me-Im. The auxochromic shift for PBG-Im and the bathochromic shift for PBG-N-Me-Im indicated the occurrence of reactions.

Methylation of PBG

A series of experiments involving the methylation of PBG with CH_3I at two pH's were performed (pH 7.4 and 9.5). The methylation steps are postulated to proceed as follows:



Methylation occurred more readily at pH 9.5 than at pH 7.4 because more of the uncharged α-amino species was present. The enzymatic results where methylated PBG (CH_3-PBG) was used as the substrate indicated that methylation at pH 9.5 resulted in a PBG derivative that was almost completely enzymatically converted to uroporphyrin III (Table 4).

Paper electrophoresis at pH 9.2 (Fig. 9) and thin layer chromatography in two different solvent systems (Fig. 13, 19) indicated that CH_3-PBG behaved differently than PBG. On gel filtration, methylated PBG came through at V_e = 79, 83, 86 ml. indicating that the methylated PBG derivative was probably a mixture.

Fig. 24

Ultraviolet Absorption Shift of PBG versus PBG + Excess Imidazole in .05M Tris Buffer (pH 7.4).

——————— PBG
--------- PBG + Excess Imidazole

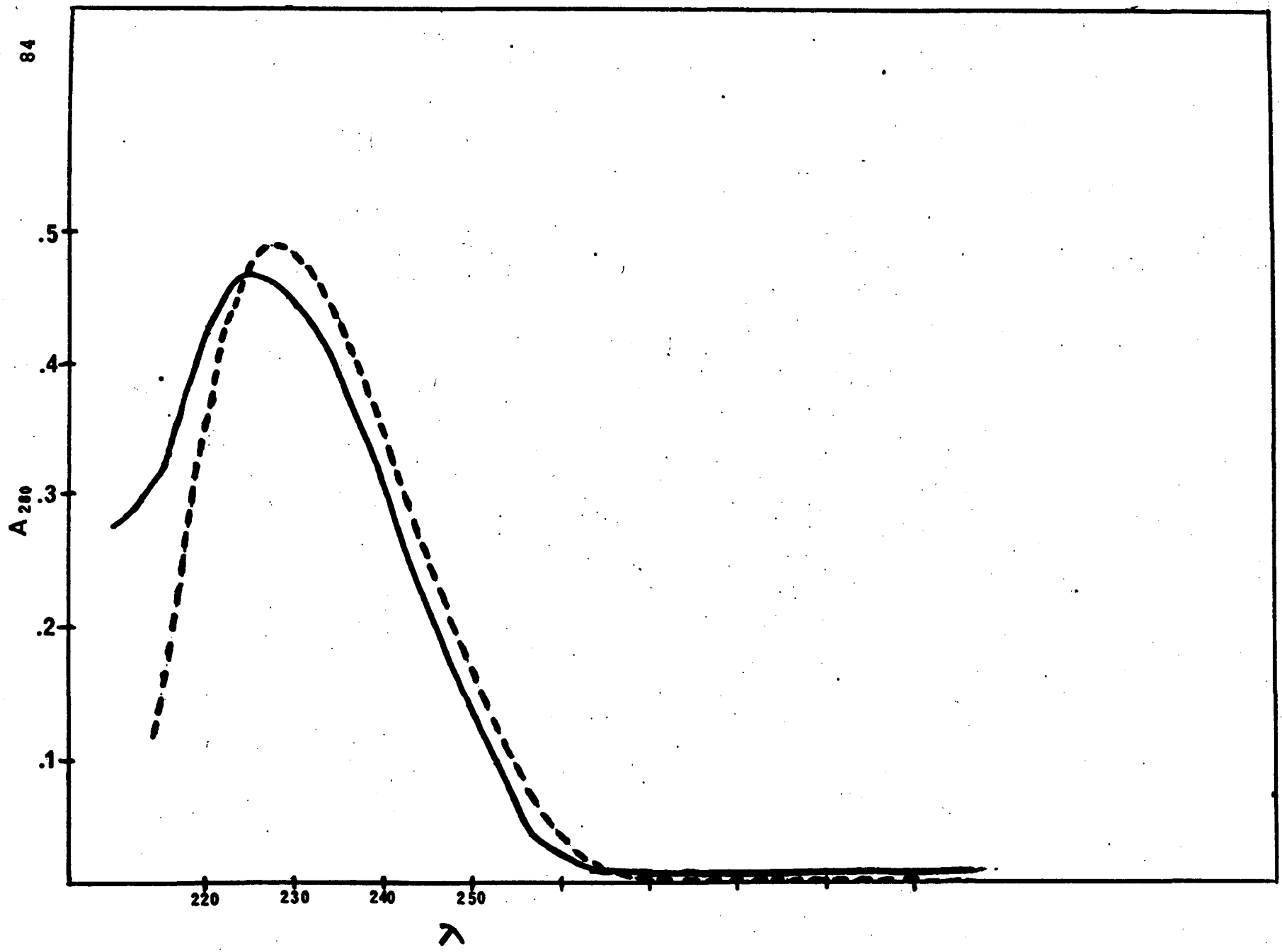
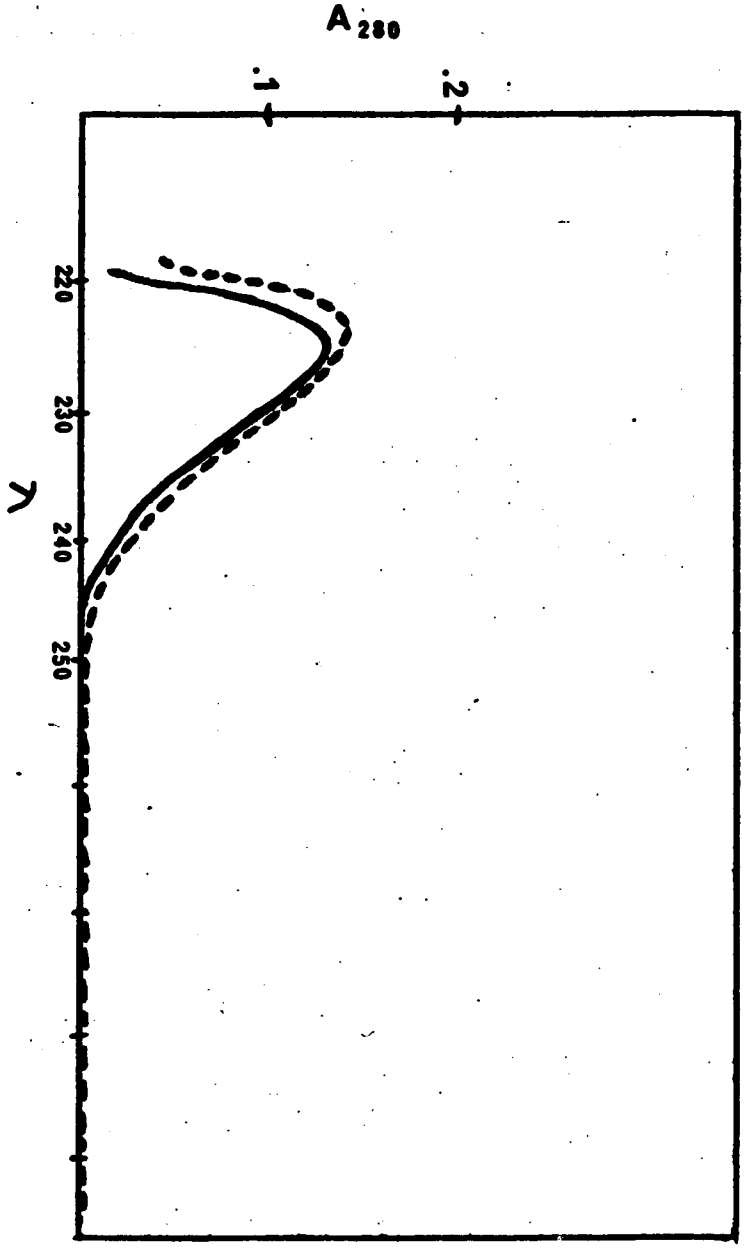

.5
.4
.3
.2
.1
A_{280}
220
230
240
250
λ

Fig. 25

Ultraviolet Absorption Shift of PBG versus PBG + Excess N-methylimidazole in .05M Tris Buffer (pH 7.4).

———— PBG
-------- PBG + Excess N-methylimidazole


A_{280}
.1
.2
220
230
240
250
λ

Enzymatic Effects of PBG Derivatives on Porphobilinogenase

When an excess of different nucleophilic imidazoles (Im, N-Me-Im, 2-Me-Im) were exogenously incubated with PBG and porphobilinogenase in .05M Tris buffer at pH 7.4, less PBG was consumed and urogen III yield was increased. The presence of 2-Me-Im did not alter substrate consumption or the yield of product when compared with PBG alone (Table 2).

PBG derivatives of Im and N-Me-Im prepared at pH 7.4 and 2-Me-Im prepared at pH 8.5 were freed of excess imidazole on Sephadex G-15 and were used as substrates for bovine liver porphobilinogenase. The effects of these purified derivatives were almost similar to those of incubation mixtures with exogenously-added imidazole and N-methylimidazole (Table 5). The percentage yields of urogen III in exogenously-added imidazoles incubations were slightly lower than those of purified derivatives. 2-Methylimidazole and PBG, however, had to react at pH 8.5 before the derivative was formed and its behavior on the enzymatic system was different than PBG alone but similar to PBG-Im and PBG-N-Me-Im. Its substrate consumption was lower than for PBG. The urogen isomer formed was mainly urogen III. When PBG-Im, PBG-N-Me-Im, and PBG-2-Me-Im were used as substrates for bovine liver porphobilinogenase, six to nine percent of urogen I was formed and the rest was urogen III. Incubation of methylated PBG (Table 4) with porphobilinogenase yielded the same trend in substrate consumption and product formation as

PBG-Im, PBG-N-Me-Im, and PBG-2-Me-Im. However, methylated PBG yielded 100% urogen III. The seeming anomaly of high PBG consumption and low urogen yield was explained by results which will be described in the section on aerobic and anaerobic experimental conditions.

Table 4. Enzymatic Effects of Methylated PBG on Bovine Liver Porphobilinogenase in .05M Tris Buffer (PH 7.4)

RUN	COMPOUND	nMOLES PBG ENZ CONSUMED	nMOLES PORPH ENZ FORMED	% PBG USED	% PORPH FORMED	% I, III
1	S^{6}_{PBG}	354	87.5	63.4	98.9	-----
1	$S^{6}_{PBG-CH_3}$	333	84.8	59.7	100	------
2	S^{3}_{PBG}	55	4.2	19.6	30.4	------
2	$S^{3}_{PBG-CH_3}$	26	5.9	13.9	91	------
2	S^{6}_{PBG}	136	16.6	48.4	48.9	0, 100
2	$S^{6}_{PBG-CH_3}$	81	16.8	43.3	83.1	0, 100

Table 5. Enzymatic Effects of Purified PBG Derivatives of Im, N-Me-Im, 2-Me-Im on Bovine Liver Porphobilinogenase in .05M Tris Buffer (PH 7.4)

RUN	COMPOUND	nMOLES PBG ENZ CONSUMED	nMOLES PORPH ENZ FORMED	% PBG USED	% PORPH FORMED	% I, III
1	S^{3}_{PBG}	61	4.5	19.6	29.6	------
1	S^{3}_{PBG-Im}	25	5.7	7.9	91.7	------
1	$S^{3}_{PBG-N-Me-Im}$	17	4.3	5.2	100	------
1	S^{6}_{PBG}	146	18.5	46.9	50.4	0, 100
1	S^{6}_{PBG-Im}	86	21.6	27.1	100.5	6.0, 94.0
1	$S^{6}_{PBG-N-Me-Im}$	69	16.9	21.0	98.1	9.6, 90.4
2	S^{3}_{PBG}	55	4.2	19.6	30.4	------
2	$S^{3}_{PBG-2-Me-Im}$	19	4.9	6.2	100	------
2	S^{6}_{PBG}	136	16.6	48.4	48.9	0, 100
2	$S^{6}_{PBG-2-Me-Im}$	67	17.2	21.9	100	8.7, 91.3

Detection and Characterization of Intermediate Polypyrroles

Bovine liver porphobilinogenase was purified approximately four-hundred and forty-fold (Table 6)(Fig. 26). The enzymatic runs where PBG was the substrate indicated that there is a lag period of about 3 hours between substrate consumption and product formation (Table 7). Experimental attempts were made to detect the presence of intermediates, especially at t = 3 hrs., by reactions of incubation mixtures with modified Ehrlich's reagent to look for rate or spectral changes. It had been reported by Bogorad[2] and Neuberger[7] that intermediate polypyrroles such as a dipyrrylmethane gave different spectra with modified Ehrlich's reagent. Thin layer chromatography of incubation mixtures on polygram and paper electrophoresis of incubation mixtures were also carried out in an attempt to detect intermediate polypyrroles. The experimental results did not indicate the presence of intermediates. The only Ehrlich-positive material detected was PBG and the only fluorescent material observed was uroporphyrin. From the stoichiometric calculations, it was apparent that the majority of the enzymatic runs did not convert all of the enzymatically-consumed PBG to uroporphyrin. Some of the enzymatically-consumed PBG apparently went to non-porphyrin material. The non-porphyrin material might be the oxidation

Table 6. Purification of Bovine Liver Porphobilinogenase.

Fraction	Mg Protein	Activity [nmoles PBG conv. to Porph]	Sp. Activity [Activity / hr.-mg]	Purification
Homogenate	9.28	7.8	.14	1
$Ca_3(PO_4)_2$ Treatment	1.24	38.5	5.17	37
Sephadex G-100 Column (Peak Activity)	.72	268.7	62.20	444

Fig. 26

Elution Profile and Specific Activity of Bovine Liver Porphobilinogenase Purified on Sephadex G-100 (1.8 x 82 cm) and Eluted with .05M Tris—.1M NaCl (pH 7.4).

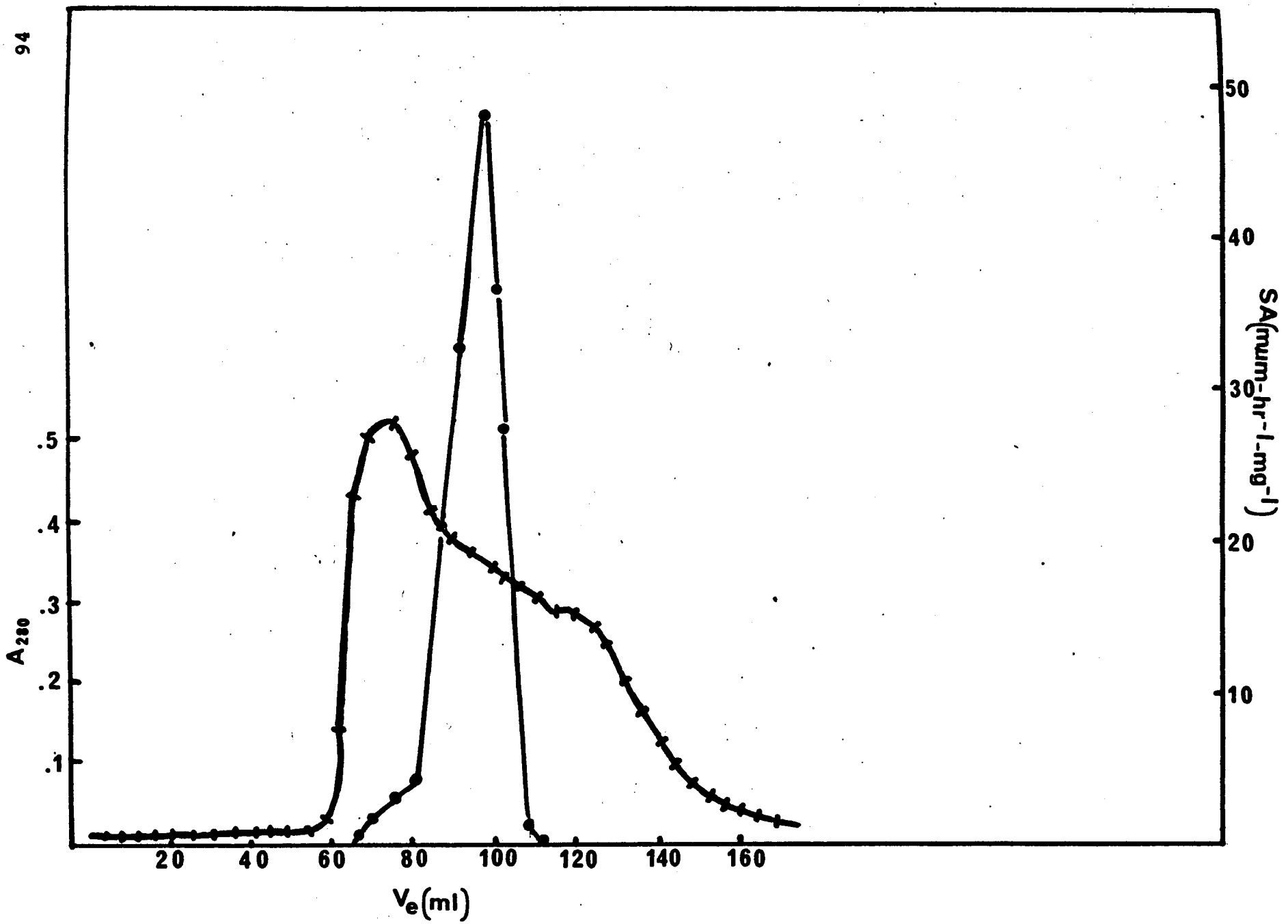

A280
.1
.2
.3
.4
.5
20
40
60
80
100
120
140
160
Ve(ml)
10
20
30
40
50
SA(mum-hr-1-mg-1)

Table 7. PBG Consumption and Uroporphyrin Formation by Bovine Liver Porphobilinogenase in .05M Tris Buffer (pH7.4).

RUN	COMPOUND	nMOLES PBG ENZ CONSUMED	nMOLES PORPH ENZ FORMED	% PBG USED	% PORPH FORMED
1	S^3_{PBG}	39	3.9	12.0	40.0
1	S^6_{PBG}	91	12.3	28.3	54.1
2	S^3_{PBG}	24	6.2	20.9	85.0
2	S^6_{PBG}	45	8.8	40.0	64.8
3	S^3_{PBG}	60	5.8	23.6	37.1
3	S^6_{PBG}	105	15.1	40.1	54.3
4	S^3_{PBG}	111	16.9	18.5	60.8
4	S^6_{PBG}	256	29.0	42.7	45.3

products of a PBG oxygenase as reported by Frydman et al.[31,32] When the thin layer polygram plate from chromatography of the incubation mixture was developed and was heated at 120°C after spraying with Ehrlich' reagent[30], an orange compound with a R_f value of .28 (approximately half way between PBG and the the origin) was observed. Frydman et al.[30] reported a compound as a result of action of pyrrole oxygenase on PBG which gave an orange color with Ehrlich's reagent and heat. The compound in their chromatography had a R_f value of .25.

Convinced that we were not able to detect the presence of intermediate polypyrroles, we concur in the current belief[5,6] that the biosynthesis of urogen III in bovine liver also occurs on the enzyme surface with no liberation of intermediates until the product is finished and cyclized.

Anaerobiosis versus Aerobiosis

The effects of anaerobiosis and aerobiosis on the enzymatic effect of bovine liver porphobilinogenase were explored. The results (Table 8) indicated that although more PBG was converted enzymatically under aerobic conditions, less urogen was formed. It was decided that anaerobic condition would be better for all enzymatic reactions although Grinstein et al.[14] have declared that aerobic conditions did not affect porphyrin yield. Other workers[33,34] have shown that anaerobiosis was much preferred for complete porphyrin formation than aerobiosis.

In many of the enzymatic runs where PBG was used as the substrate, the percent of consumed PBG converted to urogen III was about 50% (Table 7). The possibility existed that the system was not truly anaerobic. Frydman et al.[35,36] have reported the presence of pyrrolooxygenases in wheat germ, chloroplasts, and rat liver. Frydman et al.[31,32] characterized an oxygenase both in wheat germ and in rat liver which oxidizes PBG to 2-hydroxy-5-oxoporphobilinogen (2-OH-5-OXO-PBC) and 5-oxo-porphobilinogen(5-OXO-PBG). The ratio of yields of the two oxidation products in wheat germ was 4:1 of (2-OH-5-OXO-PBG) to (5-OXO-PBG) respectively. In order to "scour" for residual oxygen in our porphobilinogenase reaction, glucose (20 mg) and glucose oxidase (2 mg) were added to the incubation mixture[17]. Delwiche and co-workers were able to exclude oxygen in this manner in their

investigation of aerobic and anaerobic processes in component reactions of oxidative sterol demethylation. Glucose oxidase in the presence of oxygen converts β-D-glucose to δ-gluconolactone. The effect of glucose oxidase on the incubation mixture is shown in Table 9.

Table 8. Anaerobiosis versus Aerobiosis for Bovine Liver Porphobilinogenase in .05M Tris Buffer (pH 7.4).

RUN	COMPOUND	nMOLES PBG ENZ CONSUMED	nMOLES PORPH ENZ FORMED	% PBG USED	% PORPH FORMED
1	$S^{3.5}_{PBG}$	119	23.3	23.4	78.3
1	$AS^{3.5}_{PBG}$	177	7.6	34.8	17.2
2	$S^{3.5}_{PBG}$	300	28.2	81.7	37.6
2	$AS^{3.5}_{PBG}$	326	7.4	88.6	9.1
3	$S^{3.5}_{PBG}$	269	23.6	81.5	35.1
3	$AS^{3.5}_{PBG}$	292	5.4	85.6	7.3

Table 9. Effect of Glucose and Glucose Oxidase on PBG and Porphobilinogenase in .05M Tris Buffer (pH7.4).

RUN	COMPOUND	nMOLES PBG ENZ CONSUMED	nMOLES PORPH ENZ FORMED	% PBG USED	% PORPH FORMED	% I, III
1	S^3_{PBG}	55	4.2	19.6	30.4	------
1	$S^3_{PBG+glucose\ oxidase}$	26	5.8	9.1	88.5	------
1	S^6_{PBG}	136	16.6	48.4	48.9	0, 100
1	$S^6_{PBG+glucose\ oxidase}$	68	15.5	23.9	91.2	0, 100

Methylation of Porphobilinogenase

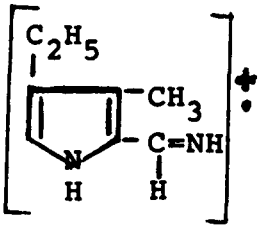
When porphobilinogenase was treated with methyl iodide at pH 7.4, its enzymatic activity dropped by 33% (Table 10). The various groups on the enzyme that might be alkylated are: -NH, -SH, -OH (His, CysH, Lys, Ser). Bovine liver porphobilinogenase is known to be inhibited by mercuribenzoate and heavy metals[14]. This inhibition is reversed by cysteine. Certainly an -SH group is essential for enzymatic activity. It is possible that methyl iodide alkylated this -SH group or one of the other groups mentioned above.

Table 10 Enzymatic Effects of Porphobilinogenase versus Alkylated Porphobilinogenase in .05M Tris Buffer (pH 7.4)

RUN	COMPOUND	nMOLES PBG ENZ CONSUMED	nMOLES PORPH ENZ FORMED	% PBG USED	% PORPH FORMED
1	$S^6_{PBG+PBGnase}$	354	87.5	63.4	98.9
1	$S^6_{PBG+CH_3-PBGnase}$	177	29.0	31.7	65.5

The mass spectra did not show the presence of molecular ions for PBG, PBG-Imidazole, and PBG-N-methylimidazole. The prominent peaks common to PBG and PBG-Im and PBG-N-Me-Im were m/e 167, 122, 108, and 94.

However, the spectrum for PBG had a peak at m/e 136 whereas PBG-Im and PBG-N-Me-Im did not. The mass at 136 may be due to to the following fragment.



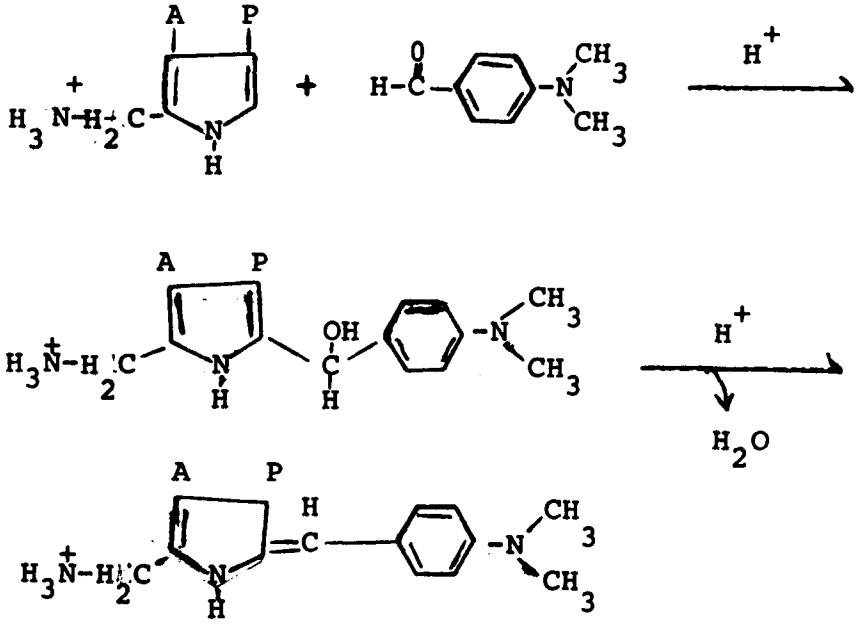
The mass spectrum of PBG-Im had a prominent peak at m/e 68 (imidazole) which was not present in the mass spectrum of PBG. The mass spectrum of PBG-N-Me-Im had a prominent peak at m/e 82 (N-methylimidazole) which was not present in the mass spectrum of PBG. From the fragment patterns of the mass spectra, we believe that derivatives of PBG were synthesized.

DISCUSSION

Imidazole or N-methylimidazole or 2-methylimidazole and PBG react non-enzymatically to form PBG derivatives. These derivatives have been purified and characterized in the following ways. a) They have been freed of excess reagent on Sephadex G-15 and eluted at a volume (lower than for PBG) compatible with the expected molecular weights which are higher than PBG. b) The samples purified on Sephadex G-15 behaved differently from PBG in electrophoresis and thin-layer chromatography in two different solvent systems, but behaved exactly like samples of PBG and excess imidazole and N-methylimidazole. c) The PBG derivatives give the same Ehrlich reaction but the color developed at a slightly slower rate. d) The derivatives are like PBG in that they are substrates for porphobilinogenase but unlike it in that they are completely converted into uroporphyrinogen. e) The mass spectra are compatible with PBG-Im and PBG-N-Me-Im derivatives. f) PBG and 2-methylimidazole at pH 7.4 where 2-methylimidazole is not a good nucleophile do not react and

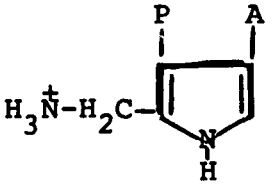
the mixture is indistinguishable from PBG in G-15 chromatography, electrophoresis, thin layer chromatography in two solvent systems and towards porphobilinogenase and oxygenase. However, when the pH was brought up to 8.5, reaction took place and the compound purified on G-15, then behaved like the other PBG derivatives.

PBG derivatives reacted with the modified Ehrlich's reagent and gave identical spectra in the visible region; the only apparent difference was in the kinetics of color development monitored at 553 mu (Fig. 23). Ehrlich's reagent is known to react with the free α-position on any pyrrole (on PBG in this case).

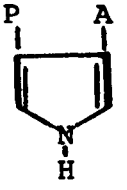


This suggests that the PBG derivatives obtained from reactions of PBG with various imidazoles have a free α position.

These derivatives are as good substrates as PBG for porphobilinogenase and produce uroporphyrinogen III. Opsopyrrole dicarboxylic acid (OPSO-PBG) is a competitive inhibitor of PBG consumption (Bogorad)[37] and isoporphobilinogen (ISO-PBG) acts similarly (Carpenter and Scott)[38].

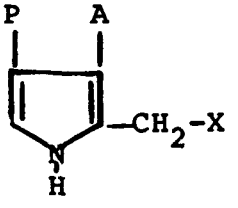


ISO-PBG



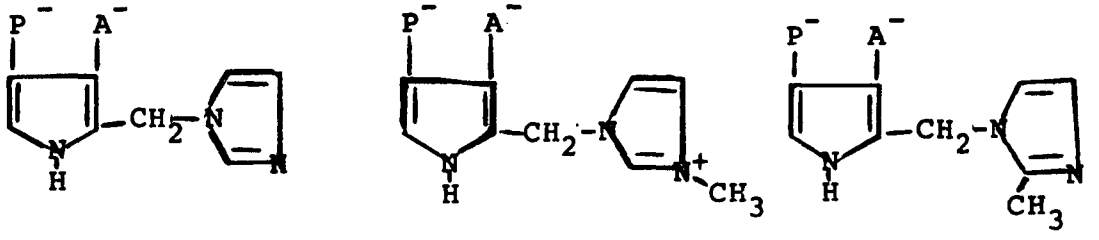
OPSO-PBG

It is concluded that the order of the acetic acid and propionic acid groups in the substrate is crucial for binding to the enzyme and for the biosynthesis of urogen III. Apparently, the enzyme recognizes the order of the carboxylic acid side chains and requires the following structure for binding



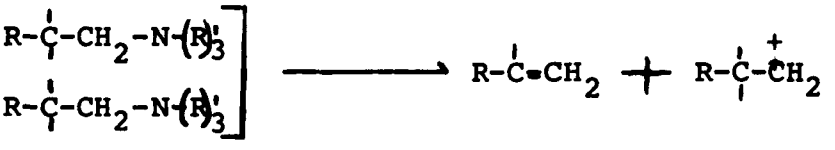
but does not discriminate between $-NH_3^+$, $-\overset{CH_3}{\underset{CH_3}{N}}-CH_3^+$, and imidazoles as leaving groups. Porphobilinogen oxygenase can discriminate between the derivatives and PBG.

The foregoing considerations suggest the following structures at pH 7.4 for the imidazole derivatives of PBG:

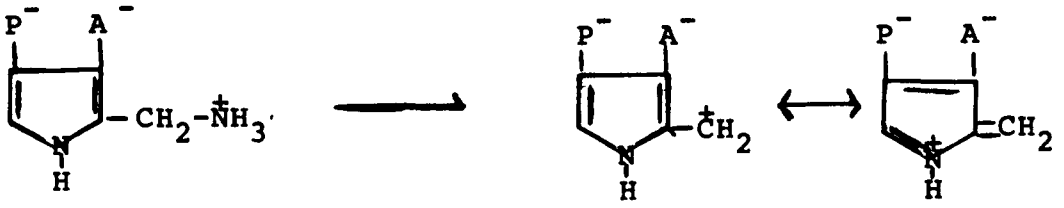


PBG-IM PBG-N-Me-IM PBG-2-Me-IM

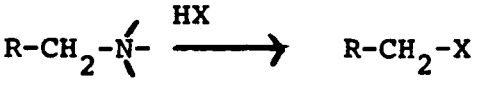
The proposed structures for the derivatives of PBG are in line with the chemical nature of PBG and various imidazoles. Porphobilinogen behaves like a Mannich base and Mannich bases can undergo deamination by cleavage of the CH_2-N- bond (Tramontini)[39]



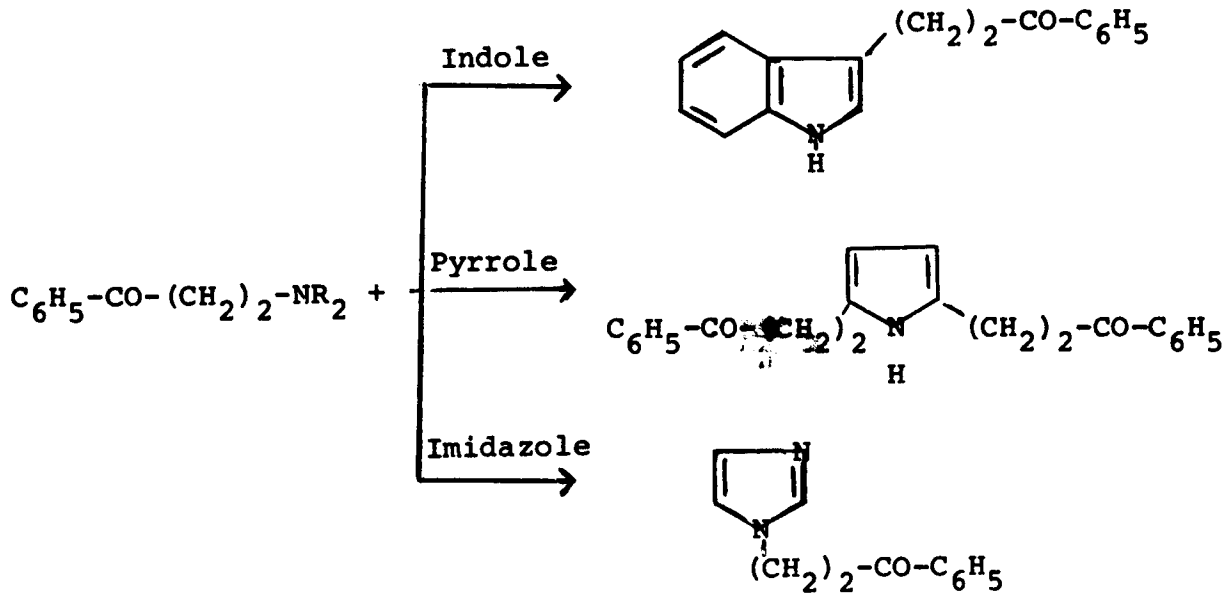
In our case, PBG or PBG derivatives may be a carbonium ion which is stabilized by the pyrrole ring or undergo displacement in which partial carbonium ion character stabilizes the transition state.



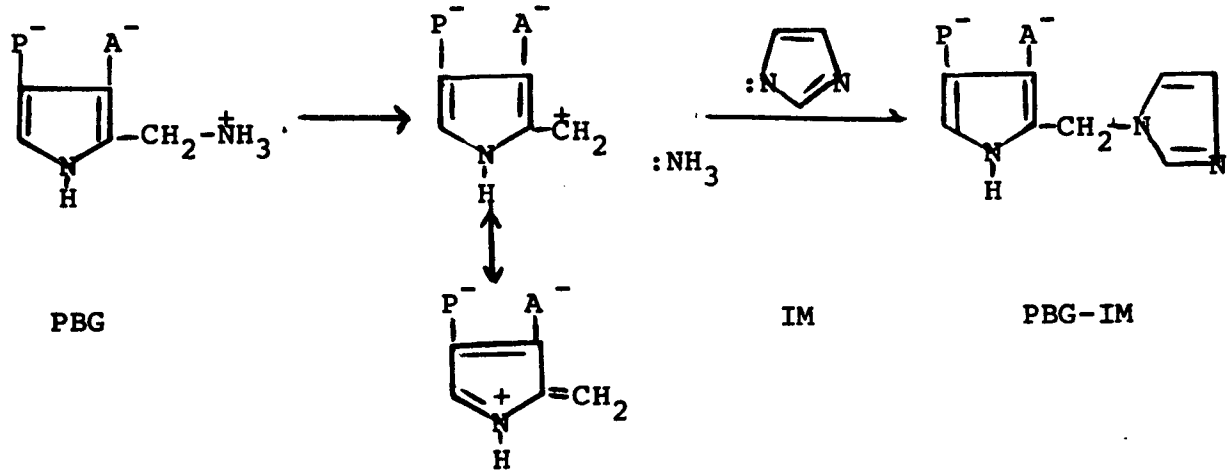
Mannich bases undergo substitution of the amino group, possibly through the carbonium ion shown above by a group X (Tramontini)[52].



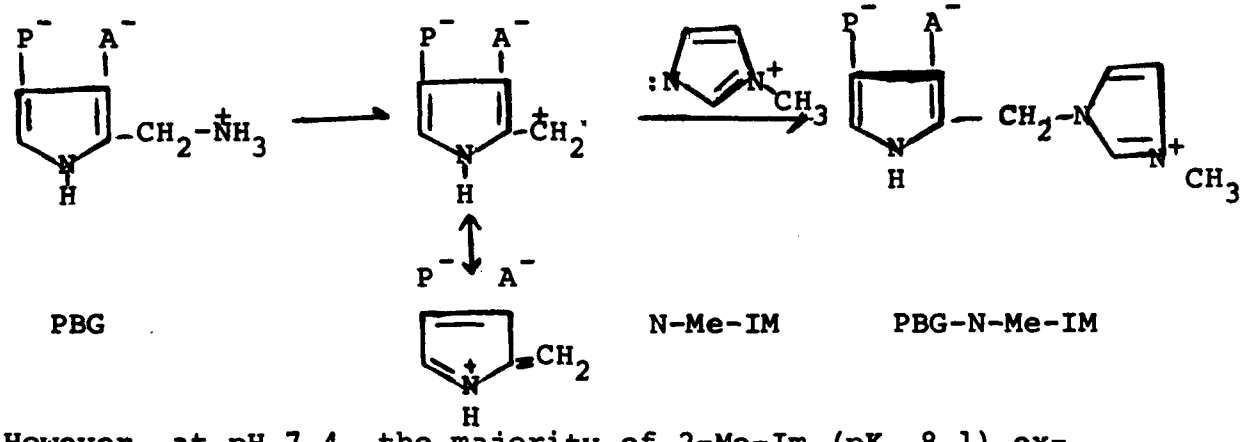
This substitution reaction (with elimination of the amino group) can be an N-alkylation on NH-heterocyclic compounds (Tramontini et al.)[40] or C-alkylation.



In our case, the reaction between PBG and a large excess of the various imidazoles was carried out in .05M Tris buffer (pH 7.4) and was complete in 30 min. Much of imidazole (pK_a 7.1) and N-methylimidazole (pK_a 7.3) existed as the non-protonated species which were excellent nucleophiles for N-alkylation by PBG.

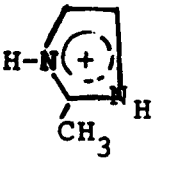


PBG IM PBG-IM



PBG N-Me-IM PBG-N-Me-IM

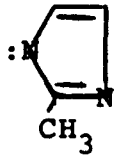
However, at pH 7.4, the majority of 2-Me-Im (pK_a 8.1) existed as the protonated species and this was a poor nucleophile for N-alkylation by PBG:



2-Me-IM at pH 7.4

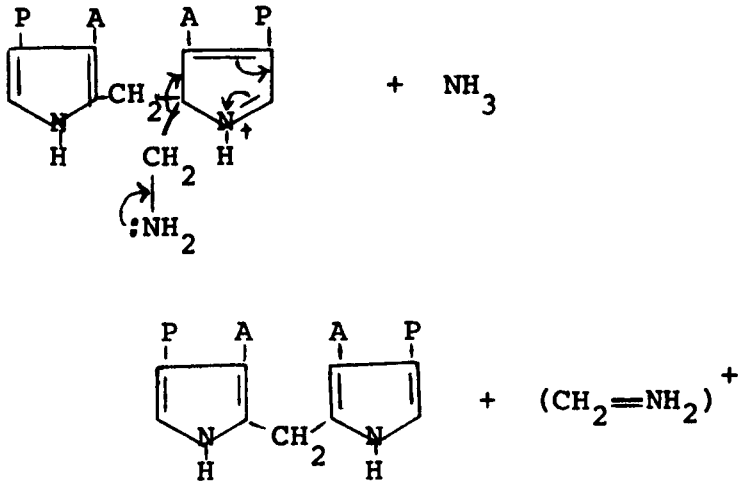
When PBG and 2-Me-Im were reacted at pH 8.5, a reaction took place which was confirmed by molecular weight estimation on gel filtration, by the dramatic change in the results in the enzymatic reaction and in electrophoresis and thin layer chromatography. PBG-2-Me-Im behaved similarly to PBG-N-Me on Sephadex G-15 (eluted off the column at an earlier fraction than PBG) in paper electrophoresis, and thin layer chromatography. Compared with PBG consumption

by porphobilinogenase, less PBG-2-Me-Im was enzymatically consumed and the yield of urogen III was higher. Enzymatically, PBG-2-Me-Im behaved like other PBG derivatives. This is to be expected because at pH 8.5, 2-Me-Im was mainly in the non-protonated form which was an excellent nucleophile for N-alkylation by PBG:



2-Me-Im at pH 8.5

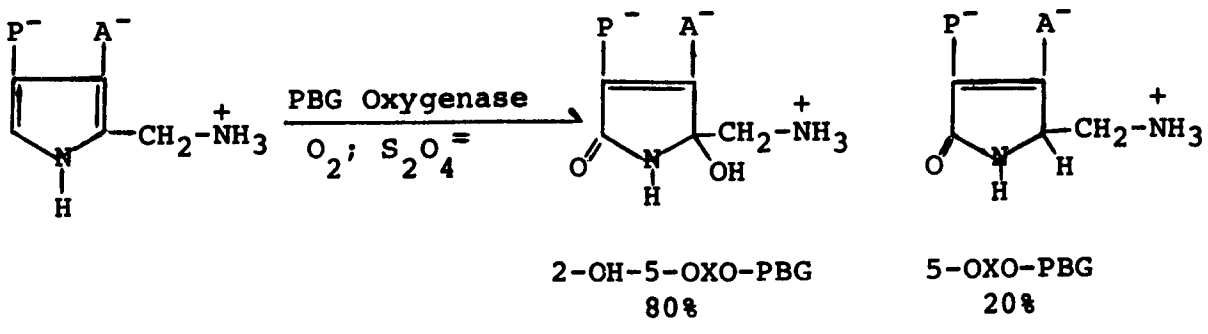
The slow in vitro decomposition of PBG in solution to form porphyrins in low yield and other products undoubtedly goes through the formation of the stabilized carbonium ion which attacks the 2 position of another PBG molecule as suggested by Frydman et al.[41] which they think is operating for PBG at neutral pH. Since PBG undergoes very slow decomposition (by solvalysis S_N1) under our experimental conditions, i.e., the blank tubes do not undergo decomposition, the mechanism is probably S_N2.



$$(CH_2{=}NH_2)^+ + H_2O \longrightarrow CH_2O + NH_3$$

Frydman et al., Biochemistry, 10, No. 7, 1154(1971).

When PBG, PBG-imidazole derivatives and methylated PBG condensed enzymatically in the presence of porphobilinogenase, the substrate derivatives gave better yields of uroporphyrinogen than PBG did. However, more PBG was consumed. In the PBG incubation mixtures, approximately 30% to 50% of the enzymatically-converted PBG could not be accounted for as uroporphyrinogen. We and others thought that intermediates were building up but getting oxidized. It seemed that PBG was being used as a substrate for some enzyme other than porphobilinogenase. Frydman et al.[30] reported a porphobilinogen oxygenase detected in wheat germ, spinach chloroplasts, Swiss chard, rat liver, and rat brain. PBG oxygenase has been further isolated and characterized in wheat germ and rat liver systems (Frydman et al.)[31,32]. The enzyme belongs to the general type of the pyrrolooxygenases, a new group of enzymes recently described by Frydman et al.[35,36]. PBG oxygenase has an absolute requirement for oxygen and a reducing agent and it oxidizes PBG to two products[30], neither of which can form urogen:



It was also reported by Frydman et al.[32] that PBG deaminase activity and PBG oxygenase activity occurred together during the first stages of the purification process of PBG oxygenase. A proteic inhibitor of PBG oxygenase was present and could only be removed during the last stages of the purification process. The proteic inhibitor, however, was heat and cold labile and could thus be destroyed without affecting the oxygenase activity. Thin layer chromatography on cellulose-coated plates on the two oxidation products reported by Frydman et al. gave an R_f value of .25 in butanol-acetic acid-water (40:10:50)[30]. The oxidized compounds developed a color after spraying the TLC plates with Ehrlich's reagent followed by heating at 120°C. The developed color after heating ranged from yellow orange to deep orange (depending on the concentration of the products in the spot). The following evidence suggests that PBG oxygenas is present in our porphobilinogenase.

a) Upon spraying our TLC plates with Ehrlich's reagent and heating at 120°C, samples of PBG incubation mixture gave orange color compounds. The R_f value was .27 and the solvent system was n-butanol-acetic acid-water (63:11:26).

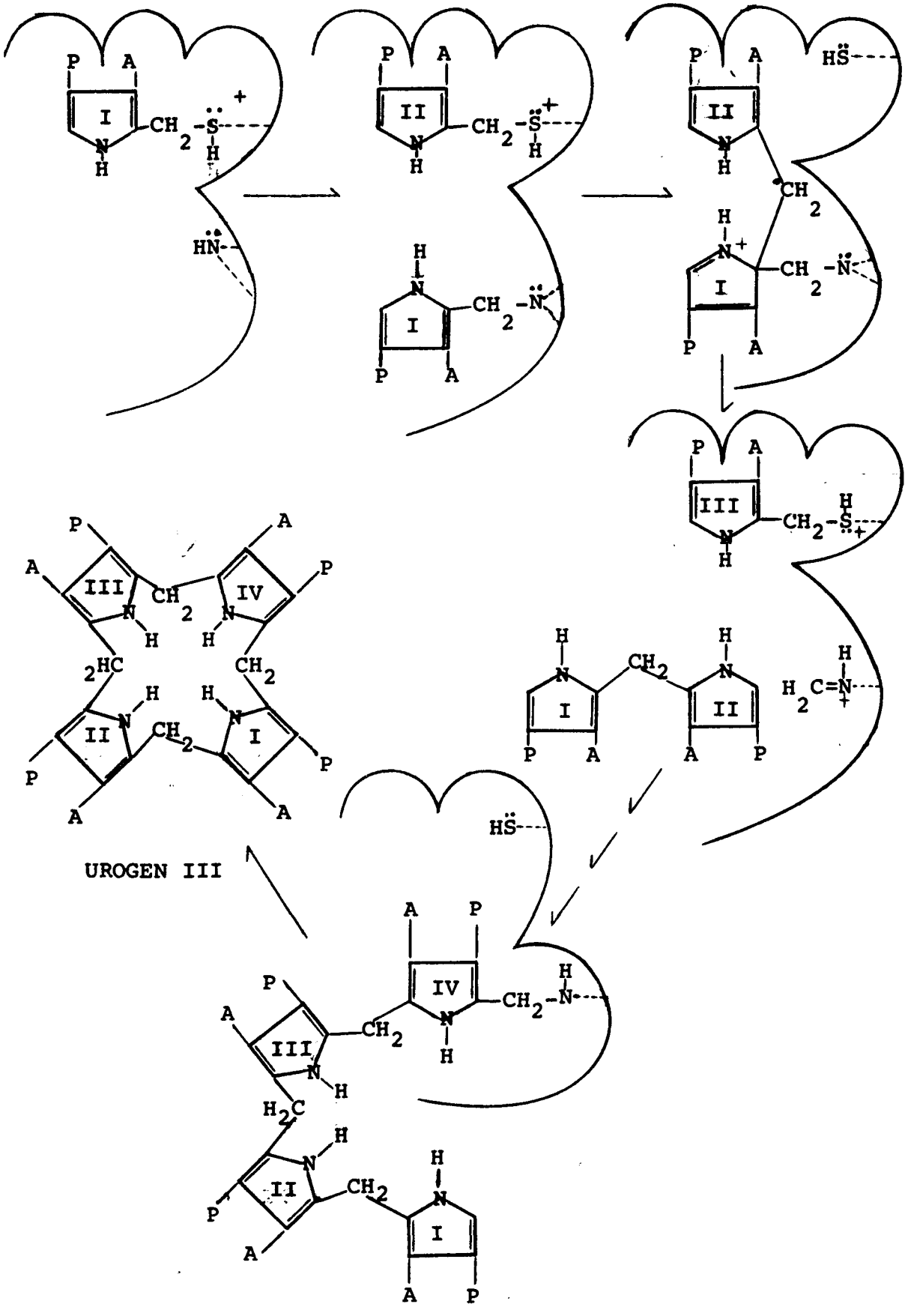
b) Enzymatic results with PBG, PBG derivatives, and PBG with glucose and glucose oxidase: It appears that PBG derivatives were not substrates for PBG oxygenase and enzymatically-converted PBG derivatives formed urogen exclusively. Data from anaerobiosis and aerobiosis showed that although more PBG was enzymatically consumed under aerobic conditions than under anaerobic conditions, a lower yield of urogen was obtained. The implication is that PBG, but not PBG derivatives, in the presence of oxygen, was enzymatically converted by a PBG oxygenase into oxidation products which could no longer form urogen. The addition of glucose and glucose oxidase to PBG and porphobilinogenase clearly reduced PBG consumption and increased the yield of urogen by removing oxygen. Bogorad[45] working with spinach porphobilinogenase reported that the yield of porphyrin was adversely affected by the presence of air. Batlle and coworkers[34] found that the activity of soybean callus porphobilinogen lowered urogen yield in presence of air. The yield of uroporphyrinogens was very low although consumption of substrated was not greatly modified. Frydman and coworkers[4] reported that strict anaerobiosis was not needed under their incubation conditions. Grinstein et al.[14], on the other hand, found that bovine liver

porphobilinogenase gave the same yield of uroporphyrinogen under anaerobic and aerobic conditions. Our work resolves these contradictory reports.

The work described here and results obtained by other workers can be incorporated into a proposed picture of the active site on bovine liver porphobilinogenase (Fig. 27). The model should account for at least two sites. One site is for the binding of incoming PBG units with steric restrictions such that binding is specific for propionate and acetate sidechains and for the methylene of PBG or PBG derivatives. Binding might be on a nucleophilic group such as HS-Enzyme or H_2N-Enzyme. After the first PBG is bound at site I, the enzyme behaves like a transferase and "swings" the first PBG unit to the second site where it is held only at the methylene group, by an H-N group on the enzyme (His or Lys). Upon transferring PBG from site I to site II, site I becomes vacant for a second PBG molecule which is held in an identical manner as the first PBG unit. The second PBG attacks the C-2 of the first PBG unit, resulting in a "head to head" condensation. Frydman et al.[41] have shown that at neutral pH, PBG prefers to attack non-enzymatically at the C-2 position of a second PBG. The 1-carbon fragment from the first PBG unit would move to the free side of the second PBG ring. Two more identical steps would produce an enzyme-bound tetrapyrrole. The above postulate was proposed by Russell[42]. By the time the tetrapyrrole is

Fig. 27

Proposed Picture of the Active Site of Bovine Liver Porphobilinogenase. Site I and Site II Discussed in Text.



UROGEN III

formed, it is proposed that the chain has curled around and placed ring I back to the site where the chain is anchored to enzyme.

The above hypothesis invokes the presence of covalent enzyme-substrate intermediates and such a mechanism has been proposed by Spector[43] for a group of enzymes he calls transferases. The best known examples of enzymatic reactions for which there is evidence for the formation of covalent intermediate compounds of enzymes and a part of the substrates are the proteases and esterases that catalyze the hydrolysis and transfer of acyl groups with the intermediate transfer of an acyl group to the hydroxyl or sulfhydryl group of a serine or cysteine residue on the enzyme[44]. There is evidence for the formation of phosphoryl-enzymes with a covalent link to the hydroxyl group of a serine or the imidazole group of a histidine residue as intermediates in the action of several phosphate-transferring enzymes. In this mechanism a catalytic group (or atom) within the active site of the enzyme reacts with the substrate and forms a covalently-linked enzyme-substrate intermediate. In our case, the substrate may be bound to a -SH or a -NH (His or Lys) on the enzyme. The covalent enzyme-substrate intermediate enables the enzyme to avoid

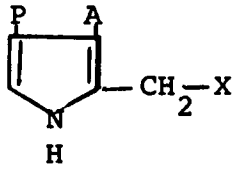
the entropic difficulty of bringing two substrates to the active site at the same time. Porphobilinogenase appears to be one of a group of transferases discussed by Spector who contends one of the main features of enzyme catalysis is the formation of a covalently-linked enzyme-substrate intermediate.

The above mechanism of action for bovine liver porphobilinogenase is consistent with the following evidence. a) Two sites for bovine liver porphobilinogenase have been proposed by Grinstein et al.[14]. From the Hill plots, n was found to be two for porphobilinogenase and one for deaminase. b) Urogen III must have a rearranged DPM (DPM-III) from the start of the polymerization (Frydman et al.)[4]. The enzymatic results of deaminase or porphobilinogenase with synthetic dipyrrylmethanes and tripyrrylmethanes by Frydman and coworkers[3,4,6] clearly showed that the biosynthesis of urogen I and urogen III must originate by a different pathway from the start of the enzymatic polymerization. Bogorad's isolation of dipyrrylmethane[2] (DPM) and tetrapyrrylmethane[5] in deaminase incubations in the presence of hydroxylamine and ammonium ions respectively corroborated the findings of Frydman and coworkers. The conclusion is that only DPM-III, not DPM, TRPM, TEPM, could

get incorporated into urogen III. Most importantly, Battersby [9] and Scott [1], using ^{13}C-NMR measurements showed that only one of the pyrrole rings was rearranged in the urogen III nucleus of protoporphyrin IX. Since only one apparent rearrangement of the PBG units took place and since DPM-III, not DPM, went to form urogen III, it can be concluded that urogen I and urogen III must originate by different pathways from the beginning of the tetramerization process. In our proposal, rearrangement begins with the second unit of PBG and follows through for the third and fourth unit of PBG. Upon cyclization, urogen III was formed. c) Porphobilinogenase must have groups that are easily alkylated which caused its inactivation of its enzymatic activity Bogorad[37] showed that formaldehyde as well as Aq^{+} and Hg^{++} ions and p-chloro-mercuric-benzoate (PCMB) are very effective inhibitors of deaminase. Our alkylation experiment with CH_3I on porphobilinogenase decreased the enzymatic activity by 30% and it probably alkylated the enzyme irreversibly in the following manner.

$$\begin{matrix} \text{Enz-NH}_2 \\ \text{-(SH)} \end{matrix} + \text{I-CH}_3 \longrightarrow \begin{matrix} \text{Enz-}\overset{\overset{|}{}}{\text{N}}\text{-CH}_3 \\ \text{Enz-S-CH}_3 \end{matrix} + \text{HI}$$

This all suggests that an -SH group and probably an $-NH_2$ are essential for activity and may be the nucleophile at site I or II or at a binding site removed from the active site. d) Binding site I is specific for the order of propionate, acetate, and methylene sidechains and non-specific for the amino group. The substrate must have the following structure, X being a good leaving group, such as $-NH_3^+$, imidazolium$^+$, N-methylimidazolium$^+$, and 2-methylimidazolium$^+$.



The fact that PBG and PBG derivatives enzymatically formed urogen III at approximately the same rate indicated that good leaving groups such as $-NH_3^+$, $-N(CH_3)_3^+$, imidazolium$^+$, -N-methylimidazolium$^+$, and 2-methylimidazolium$^+$ were easily displaced by a nucleophilic group on the enzyme. Catalysis then proceeds by a transferase-like reaction with the formation of covalent enzyme-substrate intermediates and migration of 1-carbon unit held by the enzyme. The enzyme binding site II must be able to accomodate the transient

methylene group by supplying a pair of electrons. The ϵ-amino group of lysine, -SH group of cysteine, or imidazole group of histidine might perform this function.

APPENDIX*

* Published in Journal of Chromatography, 87, 562 (1973).

An Improved Method for Separation of Coproporphyrins I an III

Some of the procedures in the literature[1,2,3] for the separation of coproporphyrins I and III do not seem to give clear-cut results in our hands. This Appendix describes a method which gives reproducible separations and utilizes commercially available cellulose polygram sheets. The chromatograms are developed for a long period of time (18 to 24 hours) and a clear separation of the isomers is achieved. "Distance ratio" (cm/hr) is used to characterize the movement of sample spots because the solvent front has been lost. Commercial preparations of coproporphyrins I and III and the mixture of isomers obtained by decarboxylation of uroporphyrins I and III were used. Their mobilities were compared with two dicarboxylic porphyrins, mesoporphyrin IX and deuteroporphyrin IX, and octacarboxylic porphyrins, uroporphyrins I and III. The "distance ratios" of the decarboxylated uroporphyrins correspond to those of standard coproporphyrins I and III respectively. The dicarboxylic porphyrins moved far ahead of the coproporphyrins and the uroporphyrins remained at the origin.

Polygram cel 300, plastic sheets pre-coated with cellulose, 20 x 20 cm (Brinkmann Instruments, Inc.) were used. Polygram sheets were heated at 100°C for fifteen minutes before and after spotting samples. Coproporphyrin I-tetramethyl ester, coproporphyrin III-tetramethyl ester, uroporphyrin I-octamethyl ester, and uroporphyrin I,III ester (Waldenstrom Type) were purchased from Sigma Chemical Company. Deutero-porphyrin IX was prepared by the method of Falk[4] and purified by the method of Caughey[5] and co-workers. Mesoporphyrin IX was prepared by the method of Falk[4], catalytic hydorgenation over PD in formic acid. The porphyrins were prepared from their corresponding methyl esters by hydrolysis in concentrated HCl in the dark for at least 24 hours. Decarboxylation of uroporphyrin I and uroporphyrin I, III to coproporphyrin I and coproporphyrin I,III was carried out by the method of Edmondson and Schwartz[6]. The porphyrins were spotted from a solvent mixture suggested by Eriksen[7] but with one drop of .1M EDTA added to the following: ammonium hydroxide (30%) + water + acetone: 1 + 2 + 7. The porphyrins were detected by observing their fluorescences in a BLE Spectroline (Black Light Eastern) viewing cabinet by excitation at 366 mu.

A mixture of 2,6-lutidine-ammonia-water-.1M EDTA (10:4.2:2.8:.02) as suggested by Bogorad[8] was placed in

the developing chamber at 25°C for equilibration for one hour. The polygram sheet was heated for fifteen minutes, spotted, and heated for fifteen minutes. Development took place at 25°C in the dark for different time periods: 18 hrs, 21 hrs., 24 hrs., and 48 hrs. Such long time periods of development result in the solvent front going off the chromatogram, but the coproporphyrin isomers remain on the polygram and are continually being separated. However, the forty-eight hour run was too long and extensive diffusion of spots occurred. The optimum time in our experience is 18 hrs. to 21 hrs. Under these conditions, uroporphyrin I and III do not leave the origin. R_f's cannot be used to characterize the sample spots. Instead, we would like to use the term "distance ratio" which is defined as the distance in cm travelled per unit time (cm/hr). The D_r values obtained are listed in Table I. Fig. 1 shows the variation of distance travelled with time for the isomers of coproporphyrin and decarboxylated uroporphyrin.

The separation of coproporphyrin I and coproporphyrin III by allowing the polygram to develop for 18 to 21 hrs., even though the solvent front goes off the upper edge of the chromatography sheet, results in a distinct separation and is reproducible. The availability of commercial cellulose polygram sheets renders a greater convenience for the separa-

tion of coproporphyrin I and coproporphyrin III.

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Table I. Distance Ratios of Porphyrin Isomers

PORPHYRIN	D_R			
	18h	21h	24h	48h
Copro I	0.306	0.329	0.350	Diffused
Decarboxylated Uro I	0.306	0.329	0.346	Diffused
Copro III	0.394	0.4000	0.425	Diffused
Decarboxylated Uro I	0.306	0.333	0.346	Diffused
Decarboxylated Uro III	-----	0.405	0.426	Diffused
Deutero IX	-----	0.786	-----	--------
Meso IX	------	0.800	-----	--------

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