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PURIFICATION AND ACTIVE SITE MODIFICATION OF WHEAT GERM UROGEN I SYNTHASE

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-by-

STUART E. POLLACK

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June, 1980
date

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Supervisory Committee

A B S T R A C T

PURIFICATION AND ACTIVE SITE MODIFICATION
OF WHEAT GERM UROGEN I SYNTHASE

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STUART E. POLLACK

Advisor: Professor Charlotte Russell

Urogen I synthase was purified from wheat germ. The purification consisted of acid precipitation, ammonium sulfate precipitation, heat denaturation, chromatography by affinity on mercuriphenylagarose followed by DEAE cellulose column chromatography. The molecular weight of the enzyme is 40,000 as judged by calibrated molecular sieve chromatography. The enzyme was inactivated by the arginine specific reagents butanedione and phenylglyoxal and by the lysine specific reagent pyridoxal-5'-phosphate. The stoichiometry of the reaction was 1:1 and the inhibition was lifted by substrate or competitive inhibitor in each case, indicating that the enzyme's active site contains 1 arginine and 1 lysine residue necessary for catalysis.

A C K N O W L E D G M E N T S

I am very grateful to my parents for their support and help during my thesis research.

I would like to thank my mentor, Dr. Charlotte Russell, for her encouragement and help in entering the Ph.D. Program and for her help throughout all phases of the work, and for introducing me to my wife.

I appreciate the help and advice of the members of my committee during the course of my research.

I am also indebted to my many colleagues, too numerous to mention, who made my stay at City College very pleasant.

D E D I C A T I O N

This thesis is dedicated with love and respect to my parents for their longstanding support and help in my educational pursuits since kindergarten.

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LIST OF ABBREVIATIONS

ALA, aminolevulinic

ATP, adenosine triphosphate

BD, butanedione

bicine, N, N-bis (2-hydroxyethyl) glycine

CoA, coenzyme A

coprogen, coproporphyrinogen

DNA, deoxyribonucleic acid

DPM, dipyrrolmethane

E, enzyme

E. coli, Escherichia coli

HCl, hydrochloric acid

HEPES, N-2-hydroxyethylpiperazine - N'-2-ethanesulphonic acid

I, inhibitor

LDH, lactate dehydrogenase

NADP, nicotinamide adenine dinucleotide phosphate

OPDC, opsopyrroledicarboxylic acid

P, phosphate

pp, pyrophosphate

PBG, porphobilinogen

PGO, phenylglyoxal

PLP, pyridoxal-5'-phosphate

protogen, protoporphyrinogen

RNA, ribonucleic acid

S, substrate

Tris, trishydroxymethylaminomethane

d-UMP, deoxyuridine monophosphate

urogen, uroporphyrinogen

INTRODUCTION

Porphyrin derivatives play an important part in the biochemistry of all living systems. The hemoglobins, chloroplasts, the cytochromes, vitamin B_{12}, catalases, peroxidase and the prosthetic groups of many hemoproteins demonstrate the deep involvement of porphyrins in many types of metabolic phenomena. Hemes that have iron porphyrins as the prosthetic group include the hemoglobins, cytochromes, catalases, and peroxidases. Vitamin B_{12} contains cobalt corrins. The chlorophylls contain magnesium pheophytins.

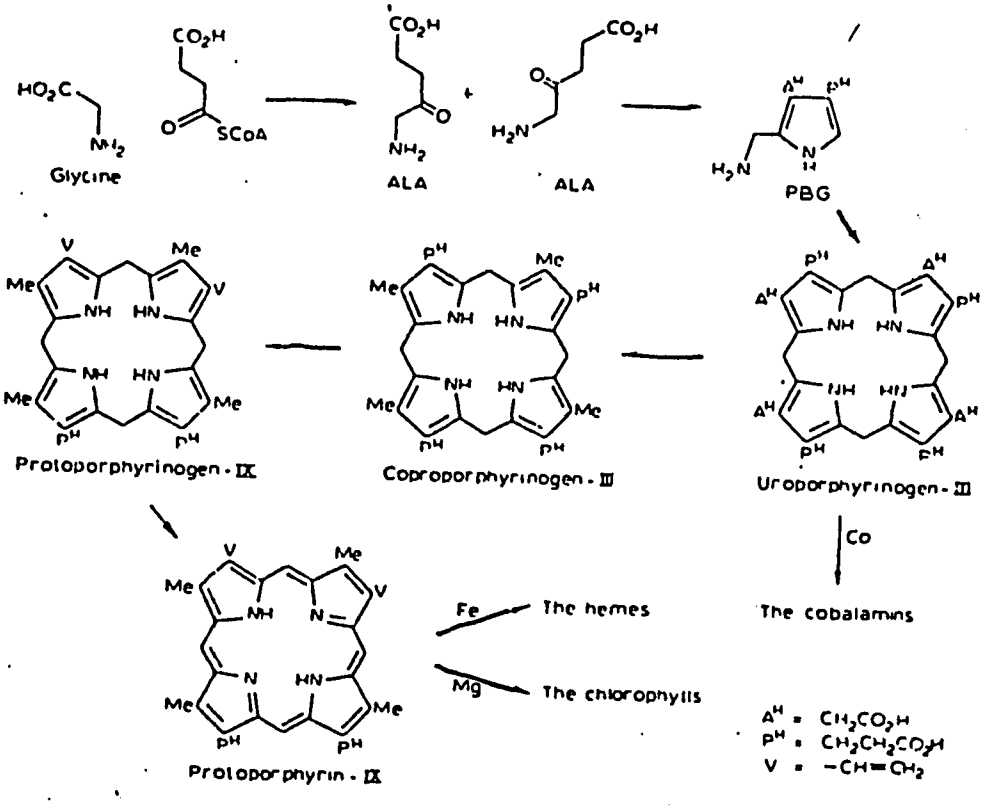
The various tetrapyrroles have several biosynthetic precursors and reactions in common (Figures 1 and 2).

Glycine was first shown to be a precursor of porphyrins in 1945 by Shemin (1). Glycine and monosuccinoyl CoA condense to form delta-aminolevulinic acid (δ-ALA). This reaction is catalyzed by the enzyme δ-ALA synthase.

The enzyme δ-ALA dehydrase catalyzes the condensation of two molecules of δ-ALA to the pyrrole, porphobilinogen (PBG).

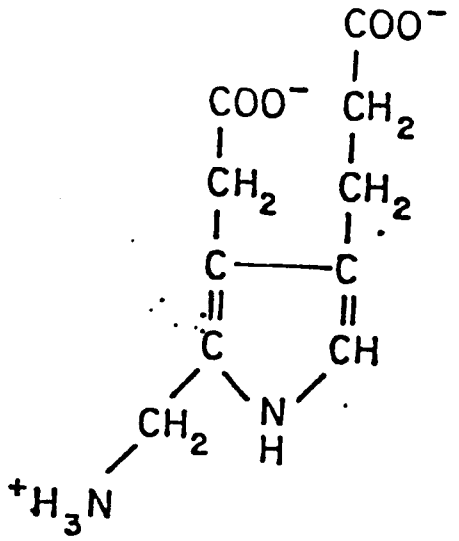
Bogorad (2) showed that uroporphyrinogen I synthase (E.C.#4.3.1.8) catalyzes the condensation of four moles of PBG to form uroporphyrinogen I (urogen) which can only be converted <u>in vivo</u> to the "dead end" porphyrin urophorphyrin I.

Figure 1. Biosynthetic pathway of porphyrins.



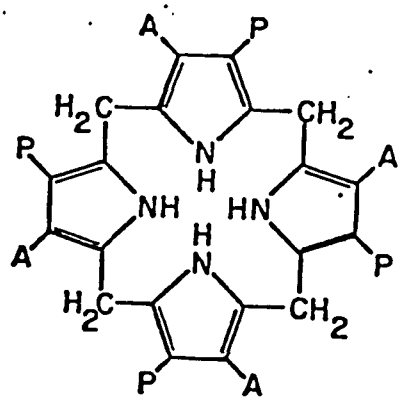
Porphyrins and Metalloporphyrins, ed. Kevin M. Smith

Figure 2. Urogen I and III isomer formation.

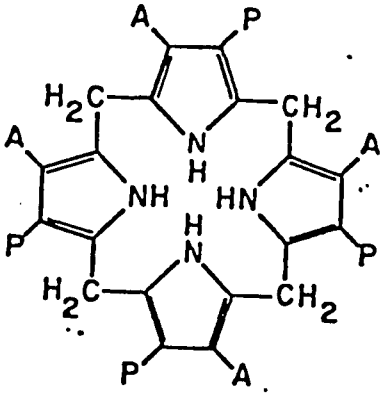


Uroporphyrinogen I synthase *via* Pre-Urogen

Uroporphyrinogen I synthase + Cosynthetase



Uroporphyrinogen I



Uroporphyrinogen III

A = CH_2COO^-

P = $CH_2CH_2COO^-$

Urogen I synthase has been isolated from spinach and from wheat germ by Bogorad (2, 3). The enzyme has been prepared from a variety of other sources including Euglena gracilis (4) and Rhodopseudomonas spheroides (5). A table of the properties of partially-purified preparations is presented in Table 1. In general, the enzyme urogen I synthase from a variety of sources appears to be single protein of molecular weight somewhat under 40,000 with a pH optimum near 8.0. K_m values are in the range of 30 μm (1).

From wheat-germ Bogorad isolated a second enzyme which he called urogen III isomerase or cosynthase (2) and which did not consume PBG. When added to urogen I synthase the system formed urogen III. The distribution of both enzymes was found to be ubiquitous in living systems (6). In vivo cosynthase appears to be present in excess thus assuring the formation exclusively of urogen III (7).

Urogen III is the first macrocylic substance formed on the biosynthetic pathway to the physiologically active porphyrins. Enzymatic decarboxylation of the four acetic acid side-chains of urogen III affords coprogen III. Next, the two propionate side chains of coprogen III are converted into vinyl groups. The enzyme involved in this oxidative decarboxylation is called coproporphyrinogenase (1). Oxidation of protoporphyrinogen IX leads to protoporphyrin IX. Ferrochelatase puts iron into protoporphyrin IX forming protoheme which is the prosthetic group of hemoglobins, myoglobin and the cytochromes (Figure 1).

Table 1: Properties of Urogen I Synthase from (1)

Source	Molecular weight	Isoelectric point	Optimum pH	K_m (PBG)
Wheat germ	-	-	8.2	50 μM
Rhodopseudomonas spheroides	35,000-36,000	4.46	7.8-8.0	40 μM at pH 7.8-8.0
Rhodopseudomonas spheroides	36,000-39,000	-	7.6	13-20 μM at pH 7.6
Chicken erythrocytes	-	4.6	7.4-8.2	18 μM at pH 8.0
Euglena gracilis	38,000-40,000	-	7.0-8.1	30 μM at pH 8.0 phosphate buffe
Spinach leaves	38,000-40,000	4.2-4.5	-	72 μM at pH 8.2
Human erythrocytes		-	7.4-8.2	130 μM at pH 7.4

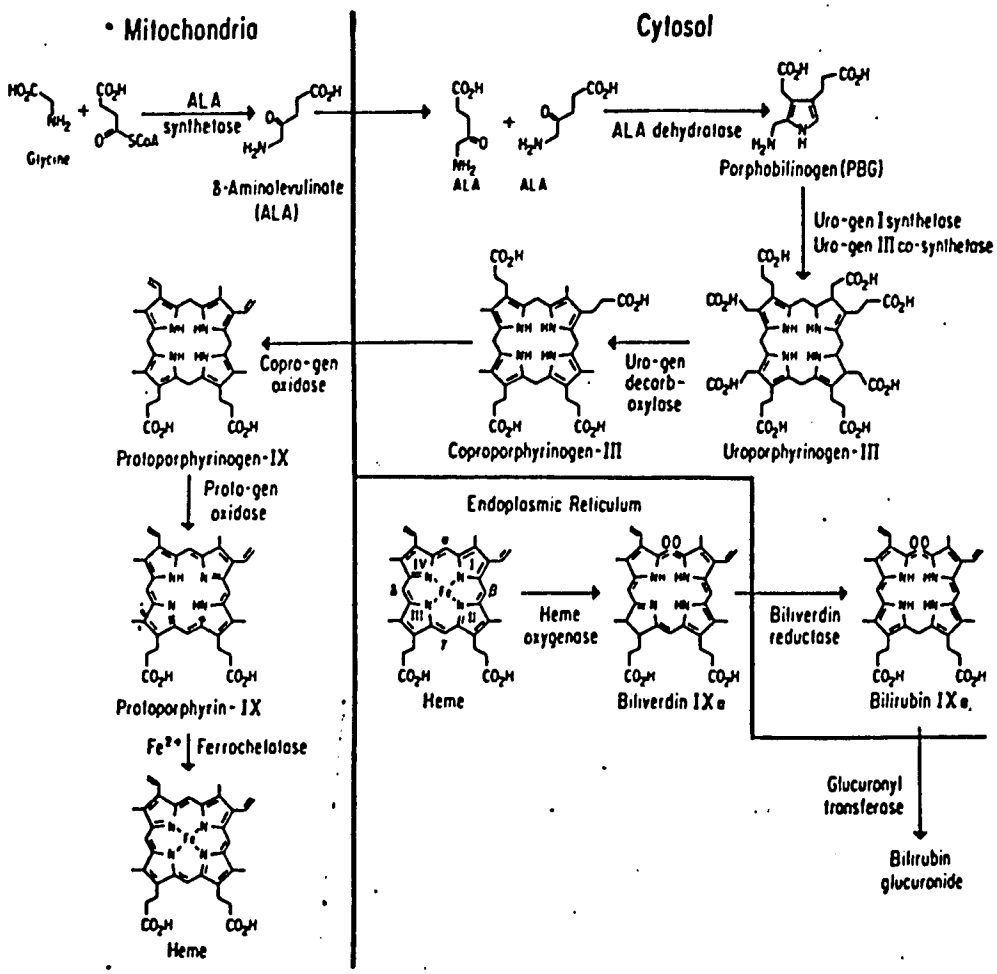
In chlorophyll biosynthesis magnesium is incorporated into protoporphyrin IX. The magnesium protoporphyrin IX undergoes reduction of a vinyl group followed by oxidative formation of the carbocylic ring. Following the reduction of this ring the propionate carboxyl group is esterified with the C_{20} alcohol phytol, forming chlorophyll-a (1).

In mammals δ-ALA synthase is located in the mitochondria. δ-ALA moves out of the mitochondria into the cytosol and is converted to PBG by δ-ALA dehydrase (δ-ALA dehydratase). PBG is converted to urogen III by the urogen I synthase urogen III cosynthase system. Urogen III is then decarboxylated to coprogen III by urogen decarboxylase. Coprogen III enters the mitochondria where coprogen oxidase, protogen IX oxidase and Fe^{2+} - ferrochelatase convert it to heme (Figure 3). The final steps in the assembly of hemoglobin molecules occur in the cytoplasm where the heme molecules attach to globin chains (8).

Porphyrias:

Disturbances in porphyrin synthesis represents a staggering burden on the human body. In acute intermittent porphyria an autosomal dominant disorder, a deficiency of urogen I synthase and high δ-ALA-synthase activity causes spilling of δ-ALA and PBG into the urine. Excreted into the urine per day is 40 - 200 μg of PBG and 20 - 40 μg of δ-ALA (7). The disease is characterized

Figure 3. The pathways and locations of mammalian heme biosynthesis and degradation.


• Mitochondria
Cytosol
Glycine
ALA synthetase
δ-Aminolevulinate (ALA)
ALA
ALA dehydratase
Porphobilinogen (PBG)
Uro-gen I synthetase
Uro-gen III co-synthetase
Copro-gen oxidase
Uro-gen decarb-oxylase
Protoporphyrinogen-IX
Coproporphyrinogen-III
Uroporphyrinogen-III
Endoplasmic Reticulum
Proto-gen oxidase
Protoporphyrin-IX
Heme oxygenase
Biliverdin reductase
Heme
Biliverdin IXα
Bilirubin IXα
Fe2+ Ferrochelatase
Glucuronyl transferase
Bilirubin glucuronide
Heme

clinically by periodic attacks of intense abdominal colic, usually accompanied by nausea and vomiting, obstinate constipation, neurotic or even psychotic behavior and neuromuscular disturbances. The mortality rate is high (9).

In congenital erythropoietic porphyria an autosomal recessive disorder, the low level of cosynthase is the basic genetic lesion. Overproduction of urogen I and coprogen I results in spillage into the urine of 100 mg of urogen I and 10 mg of coprogen I each day. Also there is a deposition of uroporphyrin I and copro-porphyrin I in the bone marrow, teeth, bones and skin (7). Cutaneous photosensitivity, severe scarring, hirsutism, and hemolytic anemia plague these victims.

Other hereditary porphyrins include porphyria variegata, porphyria cutanea tarda and coproporphyria. Porphyrins may also be chemically induced. Drugs such as barbiturates, which induce δ-ALA synthetase and thereby enhance the synthesis of PBG are capable of provoking porphyric attacks (9).

Mechanism of urogen III formation:

The enzymatic formation of the urogen III isomer presents a fascinating problem. It is not surprising that over 20 hypothetical schemes have been proposed to account for it (1, 10, 11, 12).

One theory (10, 13) postulates head to head condensation of two units of PBG, followed by a 2-aminomethyl migration. This

leads to rearrangement at the first step with chain building continuing on the rearranged dipyrrylmethane (Figure 4a) without further rearrangement (14, 15). Furthermore, Frydman and Frydman and coworkers synthesized this [^{14}C]-labelled head to head rearranged dipyrrylmethane and have shown that it is incorporated into urogen III only. The [^{14}C]-dipyrrylmethane formed by head to tail condensation of two PBG units (Figure 4b) was shown to be incorporated only into urogen I (1, 10, 14, 15).

These results have been criticized because the duration of the incubation was short (1 hour), a low concentration of dipyrrylmethane was used and a small conversion about 10% was measured by subtracting a large chemical blank (16).

Battersby using [^{13}C]-PBG has shown that the first three PBG units are incorporated intact without rearrangement. The fourth PBG unit is built in with rearrangement which is intramolecular with respect to that PBG unit (1, 12, 16, 17, 18, 19).

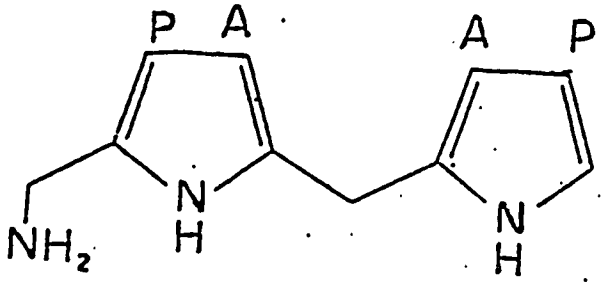
Furthermore, Battersby and coworkers have results which show that the key rearrangement step occurs after the unrearranged linear tetrapyrrole (Figure 5) or the enzyme bound form has been built by the head to tail condensation of four PBG units (16, 17).

The unrearranged linear tetrapyrrole (Figure 5) was incubated at pH 7.2 for 4 hours at 37°C with purified urogen I synthase plus cosynthase from Euglena gracilis and a parallel blank was run lacking the enzymes. Both reaction mixtures were quenched with iodine. The uroporphyrin isomers were converted to the

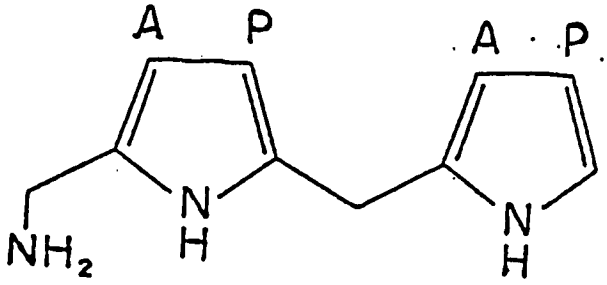
Figure 4a. The dipyrrylmethane formed from the head to head condensation of two PBG molecules followed by a 2-aminomethyl migration.

Figure 4b. The dipyrrylmethane formed from the head to tail condensation of two PBG molecules.

a)



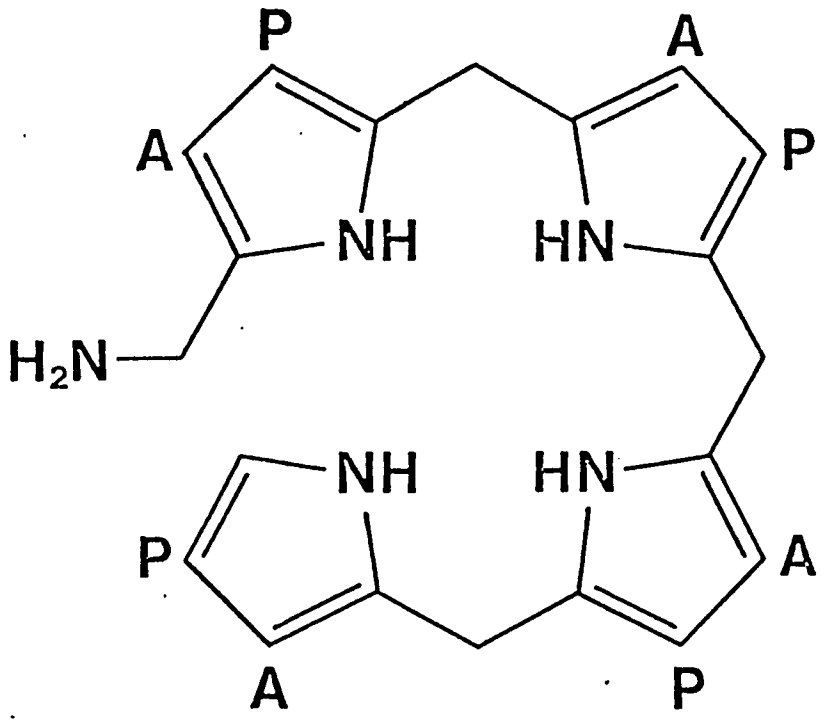
b)



A = CH_2COO

P = CH_2CH_2COO

Figure 5: The unrearranged linear tetrapyrrole.



A = CH_2COO^-

P = $CH_2CH_2COO^-$

coproporphyrin esters which could be separated using high pressure liquid chromatography, on a C_{18} reverse phase column. The product from the blank was pure type-I isomer. The striking result for the enzymatic run showed that 70% of the product was type-III, the rest being type-I produced in a competitive non-enzymatic cyclization (16, 17).

In similar experiments Battersby and his coworkers have shown evidence that the unrearranged dipyrrylmethane (Figure 4b) is converted by urogen I synthase cosynthase system into urogen III and that the product is mechanistically equivalent to what is seen for PBG (18, 20).

Also, synthesis of doubly [^{13}C]-labelled forms of the unrearranged linear tetrapyrrole (Figure 5) show that it is converted by urogen I synthase cosynthase system into urogen III by an intramolecular process, without scrambling (18, 19).

Urogen I synthase without cosynthase makes only the urogen I isomer. Cosynthase must bring about this rearrangement either by operating on an intermediate produced by the urogen I synthase or by modifying the way urogen I synthase brings about one of the coupling steps.

Scott and coworkers (22, 23, 123), have used [^{13}C]-PBG and [^{13}C]-FT NMR to measure the conversion of PBG into urogen I and urogen III. The urogen I synthase and the urogen I synthase cosynthase complex were isolated from Rhodopseudomonas spheroides. Scott has isolated and observed a short-lived intermediate in

the biosynthesis of urogen I and urogen III that he calls "pre-urogen." Previous studies on the enzymatic formation of urogen have revealed little evidence for the existence of free intermediates. The pre-urogen was formed transiently ($t_{0.5}$ = 5 min.) by the addition of urogen I synthase. In the absence of cosynthase it forms urogen I, possibly non-enzymatically. The formation of urogen I can occur in the absence of urogen I synthase. Pre-urogen was converted into urogen III in high yield by cosynthase in the absence of urogen I synthase. PBG is not consumed during the conversion of pre-urogen to urogen III, nor does urogen I act as a substrate for cosynthase. This data suggests that urogen I synthase and cosynthase act sequentially and independently as shown in Figure 6.

A likely mechanism consistent with both Battersby's and Scott's work was proposed by Mathewson and Corwin. in 1961 (11). They proposed that head-to-tail condensation of four PBG units leads to the unrearranged linear tetrapyrrole (Figure 5) which then underwent ring closure not to the α-free position of the terminal pyrrole unit (which would yield the type I isomer) but to the substituted α-position of that pyrrole unit (Figure 7).

Inhibitors:

No intermediates of urogen formation have ever been isolated

Figure 6. Pre-urogen, the precursor of both urogen I and urogen III.

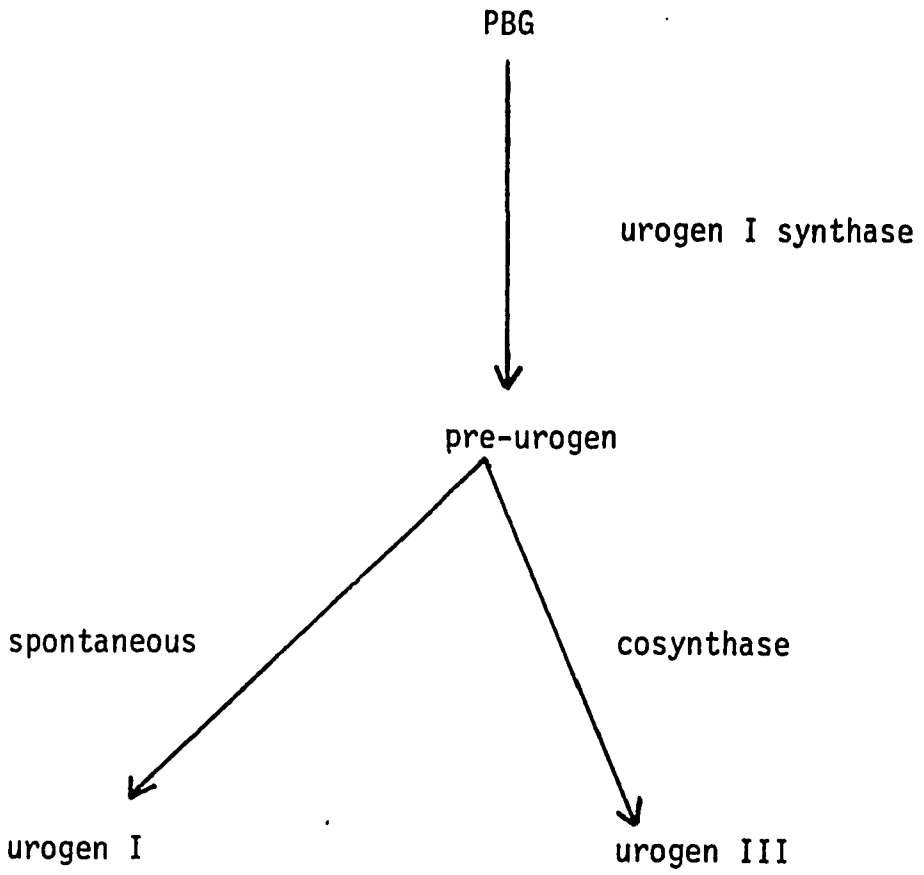
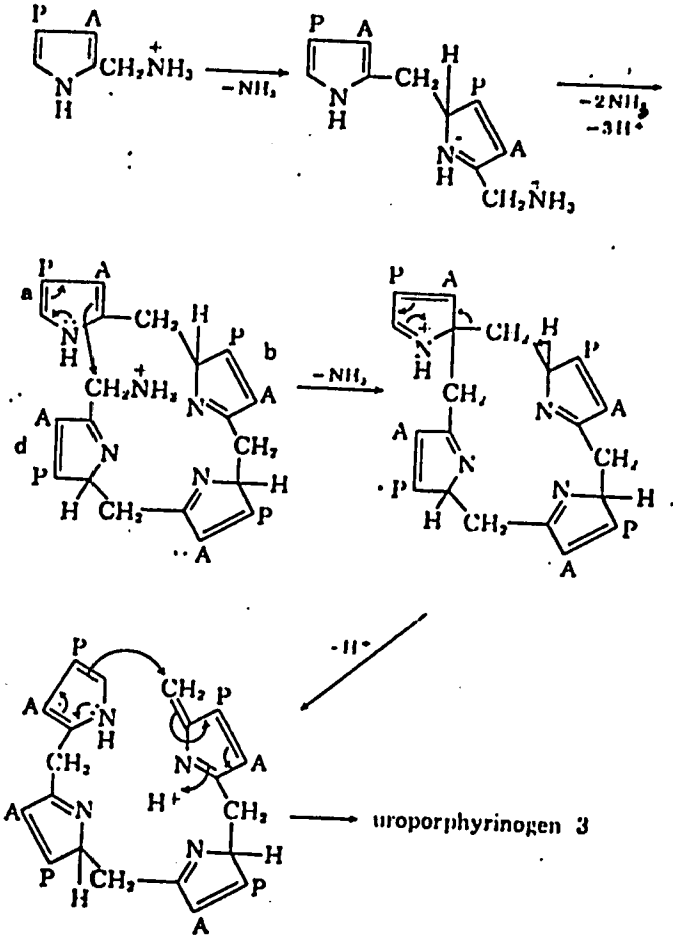

PBG
urogen I synthase
pre-urogen
spontaneous
cosynthase
urogen I
urogen III

Figure 7: The Mathewson and Corwin proposal.


P
A
CH₂NH₃
−NH₃
H
−2NH₃
−3H
a
b
d
uroporphyrinogen 3

from normal incubation mixtures prior to Scott's previously mentioned work. However, incubation of urogen I synthase with PBG in the presence of hydroxylamine caused a decrease in the formation of urogen I although PBG consumption was normal. It was shown that the unrearranged dipyrrylmethane (DPM I), shown in Figure 4b, was being formed at the expense of urogen I. Synthetic [^{14}C]-DPM I and DPM I obtained from hydroxylamine inhibited mixtures were both convertible to urogen I by urogen I synthase but only if PBG was present (24).

Inhibition of urogen I synthase by ammonium in the presence of PBG caused the accumulation of a material assigned the bilane structure shown in Figure 5, on the basis of its non-enzymatic conversion to urogen I with release of ammonia and a positive Ehrlich reaction suggesting a free α-position on a pyrrole ring (25).

It is possible that the normal enzyme-bound intermediates, in the conversion of PBG to urogen I by urogen I synthase, are removed from the enzyme by ammonia and hydroxylamine by nucleophilic displacement.

Opsopyrrole dicarboxylic acid (OPDC) shown in Figure 8a, is a competitive inhibitor of PBG for urogen I synthase. In an experiment in which urogen I formation was measured a K_I of 0.28 mM was found for OPDC (17, 26).

Isoporphoblinogen, shown in Figure 8c, is also a competitive inhibitor of urogen I synthase. When urogen I formation was

measured a K_I of 0.51 mM was determined for isoporphobilinogen (26).

A number of synthetic pyrroles (Figure 8) structurally related to PBG were assayed as substrates of urogen I synthase (33). None were found to act as such, even at the slowest rate. A free (non-esterified) propionic acid residue at C-4 was essential to obtain an efficient inhibition of urogen I synthase. Isoporphobilinogen (Figure 8c) and 2-methylopsopyrrole dicarboxylic (Figure 8b) inhibited the activity by addition to the incubation mixture, while 2-aminomethyl-3-pyrrole acetic acid (Figure 8e) and 2-aminomethyl-4-ethyl-3-pyrrole acetic acid (Figure 8d) exerted their effect only by pre-incubation.

Wheat germ urogen I synthase is inhibited by the sulfhydryl reagents, $HgCl_2$ and p-chloromercuribenzoate (7). Inhibition by $HgCl_2$ and p-chloromercuribenzoate was reversed by cysteine. Iodoacetate had no effect on urogen I synthase activity while iodoacetamide was inhibitory. Frydman and coworkers reported that N-ethylmaleimide inhibited urogen I synthase activity and that porphyrin formation was inhibited more than PBG consumption (13, 27). The inhibition was lifted by dithiothreitol. It was concluded that a cysteine residue was required for enzymatic activity.

Bogorad reported that formaldehyde inhibited the enzymatic conversion of PBG to porphyrins (7). Bogorad and Marks presented evidence that formaldehyde was neither a stoichiometric by-product

Figure 8: Structure of some urogen I synthase inhibitors:

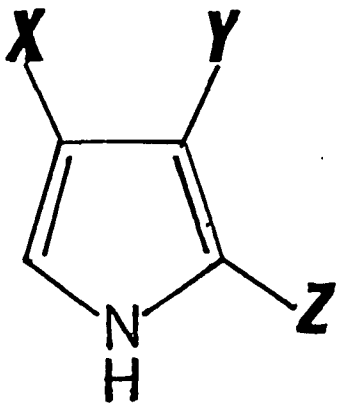
a. opsopyrroledicarboxylic acid

b. 2-methylopsopyrroledicarboxylic acid

c. isoporphoblinogen

d. 2-aminomethyl-4-ethyl-3-pyrrolacetic acid

e. 2-aminoethyl-3-pyrrolacetic acid



a. X = CH_2COOH, Y = CH_2CH_2COOH, Z = H

b. X = CH_2CH_2COOH, Y = CH_2COOH, Z = CH_3

c. X = CH_2COOH, Y = CH_2CH_2COOH, Z = CH_2NH_2

d. X = C_2H_5, Y = CH_2COOH, Z = CH_2NH_2

e. X = H, Y = CH_2COOH, Z = CH_2NH_2

nor a reactant in this process (28) although it was readily incorporated into urogens in the non-enzymatic condensation of PBG. Formaldehyde was never preincubated with urogen I synthase and removed before assaying for enzymatic activity.

Frydman and Frydman observed that urogen I synthase was inhibited by preincubation with N-bromosuccinimide followed by dialysis. Photoxidation as well as oxidation by pyrrolooxygenase inhibited urogen I synthase (2). These results point to essential tryptophan residues.

Inhibition by Butanedione:

The first inhibitor considered was butanedione (BD).

BD was first shown to react with arginyl residues of proteins almost 70 years ago by Harden and Norris (30) but this observation received surprisingly little attention.

In 1970 Yankeelov showed that in sodium phosphate buffer, pH 7.0, BD reacts 100-fold slower with lysine than with arginine. Under these conditions no other amino acid is modified. However, complete modification requires more than 48 hours (31).

In 1973 Riordan (32) showed that borate buffer is the best buffer for the modification of arginines in proteins by BD. The reaction in this buffer is much faster than under the conditions employed by Yankeelov. In this study amino acid

mixtures were modified with BD in borate buffer for 15 minutes and then applied to an amino acid analyzer. Arginine was 96% modified and all other amino acids were recovered with an average yield of 80%. A similar procedure was used to study the modification of arginine, lysine, and an equimolar mixture of the two. Lysine was not modified, nor did its presence interfere with the modification of arginine.

Riordan (32) has proposed that BD first reacts reversibly with arginine to form the cis-diol dihydroxyimidazoline derivative (see Figure 9). This would then complex rapidly with borate. Thus, in the presence of borate the reaction proceeds faster due to product stabilization by borate. The product is further stabilized in acid which prevents regeneration of free arginine. Arginyl residues functioning in (a) substrate-binding to the active site, (b) allosteric effector binding to an allosteric site different from the active site, and (c) cofactor binding at a separate site have been identified in enzymes using BD in borate buffer. A summary of a large and representative group of enzymes which have been treated with BD in borate buffer is shown in Table 2. In those studies where the authors did not do protection experiments with substrates allosteric effectors or cofactors the site of inhibition could not be inferred from their work. These studies are noted with a + in the protection experiments not done column in Table 2. In those studies where protection experiments were done and the

Figure 9. Reaction of BD with arginine in borate buffer.

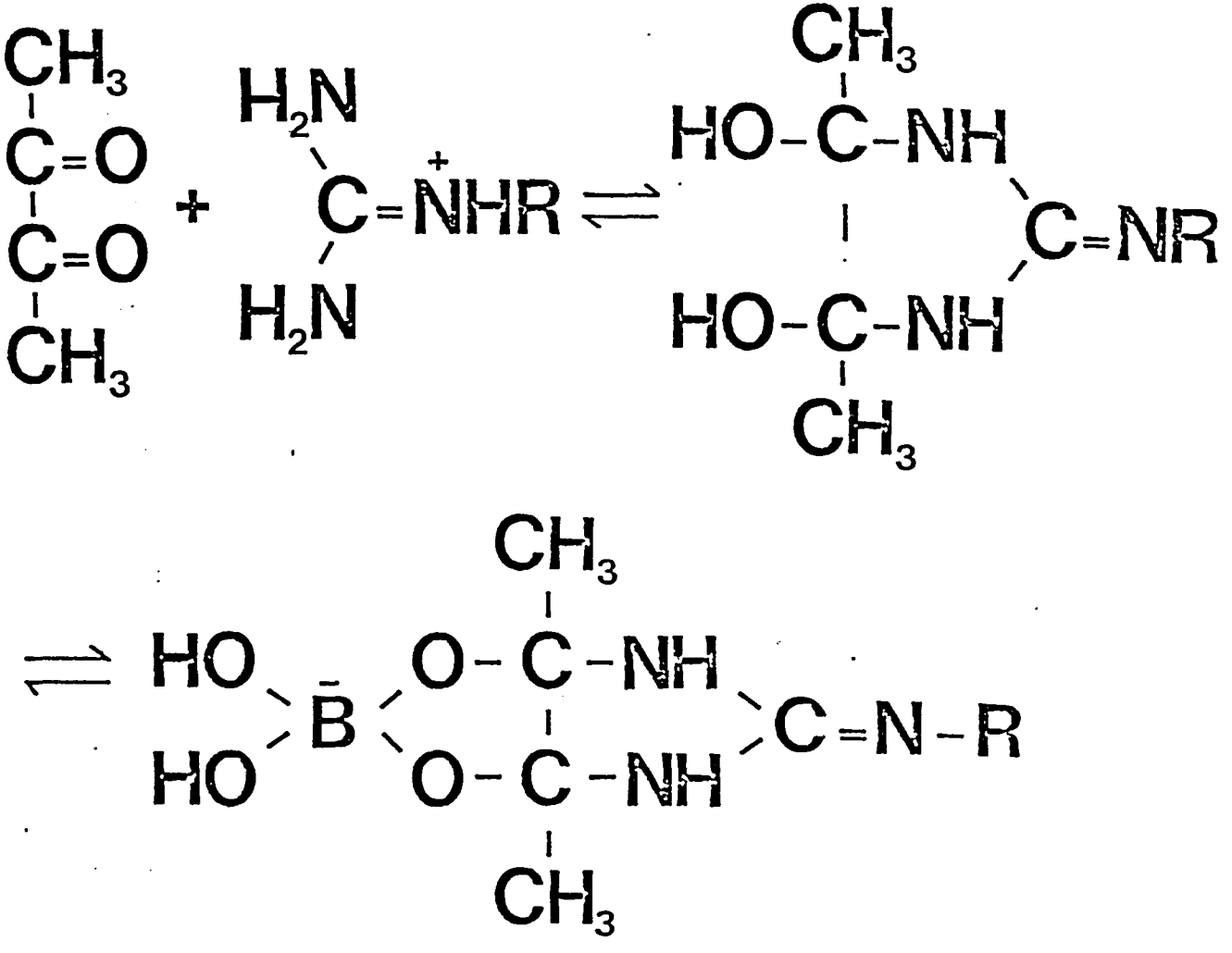


Table 2: Enzymes Shown to Require Arginine Residues, Involved in Substrate or Cofactor Binding, by Modification With BD in Borate Buffer

Enzyme	Source	Cofactor Binding	Allosteric Effector Binding	Substrate Binding	Protection Not Done	Reference
Enolase	yeast	--	--	L-phospho-glycerate	--	34
D-serine dehydratase	Escherichia coli	pyridoxal-phosphate	--	--	--	35
fructose 1,6-bisphosphatase	pig kidney	--	K^+, AMP	--	--	36, 37
aconitase	pig heart	--	--	citrate	--	38
aspartokinase/ homoserine dehydrogenase	Escherichia coli	--	K^+	--	--	39
mitochondrial ATPase	bovine heart	--	--	ATP	--	40
aldehyde reductase	pig kidney	NADPH	--	--	--	41
glucose-6-phosphate dehydrogenase	Leuconostoc mesenteroides	--	--	glucose-6-phosphate	--	42
acetyl-coA: aceto-acetate CoA-transferase	Escherichia coli	--	--	--	+	43

Table 2 (continued): Enzymes Shown to Require Arginine Residues, Involved in Substrate or Cofactor Binding, by Modification With BD in Borate Buffer

Enzyme	Source	Cofactor Binding	Allosteric Effector Binding	Substrate Binding	Protection Not Done	Reference
isocitrate dehydrogenase	pig heart	--	ADP	--	--	44
carboxypeptidase A	bovine	--	--	binds C-terminus of peptides	--	32
carboxypeptidase B	porcine	--	--	binds C-terminus of peptides	--	32
prostatic acid phosphatase	human	--	--	--	+	45
D-ribulose-1, 5 bisphosphatase	barley	--	--	--	+	46
cystathionase	rat liver	--	--	L-homoserine L-cysteine	--	47
ATPase	spinach chloroplast	--	--	ATP	--	48
glycogen phosphorylase	rabbit muscle	--	--	glucose-1-phosphate	--	49
thymidylate synthetase	Lactobacillus casi	--	--	d-UMP	--	30

Table 2 (continued): Enzymes Shown to Require Arginine Residues, Involved in Substrate or Cofactor Binding, by Modification With BD in Borate Buffer

Enzyme	Source	Cofactor Binding	Allosteric Effector Binding	Substrate Binding	Protection Not Done	Reference
RNA dependent DNA polymerase	avian, feline and simian type CRNA viruses	--	--	RNA template	--	51
angiotensin converting enzyme	rabbit lung	--	--	C-terminus of peptide	+	52
stearylcoenzyme A reductase	rat liver microsomes	--	--	stearyl CoA	--	53
creatine kinase	rabbit muscle	--	--	Mg-ATP	--	54
ornithine transcarbamylase	human and bovine liver	--	--	carbamyl phosphate	--	55

authors were able to infer at which site the inhibitor acted on; I have noted this by entering their conclusion in the cofactor binding, allosteric effector binding or substrate binding column of Table 2.

In all of the studies where modification by BD in borate buffer was followed by amino acid analysis the only amino acid modified was arginine (23, 42, 45, 46, 49, 56, 57, 58, 59).

In light of these data Riordan (32, 37) has suggested as a general rule that enzymes acting on anionic substrates or cofactors will probably contain arginyl residues as components of their ligand binding sites--or lysine or a metal cation.

It is of interest that in the instances where stoichiometry has been established arginine modification generally appears to be quite selective. Only one or two of the enzyme's total arginines react with BD (60).

A comparison of the rate of modification of the essential arginyl residue of creatine kinase by BD in borate buffer with that of free arginine under the same conditions reveals that the enzyme reacted ten to fifteen times faster than the free amino acid (54, 60). This hyperreactivity, coupled with a decreased reactivity of all the other arginyl residues in the enzyme probably reflects a structural environment that enhances the binding capabilities of the active site arginyl residues. A hydrophobic micro-environment as proposed by Powers and Riordan (44) enhances the positive charge character of the active site arginyl residues and makes

them more attractive to anionic substrates. Since arginyl residues serve as positively-charged binding loci their electrostatic interaction would be enhanced in an environment of low dielectric constant. Also, the high pk_a (12.48) would make the arginyl residue reliably cationic under most conditions. If the active site arginine is to attract substrate to the active site, it must be more reactive or substrate would bind to the wrong arginine residue!

As can be seen from Table 2, the two anionic moieties in ligands that arginine most often binds to are phosphate and carboxylate.

It is instructive to summarize results for other enzymes which have carboxyl group(s) in their substrates. Carboxypeptidase catalyzes the hydrolysis of carboxy-terminal peptide bonds. The presence of a free terminal α-carboxyl group of the substrate is a strict specificity requirement of this enzyme.

Lipscomb and coworkers showed that gly-tyr binding to the enzyme caused the guanidinium group of arg 145 to move by 2Å. This movement is believed to be influenced by the formation of a salt link between arg 145 and the terminal carboxylate group of gly-tyr (62). Riordan (32) showed that one arginine residue was essential for substrate binding to the active site.

BD inhibits human serum transferrin activity (63). The authors suggest that an arginine probably serves as a cationic residue in the anion (normally carbonate or bicarbonate)--binding site.

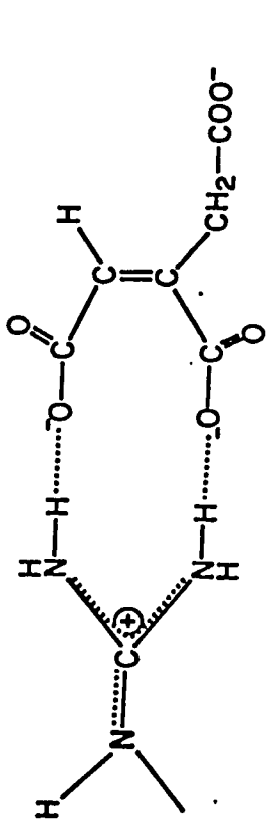
Aconitase is inhibited by BD (38) and cis-aconitate protects against the inhibition. A model which demonstrates hydrogen bonding between vicinal carboxyl groups and the guanidinium group, is proposed by Gawran and Jones (38) in Figure 10a. The analogous model for urogen I synthase and PBG is shown in Figure 10b. Another equally possible model is shown in Figure 13b.

The difference Fourier method was used to look for conformational changes in dogfish lactate dehydrogenase (LDH). A comparison of the preliminary 3.0 A° resolution structure of LDH:NAD-pyruvate ternary complex with the more complete 2.0 A° resolution structure of the apoenzyme provided inferred information on conformational changes during catalysis. In forming the ternary complex the quanidinium group of arginine 101 moves 13 A° with respect to its position in the apoenzyme and binds to the exposed side of both phosphates in NAD, as shown in Figure 11. Pyruvate is close to histidine 195 and interacts with arginine 171 and possibly arginine 109. Arginine 171 can interact with the carboxyl group of the pyruvate molecule, as also shown in Figure 11. Using PGO an arginine was shown to be involved in substrate binding. When LDH was treated with BD three arginines were implicated as essential for catalytic activity of the enzyme. These might be arginine 171, 109, and 101 (64).

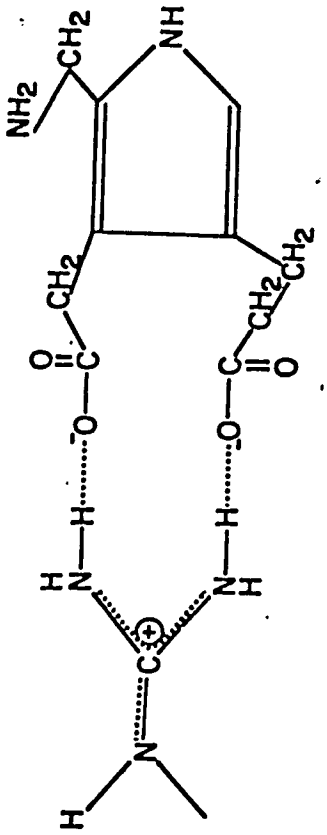
Kiner et al. (59) reported the effect of guanidinium ions on the rate of reaction of a carboxylate anion. They investigated the hydrolysis of the monophenyl ester of succinic acid which

Figure 10a. Aconitase cis-aconitate complex proposed by Gawron and Jones (38).

Figure 10b. Analogous model for urogen I synthase and PBG.

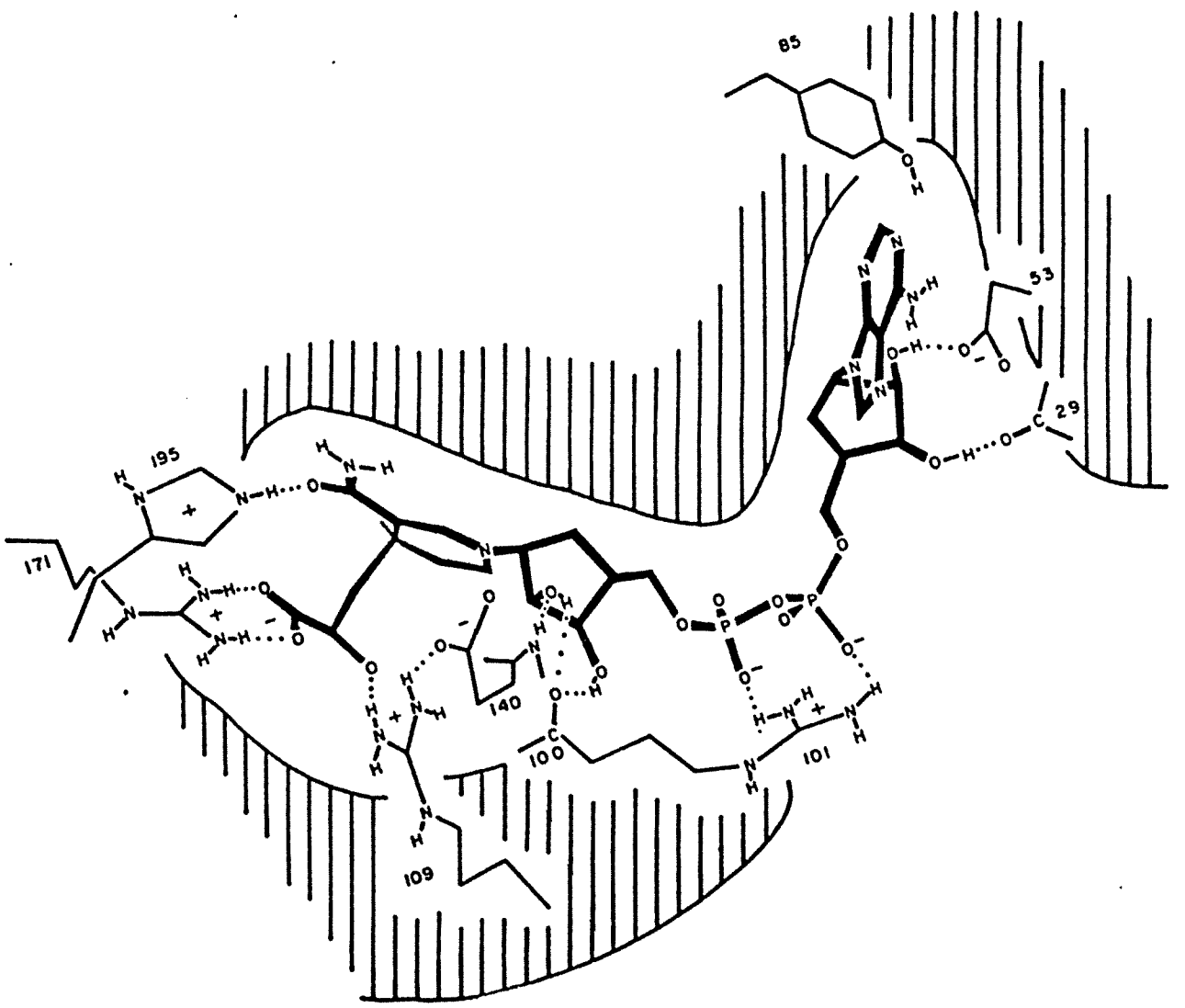


a)



b)

Figure 11. The lactate dehydrogenase-NAD-pyruvate complex (64).


85
53
29
195
171
140
100
101
109

involves intramolecular nucleophilic attack of the succinate carboxylate anion, as shown in Figure 12. The rate constants for the hydrolysis of the monophenyl succinate demonstrates inhibition by guanidinium ion and suggests the nature of the guanidinium carboxylate interaction. The model that the authors put forward is seen in Figure 13a. The analogous model for urogen I synthase-PBG binding is shown in Figure 13b.

In summary, the arginine might bind with either or both of the two carboxyl groups of PBG. The planar guanidinium group of arginine is well suited to interact with the planar carboxyl group of PBG possibly via two hydrogen bonds as well as by electrostatic interactions.

Inhibition by Phenylglyoxal:

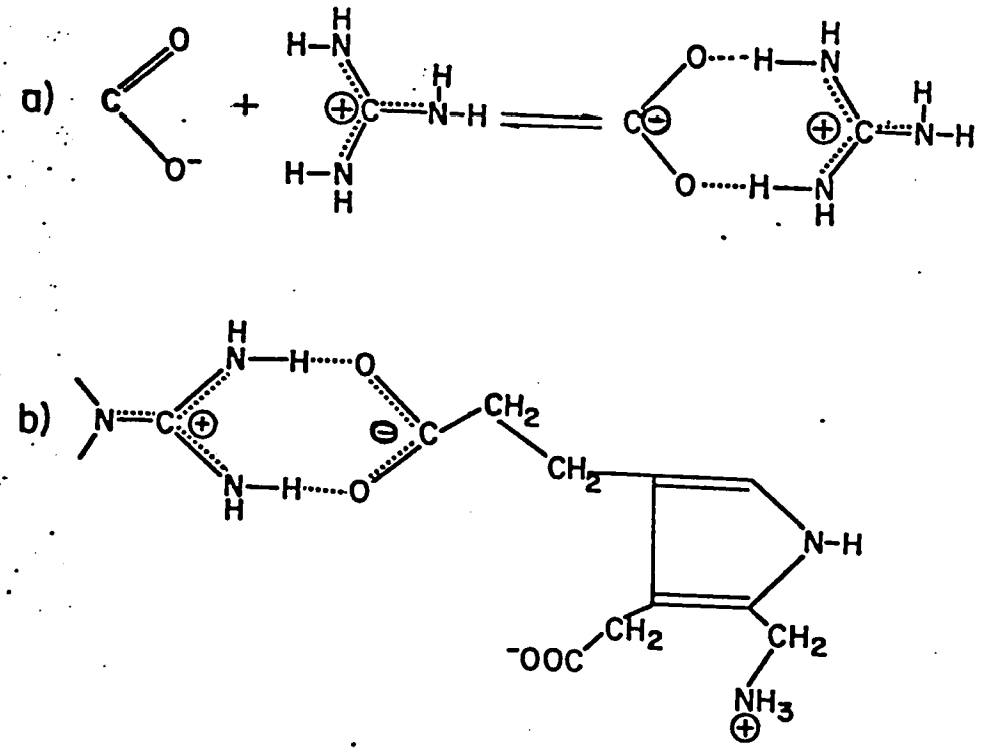
Phenylglyoxal (PGO) in a variety of buffers has been shown to be specific for the modification of arginyl residues in enzymes. Takahashi (65) has shown that N^{α}-acetyl-L-arginine and L-arginine reacts with PGO in N-ethylmorpholine acetate buffer, pH 8.0. Two molecules of PGO condensed with one molecule of arginine. The results were confirmed by elemental analysis of the product and incorporation of [^{14}C]-PGO into di(phenylglyoxal)-N^{α}-acetyl-L-arginine and di(phenylglyoxal)-L-arginine. Takahashi proposed the first molecule condenses in the rate limiting step with the

Figure 12. Intramolecular hydrolysis of the phenyl ester of succinic acid (59).

O=C
O-
CO2C6H5
O=C
C
O-
O
OC6H5
O=C
C=O
O
+ C6H5O-

Figure 13a. A model depicting the association of carboxylate anion and guanidinium cation, as proposed by Kiner (59).

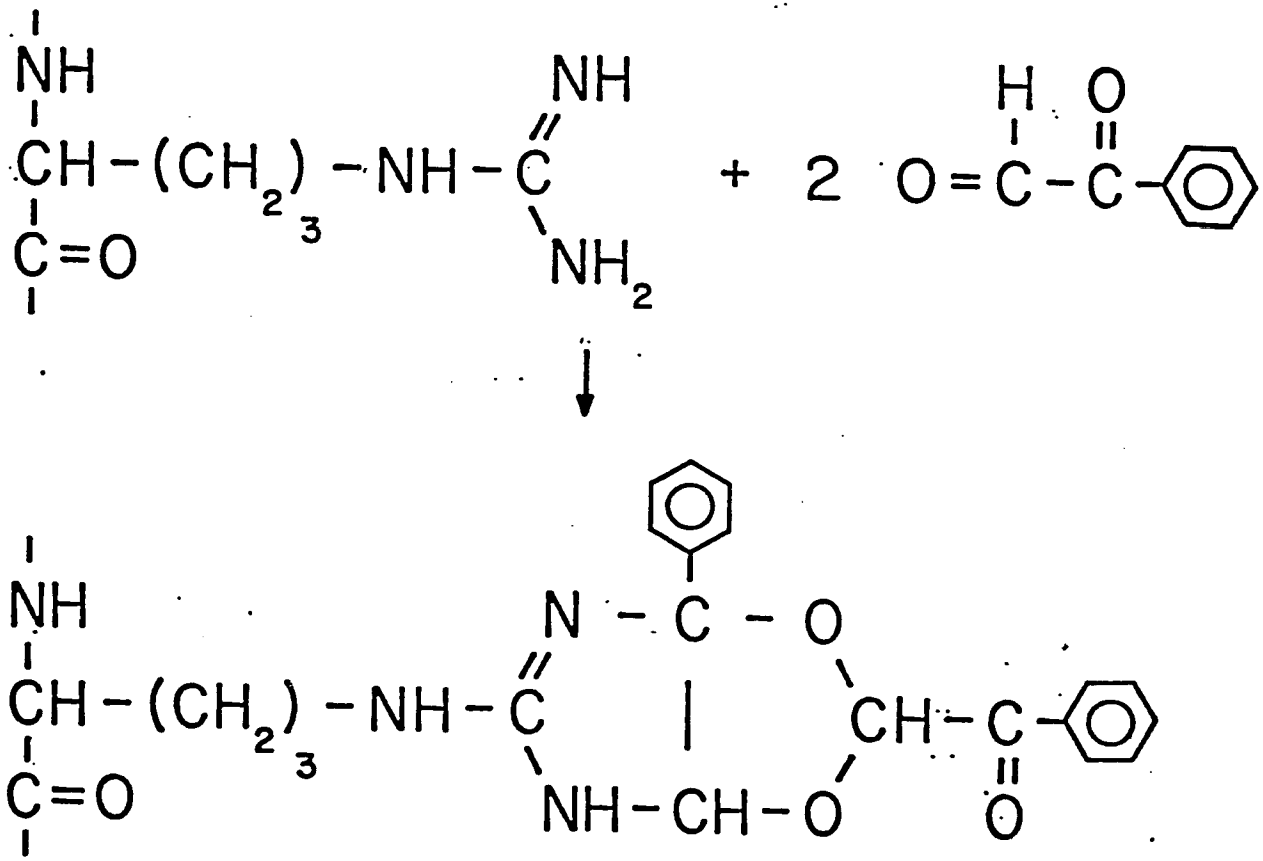
Figure 13b. A model showing the association of a carboxylate ion of PBG and urogen I synthase.


a)
b)

guanidino group (Figure 14) to form a glyoxaline ring which then reacts rapidly with a second molecule to form the final product. When proteins were treated with the reagent for a long period of time (24 hours) a reaction could also occur with the ε-amino group of lysine molecules. The N-terminal amino groups of proteins are also very susceptible to PGO modification.

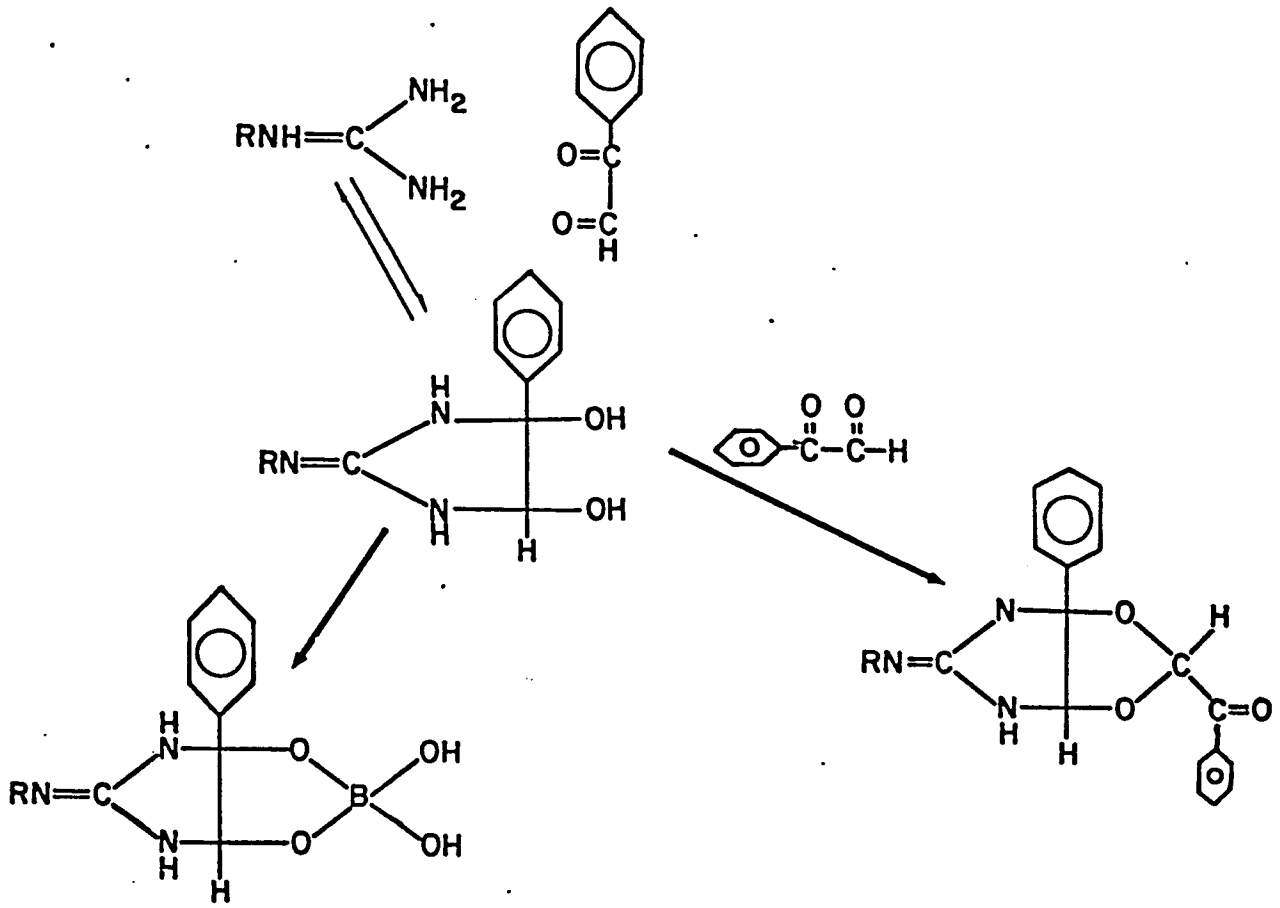
Cheung and Fonda (66, 67) investigated the reaction of PGO with arginine. They followed the reaction of N^2-acetylarginine or L-arginine with PGO spectrophotometrically, by the Sakaguchi reaction, and by amino acid analysis. PGO reacts much more rapidly with N^2-acetylarginine than with N^2-acetyllysine or N^2-acetylcysteine. The rate of the reaction of PGO with either N^2-acetylarginine or arginine increases with increasing pH from 7.0 to 11.5. The model reaction with arginine is much faster in bicarbonate-carbonate buffer, than in N-ethylmorpholine, borate, phosphate or Tris buffers. Spectroscopic measurements indicate that PGO may interact with borate, phosphate, and Tris, thus leading to the decreased rate in reaction. Gel chromatography and kinetic studies indicate that bicarbonate-carbonate buffer forms a complex with N^2-acetylarginine. In addition, bicarbonate-carbonate lowers the pk_a of the guanidinium group of N^2-acetylarginine. It was concluded that bicarbonate-carbonate catalyzes a nucleophilic attack by the guanidinium group against the carbonyl carbon of PGO.

Figure 14. Reaction of PGO with a guanido group as proposed by Takahashi (65).



Porcine carboxypeptidase B was modified with [^{14}C]-PGO in 0.3 M borate buffer. The modified protein was separated from excess reagent on a Biogel column. Loss of enzymatic activity correlated with the incorporation of about two moles of [^{14}C]-PGO per mole of enzyme. This incorporation might correspond to the modification of one arginyl residue since from Takahashi's model reactions it is known that two molecules of PGO condense with one mole of arginine. Amino acid analysis indicated a loss of about 1.5 arginyl residues while no other differences were found between the amino acid compositions of the native and modified enzymes. The correlation of the changes in peptide activity with the loss of about 1.5 arginyl residues in the enzyme would usually indicate that modification of two arginyl residues was responsible for the activity changes in carboxypeptidase B. This apparent discrepancy is rationalized by the authors in the following way, depicted in Figure 15. Thus, PGO reacts with the guanido group of arginine to yield a cis-diol derivative as suggested for the condensation of BD with arginine (68). Following this the diol derivative will form a complex with borate, and the formation of this complex would prevent further condensation with another molecule of PBG. Borders and Riordan (54) have observed a 1:1 stoichiometry for the PGO-arginine complex, for creatine kinase, in bicarbonate buffer. Philips and coworkers have deduced a 1:1 stoichiometry for the PGO-arginine complex, for 3-phosphoglycerate kinase, in veronal buffer.

Figure 15. PGO arginine binding modes possible in borate buffer (68).


RNH=C
NH2
NH2
O=C
O=C
H
RN=C
N
H
OH
OH
H
O
C
C
H
RN=C
N
H
O
B
OH
OH
H
RN=C
N
H
O
O
H
C
C=O

Arginyl residues functioning in substrate-binding to the active site, allosteric effector-binding to an allosteric site different from the active site, and cofactor-binding at a separate site have been identified in enzymes, using PGO in a variety of buffers. A summary of a large representative group of enzymes which have been treated with PGO is shown in Table 3. In those studies where the authors did not do protection experiments with substrates, allosteric effectors or cofactors the site of inhibition could not be inferred from their work. These studies are noted with a + in the protection experiments not done column in Table 3. In those studies where protection experiments were done the authors were able to infer at which site the inhibitor acted on; I have noted this by entering their conclusions in the cofactor binding, allosteric effector binding or substrate binding column of Table 3.

Takahashi (84) has used amino acid analysis to monitor PGO incorporation into ribonuclease A in bicarbonate-carbonate buffer. α-Chymotrypsin and trypsin were modified by PGO in NEM, phosphate and acetate buffers. In each case, a marked loss of arginine residues was observed but the other amino acid residues did not change significantly with the exception of the amino-terminal residue(s). In the case of ribonuclease A about 2 of the 4 arginine residues were fairly readily modified, together with the N-terminal lysine. Incorporation of [^{14}C]-PGO agreed with amino acid results assuming two PGO molecules complex with

Table 3: Enzymes Shown to Require Arginine Residues, Envolved in Substrate or Cofactor Binding, by Modification with PGO

Enzyme	Source	Buffer	Cofactor	Effector	Substrate	Protection Not Done	Refe
carboxypeptidase B	porcine	borate	--	--	--	+	68
hexokinase P II	yeast	bicine	Mg-ATP	--	--	--	69
aspartate transcarbamylase	E. coli	bicarbonate	--	ATP, CTP	carbamyl-P	--	70,
dihydrofolate reductase	Lactobacillus casei	phosphate	NADPH	--	--	--	72
d-aspartate oxidase	beef kidney	borate	--	--	d-aspartate	--	73
alcohol dehydrogenase	horse liver	bicarbonate	NADH	--	--	--	74
tryptophanase	E. coli	phosphate	--	--	tryptophan	--	75
glutamine synthetase	ovine, brain	bicarbonate	--	--	ATP	--	76
transferrins	chicken egg white	bicarbonate	--	--	bicarbonate	--	63
yeast 3-phosphoglycerate kinase	yeast	veronal	--	--	3-phospho-D-glycerate	--	77
prenyl transferase	pig liver	borate	--	--	isopentenyl pyrophosphate	--	78

Table 3 (continued): Enzymes Shown to Require Arginine Residues, Envolved in Substrate or Cofactor Binding, by Modification with PGO

Enzyme	Source	Buffer	Cofactor	Effector	Substrate	Protection Not Done	Reference
creatine kinase	rabbit muscle	veronal	--	--	MgATP	--	54
ribulose bisphosphate carboxylase	spinach	not given	--	--	--	+	80
propionyl CoA carboxylase	human	not given	--	--	ATP, propionyl CoA	--	81
ornithine transcarbamylase	human	not given	--	--	carbamyl phosphate ornithine	--	55
chloroplast coupling factor	spinach	borate	--	--	ATP	--	82
D-serine dehydratase	E. coli	phosphate	pyridoxal phosphate	--	--	--	35
mitochondrial ATPase	bovine heart	not given	--	--	ATP	--	40
glutamate dehydrogenase	beef liver	phosphate borate	NADPH	--	--	--	83
ribonuclease A	bovine	bicarbonate	--	--	--	+	84

Table 3 (continued): Enzymes Shown to Require Arginine Residues, Envolved in Substrate or Cofactor Binding, by Modification with PGO

Enzyme	Source	Buffer	Cofactor	Effector	Substrate	Protection Not Done	Reference
ribulosebisphosphate	spinach	bicine	--	--	ribulose bisphosphate	--	85
aldehyde reductase	pig kidney	borate	NADPH	--	--	--	86

each arginine residue. Two out of three arginines react with PGO in α-chymotrypsin and one out of two in trypsin. There is no loss of lysine in either of these two proteins.

In all other cases where amino acid analysis was used to follow PGO modification only arginine residues were modified (72, 77, 87, 54).

The PGO reaction with arginine residues in proteins is accompanied by a marked spectral change. An increase in the absorbance at 250 nm is evident in PGO treated proteins. This is identical to the absorbance maximum of 250 nm seen for di-PGO-arginine. Takahashi has estimated the molar extinction coefficient of di-PGO-arginine to be about 1.1×10^4 ℓ / mole - cm (84).

Inhibition by Formaldehyde:

Many simple aldehydes and ketones inhibit enzymes by rapidly and reversibly reacting with amino groups of proteins, as illustrated in Figure 16a. Neither the initial adduct nor the Schiff base formed upon dehydration is very stable in dilute aqueous solutions but irreversible modification of protein amino groups can be obtained by reduction of the Schiff base to a stable secondary amine (88) as can be seen in Figure 16, using formaldehyde and sodium borohydride or sodium cyanoborohydride (89) reductive

Figure 16. Reductive alkylation of amino groups.

a. Schiff base formation.

b. Reduction of Schiff base by sodium borohydride.

c. Reductive alkylation using formaldehyde and sodium borohydride.

d. Reductive dehydration using formaldehyde and sodium cyanoborohydride.

a) $E - NH_2 + RR'CO \rightleftharpoons E - NH - \underset{\displaystyle R'}{\overset{\displaystyle R}{\overset{|}{\underset{|}{C}}}} - OH \overset{-H_2O}{\rightleftharpoons} E - N = C\begin{matrix} R \\ R' \end{matrix}$

b) $E - N = C\begin{matrix} R \\ R' \end{matrix} + NaBH_4 \longrightarrow E - NH - \underset{\displaystyle R'}{\overset{\displaystyle H}{\overset{|}{\underset{|}{C}}}} - R + NaH_2BO_3$

c) $E - NH_2 \xrightarrow[NaBH_4]{H_2CO} E - NHCH_3 \xrightarrow[NaBH_4]{H_2CO} E - N(CH_3)_2$

d) $E - NH_2 \xrightarrow[NaCNBH_4]{H_2CO} E - NHCH_3 \xrightarrow[NaCNBH_4]{H_2CO} E - N(CH_3)_2$

alkylation gives ε-N-methyl and ε-N, N-dimethyl lysine derivatives of amino groups as illustrated in Figure 16c and d. In moderately alkaline aqueous solutions, formation of the dimethyl derivative rapidly follows formation of the monomethyl derivative. Substitution of $NaCNBH_3$ (which reduces Schiff bases but not aldehydes) for $NaBH_4$ increases the labeling efficiency 3-fold and eliminates the side reactions caused by $NaBH_4$, the $NaCNBH_4$ is, however, much slower. Since $NaCNBH_3$ is more stable to acidic conditions, reactions can be carried out at pH values as low as 6. [^{13}C]-formaldehyde has been used with $NaCNBH_4$ to introduce a label into model proteins (88). The only groups labeled by this method were the ε-amino groups of lysine and α-NH_2 terminus. Labeling with [^{13}C]-enriched formaldehyde provides a means for studying perturbations in the chemical environment of lysyl residues by [^{13}C]-NMR spectroscopy.

Reductive methylation of bovine pancreatic ribonuclease A with formaldehyde and $NaBH_4$ produced an enzymatically inactive protein with less than a single remaining unmodified lysine residue as judged by amino acid analysis. Furthermore, amino acid analysis showed no loss of any amino acid residue other than lysine. The high yield of alkylated products, in this ribonuclease A modification study, suggest that the Schiff base intermediate is reduced at a rate many times faster than the carbonyl compound itself (90).

Bogorad (3) observed that when formaldehyde was included in the incubation mixture of spinach urogen I synthase and PBG, two things happened. First, the initial rate of PBG consumption was decreased and second, urogen III appeared in increasing proportion as the concentration of formaldehyde was increased. The urogen III appeared to be formed nonenzymatically in the presence of formaldehyde. The rate of this nonenzymatic consumption of PBG was positively correlated with the concentration of formaldehyde while the urogen I synthase reaction appeared to be inhibited by formaldehyde. Bogorad did his enzymatic assay in the presence of formaldehyde making it difficult to separate inhibition from nonenzymatic conversion of PBG to porphyrin.

Bogorad and Marks (28) presented evidence that formaldehyde was neither a stoichiometric by-product nor a reactant in this process although it was readily incorporated into urogens in the nonenzymatic condensation of PBG.

Lysyl residues have been identified using formaldehyde and either $NaBH_4$ or $NaCNBH_3$ in the enzymes shown in Table 4. In these studies protection experiments were not done, hence the site of action of the inhibitor could not be inferred from the work.

Table 4: Enzymes Shown to Have Essential Lysyl Residues by Modification With Formaldehyde in the Presence of a Reducing Agent

Protein	Source	Buffer	Reducing Agent	Reference
ovomucoid	turkey	borate	$NaBH_4$	88, 90
lysozyme	chicken	borate	$NaBH_4$	91
transferrin	human	borate	$NaBH_4$	90
insulin	bovine	borate	$NaBH_4$	89, 90
α-chymotrypsin	Worthington	borate	$NaBH_4$	90
glycogen phosphorylase b	rabbit	not given	$NaBH_4$	88
ribonuclease A	bovine pancreatic	not given	$NaBH_4$	88
chymotrypsinogen A	Worthington	borate	$NaBH_4$	90
lactate dehydrogenase	beef heart	HEPES	$NaCNBH_3$	92
alkaline phosphatase	*Escherichia coli*	HEPES	$NaCNBH_3$	92
β-galactosidase	*Escherichia coli*	HEPES	$NaCNBH_3$	92
hexokinase P I	yeast	HEPES	$NaCNBH_3$	92
glucose-6-phosphate	yeast	HEPES	$NaCNBH_3$	92

Inhibition by Pyridoxal-5'-phosphate:

Reaction of pyridoxal-5'-phosphate (PLP) followed by reduction with sodium borohydride has been shown to be specific for the modification of lysyl residues in the proteins (42, 91). The reaction of the ε-amino group of lysine and PLP is shown in Figure 17. Schiff base formation is followed by sodium borohydride reduction to a stable N^6-pyridoxyllysine. N^6-Pyridoxyllysine (reduced by $NaBH_4$) has a broad fluorescence maximum centered at about 400 nm when excited at 325 nm (91).

Essential lysine residues have been found, using PLP followed by $NaBH_4$ reduction, in the enzymes shown in Table 5.

When phospholipase A_2 from snake venom was modified with PLP and sodium borohydride, amino acid analysis established that only lysine reacted with the modifier (91).

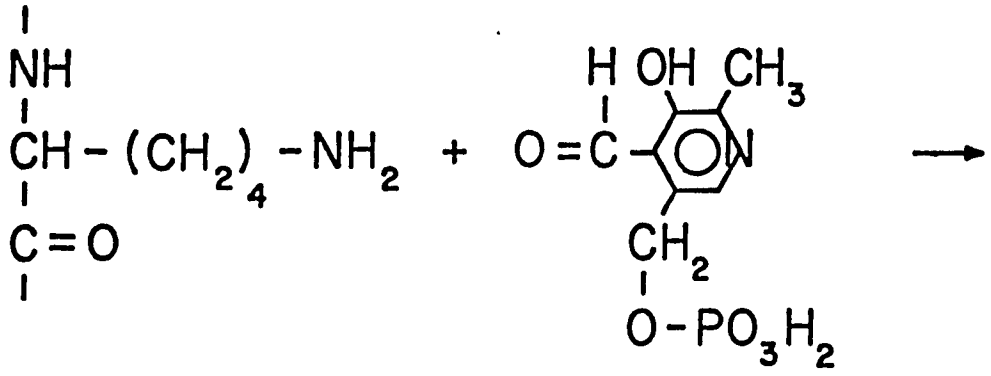
Kinetics of Inactivation by an Irreversible Inhibitor:

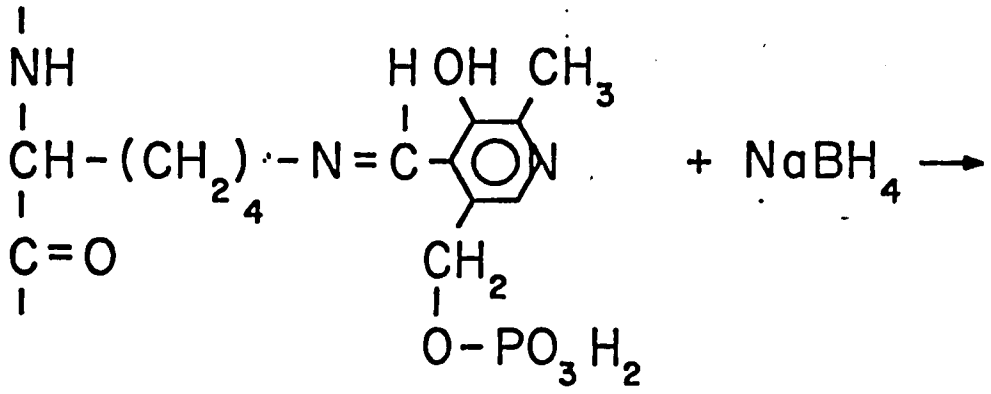
An inhibitor binds to an enzyme forming an E · I complex prior to the chemical step involving covalent linkage to the target enzyme (equation 1.1).

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot I \xrightarrow{k_2} E - I \qquad K_I = k_{-1} / k_1$$

covalent inactive complex

Figure 17: Reaction of PLP with a lysyl residue followed by reduction with $NaBH_4$.





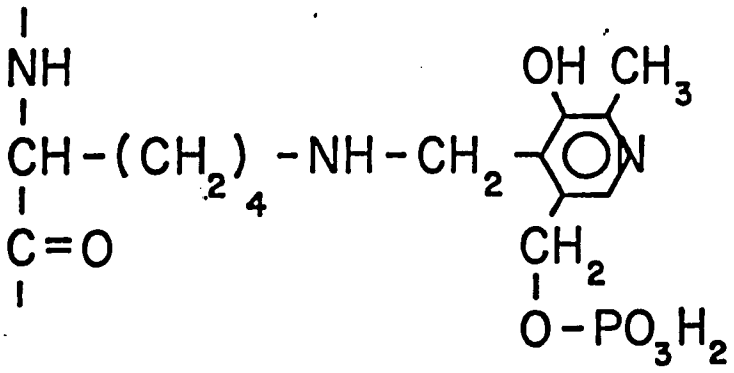


Table 5: Enzymes Shown to Require Lysine Residues, Envolved in Substrate or Cofactor Binding, by Modification with PLP

Enzyme	Source	Buffer	Cofactor	Effector	Substrate	Protection Not Done	Referen
adenosine triphosphatase	rabbit sacroplasmic reticulum	MOPS	--	--	ATP	--	93
ornithine trans-carbamylase	human	not given	--	--	carbamyl phosphate	--	55
CoA-transferase	*E. coli*	MOPS	--	--	CoA	--	42
phospholipase A2	*Bitis gabonica*	MOPS	--	--	phospholipid	--	91
glutamate dehydro-genase	bovine liver	phosphate	--	GTP	--	--	94
fructose 1, 6-diphosphate	pig kidney	borate	--	AMP	--	--	95
ribulose bis-phosphate carboxylase	*Rhodospirillum rubrum*	MOPS	--	--	ribulase bisphosphate	--	96
glucose-6-phosphate dehydrogenase	*Leuconostoc mesenteroides*	phosphate	--	--	glucose-6-phosphate	--	97
phosphoribosyl transferase	*Salmonella typhimurium*	phosphate	--	--	5-phospho-ribosyl-1-pyrophosphate	--	98

Table 5 (continued): Enzymes Shown to Require Lysine Residues, Envolved in Substrate or Cofactor Binding, by Modification with PLP

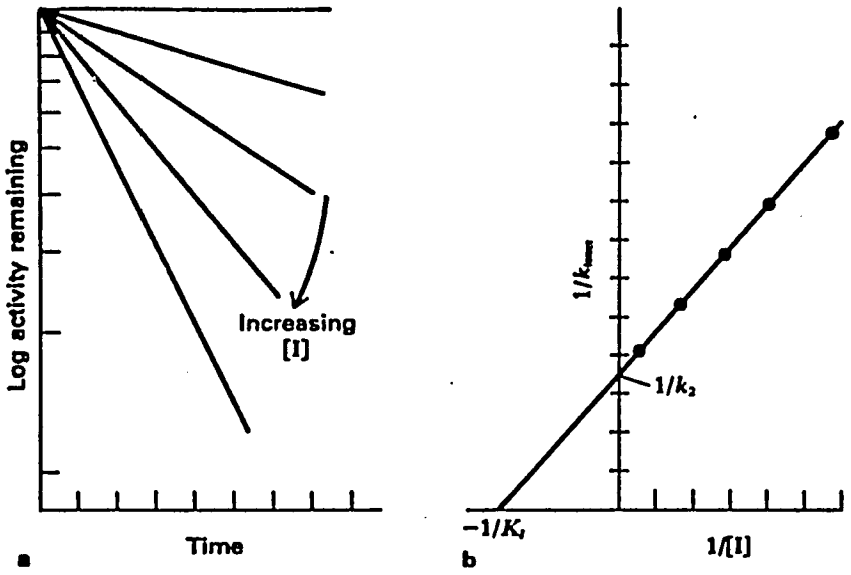
Enzyme	Source	Buffer	Cofactor	Effector	Substrate	Protection Not Done	Referen
ribuluse bisphosphate carboxylase	spinach	bicine	--	--	ribulose bis-phosphate	--	99
angiotensin	rabbit lung	HEPES	--	--	--	+	52
chloroplast coupling factor	spinach	tricine	--	--	ATP	--	115
alanine amino-transferase	pig heart	Tris	--	--	--	+	116
sequence--specific endonuclease BglI	Bacillus globigii	phosphate	--	--	DNA	--	117

This E · I complex formation is analogous to the E · S complex formed between an enzyme and a substrate molecule. If the inhibitor is binding at the active site prior to the chemical step involving covalent linkage to the enzyme there are testable kinetic consequences. First, the time dependent loss of catalytic activity should show first-order kinetics (rather than the second-order kinetics expected from a simple bimolecular collision between proteins and reagent free in solution). Experimentally, a semilog plot of the percentage of activity remaining versus time (at a fixed concentration of inactivator should yield data fitting a straight line. The half-life ($t_{0.5}$) for inactivation is related to the rate constant for inactivation by the expression $t_{0.5} = 0.693/k$ inact. (1.2).

When the experiment is performed at several fixed inhibitor concentrations, a family of lines is obtained (Fig. 18a), producing a series of rate constants of inactivation (k inact.) values. Then a secondary reciprocal plot can be constructed of 1 / k inact. versus 1 / [inhibitor] (essentially a Lineweaver-Burk plot for the inactivation shown in Figure 18b), yielding two important kinetic parameters. The vertical intercept of the reciprocal plot is $1 / k_2$ the limiting rate constant for inactivators (the observed inactivation rate if all of the enzyme is in the E · I complex). The physical significance of a finite vertical intercept is that the inactivation shows saturation kinetics and validates the idea that inactivation occurs from

Figure 18a. A plot of log of activity remaining as a function of time at a fixed concentration of inhibitors.

Figure 18b. A Lineweaver-Burk type plot of the data at several concentrations of inhibitor.


Log activity remaining
Increasing
[I]
Time
a
$1/k_{max}$
$1/k_2$
$-1/K_i$
1/[I]
b

prebound inactivator. The horizontal intercept is $-1 / K_I$ corresponding to the dissociation constant of inactivator from the E · I complex, and thus providing some measure of affinity of inactivator for enzyme (35, 38, 42, 98, 100, 106).

The Lineweaver-Burk type plot used in this study (106) was of the form shown in equation (1.3):

$$1 / k_{inact.} = \frac{K_I T}{0.693} \frac{1}{[I]} + \frac{T}{0.693} \qquad (1.3)$$

where T is the half time for inactivation at infinite concentration of I when all of the enzyme is in the E · I complex and is equal to $0.693 / k_2$ and K_I is the dissociation constant for the E · I complex. The equation could also be cast in the Eadie-Hofstee plot, a somewhat less-biased plot (108), to give the following equation:

$$k_{inact.} = -K_I \left(\frac{k_{inact.}}{[I]} \right) + \frac{0.693}{T} \qquad (1.4)$$

where K_I and T are the same as defined for the Lineweaver-Burk plot.

The order of the reaction with respect to the inhibitor can also be found. The apparent first order rate constant k app. is equal to the true rate constant for inactivation k inact. multiplied by the concentration of inhibitor to the n^{th} power

where n is the order of the reaction with respect to I. This is shown in the following equation:

$$\text{kapp.} = \text{k inact.} \; [I]^n \qquad (1.5)$$

and since kapp = 0.693 / $t_{0.5}$ then,

$$1 / t_{0.5} = \frac{\text{k inact.} \; [I]^n}{0.693} \qquad (1.6)$$

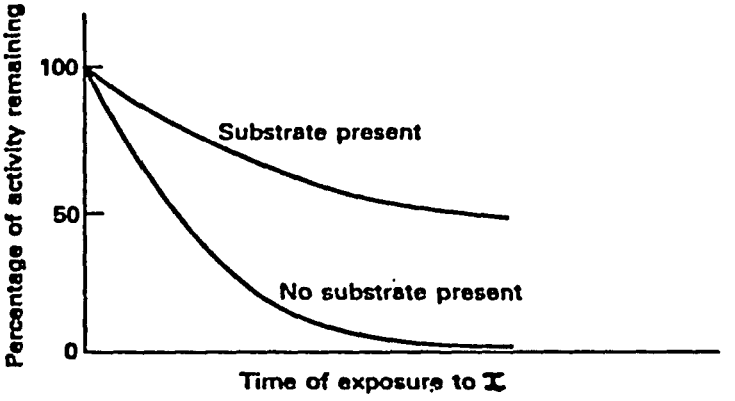
taking the log of both sides of equation (1.6) we see that,

$$\log (1 / t_{0.5}) = n \log [I] + k' \qquad (1.7)$$

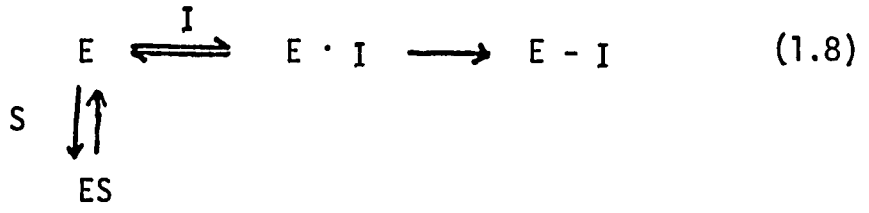
hence a plot of log (1 / $t_{0.5}$) versus log [I] will have a slope of n, the order of the reaction with respect to I (35, 38, 48, 101, 104, 105, 107, 109).

A second kinetic test that one can apply to the time-dependent loss of activity is to determine whether the addition of substrate or competitive inhibitor retards the rate of inactivation produced at a given concentration of the reagent as shown in Figure 19. This phenomenon of substrate protection arises from a binding competition between substrate and inactivation at the enzyme's active site. To the extent that substrate draws off enzyme molecules into an ES complex at any given instant, those enzyme

Figure 19 : A graph of percentage activity remaining as a function of exposure time to inhibitor in the presence and in the absence of substrate.


Percentage of activity remaining
100
50
0
Substrate present
No substrate present
Time of exposure to X

molecules cannot react with I. This inaccessibility lowers the concentration of E at those instants; a lower [E] means a slower observed rate of formation of E · I and, therefore, of the inactive covalent E - I compound. However, the formation of ES is reversible and S also gets used up, whereas covalent modification to E - I is irreversible; therefore, the presence of substrate will retard the rate of catalytic-activity loss, but cannot completely prevent it. The scheme for substrate protection is shown as follows (38, 51, 77, 98, 100):



(1.8)

The dissociation constant of a protecting ligand may be measured from rate of inactivation data (57, 102) using equation (1.9):

$$k_{inact.} - k_{min.} = \frac{k_o - k_{min.}}{1 + \frac{[S]}{K_s}}$$

where; k inact. is the observed first order rate constant, k min. is the rate constant at saturating levels of protecting ligand, ko is the rate constant in the absence of ligands, [S] is the

concentration of protecting ligand and Ks is the dissociation constant for the enzyme-ligand complex.

To determine if the covalent binding of I to E causes a conformational change in E, one can modify 50% of a population of E molecules with I. This is the same as removing 50% of the E molecules. If there is no conformation change on modification with I the K_m for substrate of the unmodified and the 50% modified population of E will be the same. The V_{max} of the 50% modified E population will be roughly half that of the native enzyme population (31, 48, 101, 103, 105, 108).

The second order rate constant for the bimolecular addition of an irreversible inhibitor may be obtained from the slope of a plot of k inact. versus the concentration of inhibitor shown in equation (2.0).

$$k \text{ inact.} = k_2 [I]^n \qquad (2.0)$$

where; k inact. is the first order rate constant for the inactivator, k_2 is the overall second order rate constant, and [I] is the concentration of inhibitor.

Significance of research:

The purpose of this research is:

1. To further purify urogen I synthase from wheat germ.

2. To study the effects of inhibitors specific for certain amino acid residues on the activity of urogen I synthase.

3. To ascertain which residues are essential for enzymatic activity.

4. To determine if the inhibition follows saturation kinetics.

5. To learn if the inhibitors are acting on active site residues by protection experiments with substrate and competitive inhibitors.

6. To determine if the residue is functioning in binding or catalysis.

Such a study of the active site of urogen I synthase will hopefully contribute to an elucidation of the mechanism of tetramerzation of PBG to a linear tetrapyrrole or urogen I.

METHODS AND MATERIALS

Reagents:

PBG was purchased from Porphyrin Products (Logan, Utah) and Sigma (St. Louis, Mo.) Butanedione and p-aminobenzaldehyde were purchased from Aldrich Chemical Co. (Milwaukee, Wi.). Wheat germ, ovalbumin, horse heart cytochrome C, α-chymotrypsinogen A, bovine serum albumin, phenylglyoxal and pyridoxal-5'-phosphate were also obtained from Sigma (St. Louis, Mo.).

Acrylamide was obtained from Bio-Rad Laboratories (Richmond, Ca.). N, N'-methylene-bis-acrylamide; N, N, N', N'-tetramethylethylenediamine ammonium persulfate; and Comassie Brilliant Blue were from Eastman Chemicals (Rochester, N.Y.). All other chemicals were reagent grade or better, obtained from standard suppliers.

DEAE-cellulose, Affi-gel 501 and Protein Assay Reagent were purchased from Bio-Rad Laboratories (Richmond, Ca.). Sephadex G-100 and Sephacryl-200 were purchased from Pharmacia (Piscataway, N.J.).

Electrophoresis was done using a Buchler (Scientific Products, Edison, N.J.) Polyanalyst Electrophoresis chamber and a Buchler D.C. Power supply model number 3-1500.

Spectrophotometeric measurements were made using the Cary 15 and Zeiss M4QIII spectrophotometers. Fluorometric measurements were made with the Perkin-Elmer MPF-2A spectrofluorometer.

Column chromatography was done using a LKB Ultrarac 7000 fraction collector with an LKB Uvicord III UV detector.

Centrifugation was done using a Beckman Model J-21 centrifuge.

PH measurements were made with corning Model 7, Corning Model 12 and Radiometer Model 26 (Copenhagen), pH meters.

Protein was determined by the method of Lowry (111) using bovine serum albumin as the standard or by measuring the absorbance at 280 nm or by using the Bio-Rad Protein Assay mixture, using the protein standard supplied with the kit (BGG, bovine γ globulin) as the standard (Bio-Rad Laboratory, Richmond, Ca.). When a microassay procedure was used for less than 5 μg of protein, the dye concentrate, 0.2 ml was added to samples of 0.8 ml. The OD at 595 nm was read 5 minutes to 1 hour after mixing.

Polyacrylamide disc gel electrophoresis was performed using the method of Davis (112).

Synthesis of 2-carboxyethyl-3-ethylcarboxyethyl-4-methylcarboxyethyl-5-carboxypyrrole.

The precursor of OPDC 2-carboxyethyl-3-ethylcarboxyethyl-4-methylcarboxyethyl-5-carboxypyrrole (123), was generously supplied by D. F. McDonald. It was converted to OPDC as follows: 100 mg was dissolved in 0.6 ml of 10% sodium hydroxide in a 20 mm tube, frozen in a dry ice-acetone bath, flushed with argon and sealed under vacuum. It was then heated in an oven for 2 hours at 175 - 178°C. The tube was frozen, opened and taken up in 6 ml of water. The material was pH adjusted to 3.1 with 2N HCl. It was lyophilized then extracted with ether. The ether extract was concentrated and the residue was crystallized from hexane, 83 mg of OPDC having a melting point of 129 - 131°C was obtained.

Enzyme assay:

Urogen I synthase activity was measured by assaying for PBG consumption and urogen formation. Incubation mixtures contained 1.0 ml of enzyme preparation, 3.5 ml of the appropriate buffer (0.05 M borate, pH 8.2; or 0.2 M bicarbonate, pH 8.2; or 0.075 M or 0.033 M phosphate, pH 8.2; or 0.1 M Tris, pH 8.2) 0.50 ml of 0.001 M EDTA and 0.1 ml of PBG (0.2 mg / ml of the

appropriate assay buffer). The PBG concentration was measured after incubation of the enzyme plus PBG at 37°C. The modified Ehrlich reagent of Mauzerall and Granick (113), (2g of p-dimethylaminobenzaldehyde added to 84 ml of glacial acetic acid and 16 ml of perchloric acid) was used to measure the PBG concentration. Equal volumes of aqueous solution and Ehrlich reagent were mixed and the color was allowed to develop for 15 minutes. Then the optical density at 553 nm ($E = 5.77 \times 10^4$ liter / mol - cm) was determined. Urogen concentration was determined by the method of Jordan and Shemin (114). Equal volumes of aqueous solution and 1N HCl solution containing 0.01% iodine are mixed. After 10 minutes in the dark, oxidation of urogen to uroporphyrin is complete. The optical density is then measured at 405 nm ($E = 5.41 \times 10^5$ liters / mole - cm), after the addition of a few crystals of thiosulfate as needed, to bind to any excess iodine. A microassay was used to measure enzymatic activity in fractions collected from a column as follows: 100 µl of the fraction to be assayed was mixed with 25 µl of a PBG solution (0.2 mg / ml). The tube was stoppered and incubated at 37°C. The assay tubes were evaluated when fluorescence appeared under the UV light in the control prepared from crude sample. The elution profile was obtained by adding 25 µl of iodine reagent to 50 µl of the assay mixture and 350 µl of buffer for 10 minutes and then reading the intensity of fluorescence at 598 nm in the spectrofluorimeter with excitation

at 410 nm. Or the elution profile could be measured by absorption spectroscopy. To the 125 µl assay mixture 200 µl of iodine reagent was added. After 10 minutes the OD at 405 nm was measured, using E = 5.41 x 10^5 liters / mole - cm.

Enzyme purification:

Urogen I synthase fraction "B" precipitate was prepared by the method of Bogorad (3).

All preparative operations including centrifugation and column chromatography were conducted at 4°C. Wheat germ (250 gm.) was extracted with 1 liter of cold distilled water for 30 minutes, with continuous mechanical stirring. This was followed by centrifugation of the slurry at 15,000 x g for 20 minutes. The sediment was discarded and the supernatant fluid was collected after filtration through cheese cloth to remove most of the fatty layer which separates out. Glacial acetic acid was then added, drop by drop with constant stirring to adjust to pH 5. A Corning Model 7 pH meter was used to monitor the pH. After 30 minutes in the cold, the material was centrifuged for 20 minutes, at 15,000 x g and the sediment was discarded and the supernatant fluid was collected. Next, solid ammonium sulfate (21.18 gm per 100 ml of fluid) was added to the supernatant fluid slowly while it was stirred mechanically.

After 30 minutes, the suspension was centrifuged, and the supernatant fluid was collected. The sediment (fraction A) was discarded after one washing with ammonium sulfate solution (21.18 gm per 100 ml). To the combined supernatant fluid and wash of fraction A was added solid ammonium sulfate (7.06 gm per 100 ml of fluid) and the procedure described above was repeated with the exception that an ammonium sulfate solution containing 28.24 gm of ammonium sulfate per 100 ml was used for the washing.

This precipitate (fraction B), which contains both urogen I synthase and cosynthase, was then suspended in a minimal amount of cold distilled water and dialyzed against 200 volumes of distilled water at 4°C for 4 hours.

The dialysate was then heat-treated by heating at 55°C for 15 minutes with gentle stirring. The suspension was centrifuged at 15,000 x g for 30 minutes. The precipitate containing the heat labile cosynthase was discarded.

Chromatography on mercuriphenylagarose was used to further purify the enzyme (112). Typically 10 ml of heat-treated enzyme (9 mg of protein / ml) was mixed with 15 ml of Affigel 501 (mercurphenylagarose) which had been washed three times with 45 ml of 0.075 M Tris buffer pH 8.0. The mixture was stirred at toom temperature for 10 minutes with a glass rod and then poured into a chromatography column (1.5 x 20 cm) and was eluted with the same buffer until the OD at 280

indicated no more protein was being eluted from the column. A large amount of protein and the yellow-brown color of the crude mixture was removed in this step. A linear gradient of Tris buffer (0.075 M, pH 8.0), containing 0.001M EDTA and the same buffer with 0.02 M mercaptoethanol was started and fractions of 3.3 ml were collected. The fractions were assayed using the microassay described in the enzyme assay section. Active fractions were pooled and stored at -70°C for long term storage, otherwise the enzyme was stored at -20°C.

Affi-gel 501 can be regenerated by washing first with 10 mM $HgCl_2$, 20 mM EDTA in 50 mM sodium acetate, pH 4.8. Excess $HgCl_2$ is removed by washing the gel with 0.2 M NaCl, 1 mM EDTA in 50 mM sodium acetate at pH 5.0. It is important to avoid introducing phosphate ions during regeneration of the gel.

Next, DEAE cellulose column chromatography was used. In a typical run, a sample of enzyme (6 mg of protein / 5 ml) was dialyzed against 25% glycerol, 10 mM mercaptoethanol in 0.02 M phosphate buffer pH 7.9 at 4°C overnight. The sample was put on a column (1 x 7 cm) and eluted with a linear gradient of 0 - 0.4 M KCl in the same buffer. Activity eluted in one peak at 0.13 M KCl. The fractions were assayed for protein using the microassay procedure described in the reagents section. The fractions were assayed for enzymatic activity using the microassay described in the enzyme assay section. The active

proteins were stored at -20°C.

Analytical polyacrylamide disc gels (112), 7.5% acrylamide pH 8.5, of the active tubes from DEAE cellulose chromatography were sliced lengthwise. One half was sliced in 0.5 cm slices, and each slice was incubated with 25 µl of PBG (0.2 mg / ml) in 0.75 M Tris pH 8.0 at 37°C for 5 hours and evaluated for pink fluorescence, due to porphyrin formation, under a UV lamp. The other half of the gel was stained for protein with Coomassie Brilliant Blue.

Calibrated Sephadex G-100 column chromatography:

One ml of Fraction "B" enzyme carried through the heat-denaturation step (12.4 mg protein / ml, specific activity 2.87 nanomoles PBG consumed / hr / mg protein) or 1 ml of fraction "B" material which was reprecipitated with ammonium sulfate (5.4 mg protein / ml, specific activity 20.1 nanomoles urogen / mg / hr) the 34-55% precipitate after dialysis against 4 liters of water were applied to a Sephadex G-100 column (36 cm x 2.6 cm) at a flow rate of 1.32 ml / minute. The elution buffer was 0.1 M Tris pH 8.2. Fractions of 4.3 ml were collected. The molecular weight standards were made as follows: (A) horse heart cytochrome C (MW 12,384) 25 mg plus egg albumin (MW 45,000) 20 mg were dissolved in 1.25 ml of 0.1 M Tris pH 8.2 and

applied to the Sephadex G-100 column. (B) α-chymotrypsinogen A (MW 25,000) 15 mg was dissolved in 1.25 ml of 0.1 M Tris pH 8.2 and applied to the Sephadex G-100 column. The column was calibrated before and after enzyme samples were put on and average values of K_{av} were used. The molecular weight was determined from the graph of K_{av} VS log molecular weight.

Calibrated Sephacryl 200 column chromatography:

Fraction "B" enzyme which was carried through the heat denaturation step and chromatography on mercuriphenylagarose was chromatographed on a Sephacryl 200 column. 3.5 ml of enzyme (5 mg protein, specific activity 36.8 nmol urogen / mg/ hr) was applied to a Sephacryl 200 column (63 cm x 1.5 cm) and eluted with 0.075 M Tris pH 8.2, containing 10 mM mercaptoethanol at a flow rate of 0.81 ml / minute. Fractions of 3.96 ml were collected. The molecular weight standards were made as follows: (A) ovalbumin (MW 45,000) 5 mg and horse heart ctyochrome-C (MW 12,384) 5 mg were dissolved in 2 ml of 0.075 M Tris pH 8.2 and applied to the column; (B) bovine serum albumin (MW 67,000) 5 mg and bovine pancreas α-chymotrypsinogen A, 5 mg were dissolved in 2 ml of 0.075 M Tris pH 8.2 and applied to the column. The molecular weight was determined from a graph of K_{av} VS log molecular weight.

Inhibition Studies:

A. Butandione.

BD solutions were prepared just before use. BD was dissolved in 50 mM borate buffer and pH adjusted to 8.2 using solid sodium hydroxide. Modification of urogen I synthase (fraction "B" carried through the heat denaturation step) was carried out at 25°C in a final volume of 8.0 ml containing 5 ml of enzyme preparation (typically containing 12.4 mg protein / ml, specific activity 2.87 nanomoles PBG consumed / hr / mg protein) 50 mM borate buffer, pH 8.2 and concentrations of BD ranging from 0 - 100 mM. Aliquots (1.4 ml) were removed after incubation from 0 - 150 minutes, chilled to 4°C and dialyzed overnight at 4°C against 50 mM borate buffer, pH 8.2. Dialysate, 1 ml, was assayed for PBG consumption and urogen formation, as described in enzyme assay. As a control, enzyme without BD was treated in the same manner for the longest incubation time and for zero incubation time and then assayed.

The ability of the substrate PBG to protect the enzyme against BD modification in 0.05 M borate pH 8.2 was tested. PBG at a final concentration of 80 μM was added to the modification mixture just before the addition of BD (10 mM). Aliquots (1.6 ml) were removed at various times and dialyzed for 24 hours at 4°C against one or more changes of 50 mM borate buffer pH 8.2,

before the enzyme was assayed. It was, however, impossible to remove all of the porphyrin. This was corrected for in the urogen assay by subtracting the initial concentration of porphyrin from the final concentration of porphyrin.

When the ability of the competitive inhibitor OPDC to prevent inhibition by BD (10 mM) was determined, 5.3 mg of OPDC was added to the modification reaction mixture 10 minutes before the addition of BD, and pH was adjusted to 8.2 using solid sodium hydroxide. The final concentration of OPDC was 2.24 mM. Modification of urogen I synthase (fraction "B" carried through heat denaturation and chromatography on mercuriphenylagarose) was carried out at 37°C in a final volume of 12.0 ml, containing 10 ml of enzyme preparation (0.81 mg protein / ml, specific acitivity 157 nmol urogen formed / hr / mg protein) in 50 mM borate pH 8.2. Aliquots of 1.8 ml each were removed after incubation at 37°C at various times, chilled to 4°C and dialyzed for 22 hours against four changes of 50 mM borate pH 8.2 at 4°C. The enzyme was assayed as described above.

Kinetic parameters were obtained using a fraction "B" enzyme preparation which had been carried through the heat denaturation step (12.4 mg protein / ml specific activity 2.87 nmol PBG consumed / hr / mg protein). Unmodified enzyme and enzyme which had been treated with 25 mM BD for 1 hour at 25°C were both dialyzed overnight at 4°C against 50 mM borate pH 8.2, and used. PBG concentrations of 25 μM through 145 μM were used

in separate incubation mixtures. Least squares analysis of data gave kinetic parameters with correlation coefficients greater than 0.97.

B. Phenylglyoxal.

PGO solutions were made just before use in either 50 mM borate pH 8.2 or 0.2 M bicarbonate and adjusted to pH 8.2 with concentrated HCl.

Modification of urogen I synthase (fraction "B" carried through heat denaturation and chromatography on mercuriphenyl-agarose) was carried out at 37°C in a final volume of 14 ml containing 10 ml of enzyme preparation, 50 mM borate buffer, pH 8.2 and concentrations of PGO ranging from 0 - 50 mM. Aliquots of 2.0 ml were removed at intervals between 0 - 90 minutes and applied to a G-25 column (15 x 1.5 cm) equilibrated and eluted with 50 mM borate, pH 8.2. The modified enzyme (1.0 ml) was assayed as described above. As a control, enzyme without PGO was treated in the same way for the longest incubation time and at zero time and passed over the same G-25 column and then assayed.

Urogen I synthase (fraction "B" carried through heat denaturation step and chromatography on mercuriphenylagarose) was also modified in 0.2M bicarbonate -0.1 M carbonate buffer pH 8.2. Modification was carried out at 37°C in a final volume

of 11.0 ml containing 7.7 ml of enzyme preparation, 0.2 M bicarbonate pH 8.2, and concentrations of PGO ranging from 0 - 4 mM. Aliquots of 1.5 ml were withdrawn and pipetted into 10 mg of sodium borohydride, to reduce the untreated PGO. The solutions were chilled to 4°C and pH adjusted to 8.2 and assayed. As a control, enzyme incubated without PGO was treated in the same way for the longest time interval and for zero time and then assayed.

When the ability of the competitive inhibitor, OPDC, to prevent inhibition by PGO was determined, OPDC (concentrations ranging from 0.11 to 8.4 mM) was added to the modification reaction mixture 10 min before the addition of PGO (concentration ranging from 2.9 to 4.0 mM). The modification (of fraction "B" enzyme carried through heat denaturation and chromatography on mercuriphenylagarose and dialysis for 12 hours against three changes of 0.2 M bicarbonate pH 8.2 at 4°C) was carried out at 37°C in a final volume of 13.0 ml containing 12.0 ml of enzyme preparation (typically containing 0.77 mg of protein, specific activity 4.62 nanomoles of urogen formed / hr / mg of protein) in 0.2 M bicarbonate -0.1 M carbonate pH 8.2.

When urogen I synthase was modified by PGO in bicarbonate buffer the enzyme was assayed as follows: 0.1 ml of PBG solution (0.2 mg / ml) was added to 1.5 ml aliquots and incubated for four hours at 37°C. Appearance of urogen and loss of PBG were measured as described above.

C. Pyridoxal-5'-phosphate.

PLP solutions were prepared just before use in 0.075 M phosphate buffer, pH 8.2. Modification of urogen I synthase (fraction "B" carried through heat denaturation) was carried out at 37°C in 0.075 M phosphate pH 8.2 in a total volume of 20 ml, containing 3.5 ml of enzyme (6.2 mg / ml, specific activity 2.87 nmol PBG consumed / hr / mg protein) PLP concentrations ranged from 0 to 25 mM. Aliquots of 2.75 ml were removed at various time intervals and added to 10 mg of sodium borohydride. The sodium borohydride reduces any Schiff base formed, prevents reversal of the reaction and reduces any unreacted PLP. Then 50 µl of PBG (0.21 mg / ml) was added to each aliquot and the sample was incubated at 37°C for 4 hours. Aliquots were removed and assayed for PBG consumption and porphyrin formation as described above. As a control, enzyme without PLP was treated in the same way for the longest incubation time and also zero time and then assayed.

When protection by PBG was done, PBG at a final concentration of 80 µm was added to the reaction mixture 10 minutes prior to the addition of PLP. Aliquots of 2.75 ml were removed at various times and added to tubes containing 10 mg of sodium borohydride. The samples were then dialyzed against 0.075 M phosphate pH 8.2 at 4°C for 24 hours and then assayed for PBG consumption and porphyrin formation as described above.

D. Formaldehyde.

Modification of urogen I synthase (fraction "B" carried through the heat denaturation step) was carried out at 37°C in a final volume of 1.2 ml containing 0.7 ml of enzyme (34.1 mg / ml, specific activity 2.87 nmol PBG consumed / hr / mg protein) 0.5 ml of 0.033 M phosphate pH 8.2 and formaldehyde at a final concentration of either 66 mM or 96 mM. The tubes were incubated at 37°C for various times between 0 and 50 minutes and then each tube was chilled to 0°C and 20 µl of 1.0 M sodium borohydride solution was added. The sodium borohydride was added to reductively alkylate lysine amino groups and to reduce unreacted formaldehyde. The sample was then dialyzed for 24 hours at 4°C against 0.033 M phosphate pH 8.2 and assayed for PBG consumption and porphyrin formation as described above. Controls were treated in the same way except that formaldehyde was omitted from the incubation mixture.

RESULTS

Enzyme purification;

Fraction "B" enzyme (2), which was dialysed to remove the ammonium sulfate and then heat-denatured, was applied to a mercuriphenylagarose column (114), as described in Appendix I. Elution with a mercaptoethanol gradient eluted one major protein peak. The fractions were assayed for enzymatic activity by adding PBG to aliquots of the individual fractions. The activity and elution profiles appear in Figure 20. A large amount of non-enzyme protein was removed. Recovery of activity was 42%.

Active fractions of mercuriphenylagarose were purified further by DEAE-cellulose chromatography in 25% glycerol and 10 mM mercaptoethanol. The enzyme was eluted with a linear KCl gradient of 0 to 0.4 M KCl. The activity and elution profiles appear in Figure 21. The low amount of protein in each tube (less than 5 µg) required a micro-protein assay. The microassay was linear over the concentration range used, as can be seen from Figure 22.

Analytical polyacrylamide gels of the most active fraction showed a protein band at R_F 0.39 (using the mobility of bromphenol blue as reference) and a faint protein band at R_F 0.33. The former band was the only one with enzymatic activity. The purificaiton scheme is presented in Table 6.

Figure 20: Typical elution profile or urogen I synthase on an Affi-gel 501 column:

- ● relative OD at 280 nm;
- ▲ nanomoles of urogen formed in 1 hour at 37°C.

The enzyme was eluted with a linear gradient of mercaptoethanol (0 - 20 mM). The gradient was started at fraction number 30.

RELATIVE OD280nm (●)
NM UROGEN/HR/MG PROTEIN (▲)
FRACTION NUMBER
90
60
30
20
40
60
20
40
60

Figure 21: Elution profile of urogen I synthase on a DEAE - cellulose column:

- ● relative OD at 280 nm;
- ▲ nanomoles of urogen formed at 37°C per hour per mg of protein.

The enzyme was eluted with a linear gradient of 0 to 0.4 M KCl. The gradient was started at fraction number 30 (shown with an arrow).

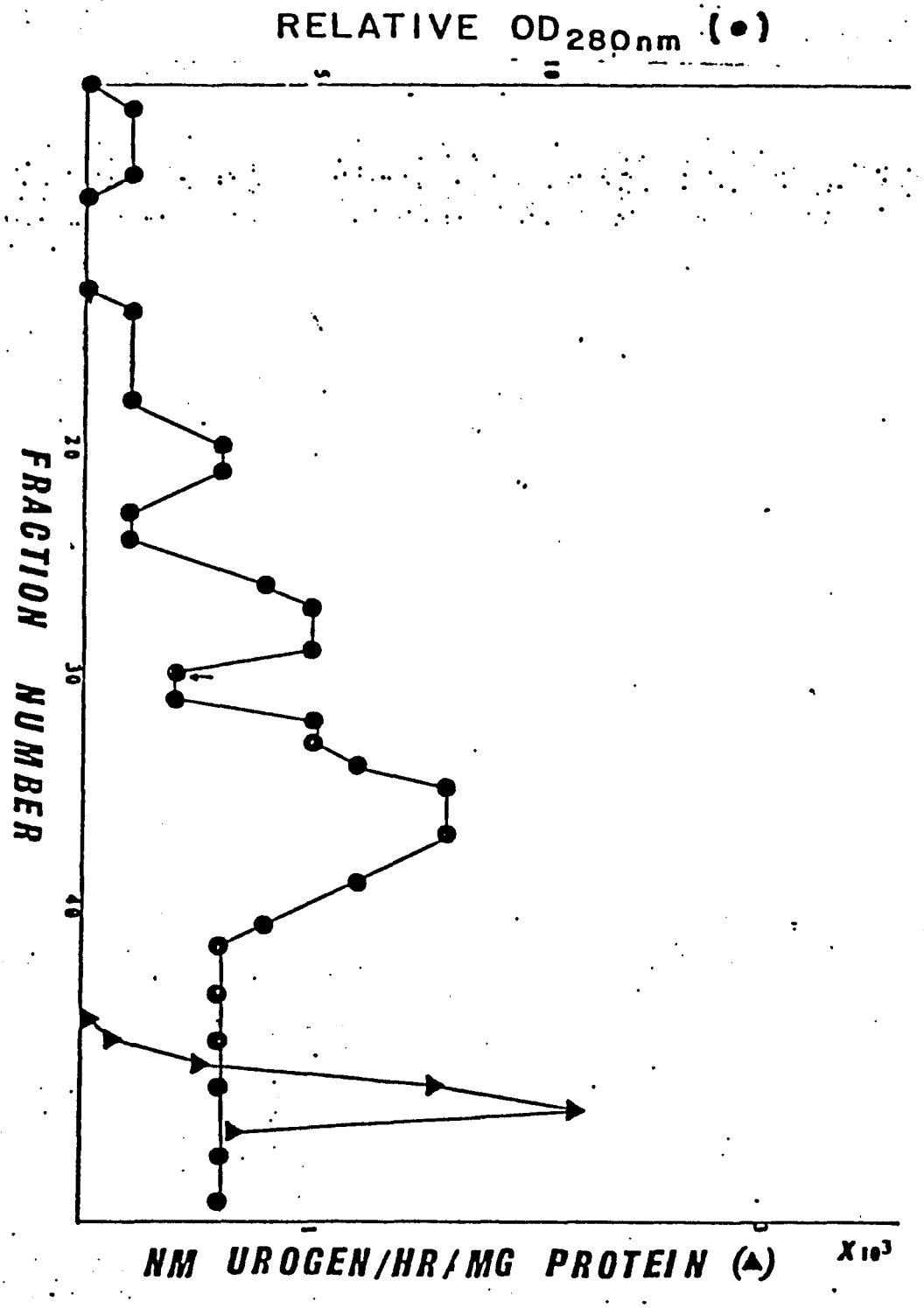

RELATIVE OD280nm (●)
FRACTION NUMBER
20
30
40
NM UROGEN/HR/MG PROTEIN (▲)
X 10³

Figure 22 : A plot of the OD at 595 versus μg of protein in standards used in a micro-Biorad protein assay.

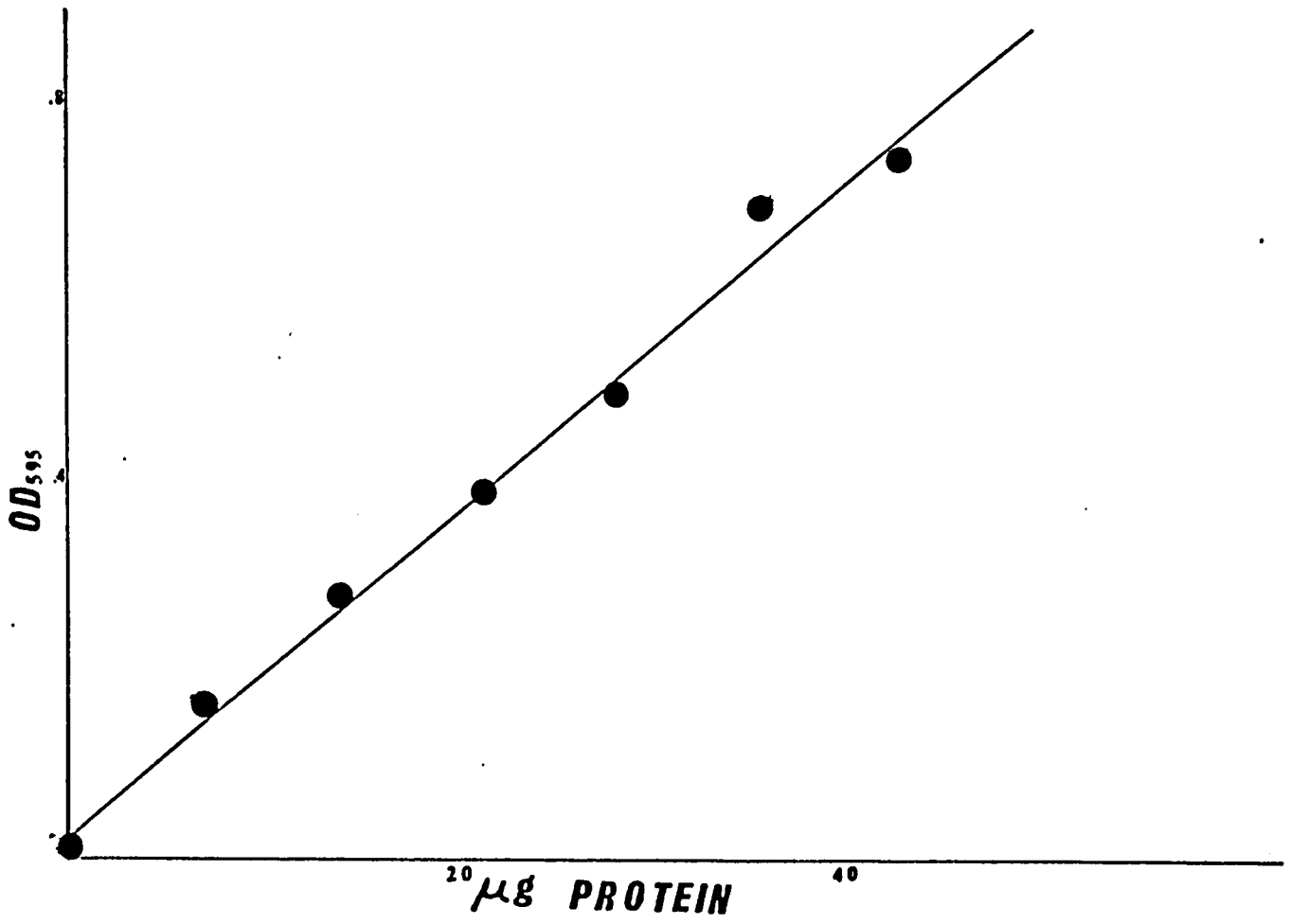

OD595
.8
.4
20
40
μg PROTEIN

Table 6: Purification of Wheat Germ Urogen I Synthase

	Total Protein (mg)	Specific Activity *	Total Units	% Recovery	Fold Purification
Fraction B	936	6.1	5,715	100	1
Heat-treated	163	25	3,995	70	4
Affi-gel 501	8.47	200	1,680	29	33
DEAE-cellulose	0.0095	2,166	21	0.4	355

*Specific activity is expressed as the nanomoles of urogen formed at 37°C per hour per mg of protein.

Gel filtration of different enzyme preparations:

Fraction "B" enzyme which was heat-denatured showed two peaks of enzymatic activity of molecular weights 30, 850 ± 2,700 and 40, 538 ± 2,700 daltons on Sephadex G-100, as shown in Figure 23. When the heat denatured preparation was refractionated with ammonium sulfate as described in the methods section, only one peak of enzymatic activity was found, which had a molecular weight of 40,346 ± 2,700 daltons, on Sephadex G-100 (shown in Figure 24).

Fraction "B" enzyme which was carried through the heat denaturation step and chromatography on mercuriphenylagarose was chromatographed on a Sephacryl 200 column. One peak of acitivity was seen which corresponded to a molecular weight of 42,135 ± 3,500 daltons.

Butanedione inhibition:

Urogen I synthase was incubated with various concentrations of BD as described in (109) included as Appendix II. The enzyme was incubated with BD in borate buffer for a series of time intervals. The enzyme aliquots were dialyzed individually against borate buffer before they were assayed for activity. The activity

Figure 23: G-100 elution profile of enzyme, showing two active peaks.

NM UROGEN/HR/MG PROTEIN (▲)
RELATIVE OD280nm (●)
FRACTION NUMBER
40
20

Figure 24: G-100 elution profile after reprecipitation with ammonium sulfate.

RELATIVE OD280nm (●)
NM. UROGEN/HR/MG PROTEIN (▲)
FRACTION NUMBER
15
10
5
1.5
1.0
0.5
20
40

of the enzyme, measured as PBG consumption (shown in Figure 25) and porphyrin formation (shown in Figure 26), was severely and rapidly reduced by BD in borate buffer. Borate buffer alone had no effect on urogen I synthase activity.

In order to determine whether the substrate can protect the enzyme from inactivation by BD, urogen I synthase was mixed with PBG (80 μM) before addition of BD (10 mM) in borate buffer. Aliquots were removed at various time intervals and dialyzed against borate at 4°C before assay. The substrate showed a pronounced ability to protect urogen I synthase from BD inactivation, as can be seen in Figure 27 (where PBG consumption was followed). For the data in the first 90 min of the reaction plotted as log % activity remaining versus time a linear relationship is obtained, as can be seen from Figure 28. The linearity of this plot implies that the modification is indeed following first order kinetics. From the semilogarithmic plot the $t_{0.5}$ for the inactivation of urogen I synthase by BD may be obtained. The $t_{0.5}$ for the inactivation in the absence of PBG was 58 minutes and in the presence PBG was 543 minutes. Urogen formation in the presence and absence of PBG in the presence of 10 mM BD is shown in Figure 29.

In order to determine if the competitive inhibitor of PBG, OPDC (K_I = 0.28 mM) could also protect urogen I synthase from

Figure 25 : Inactivation of urogen I synthase by BD in borate buffer. The enzyme was incubated with various concentrations of BD. Aliquots were removed at the indicated times dialyzed against borate buffer (50 mM, pH 8.2) and assayed for PBG-consuming activity. The incubations included BD in the following concentrations:

●, 0 mM;

▲, 10 mM;

■, 50 mM;

o, 100 mM.

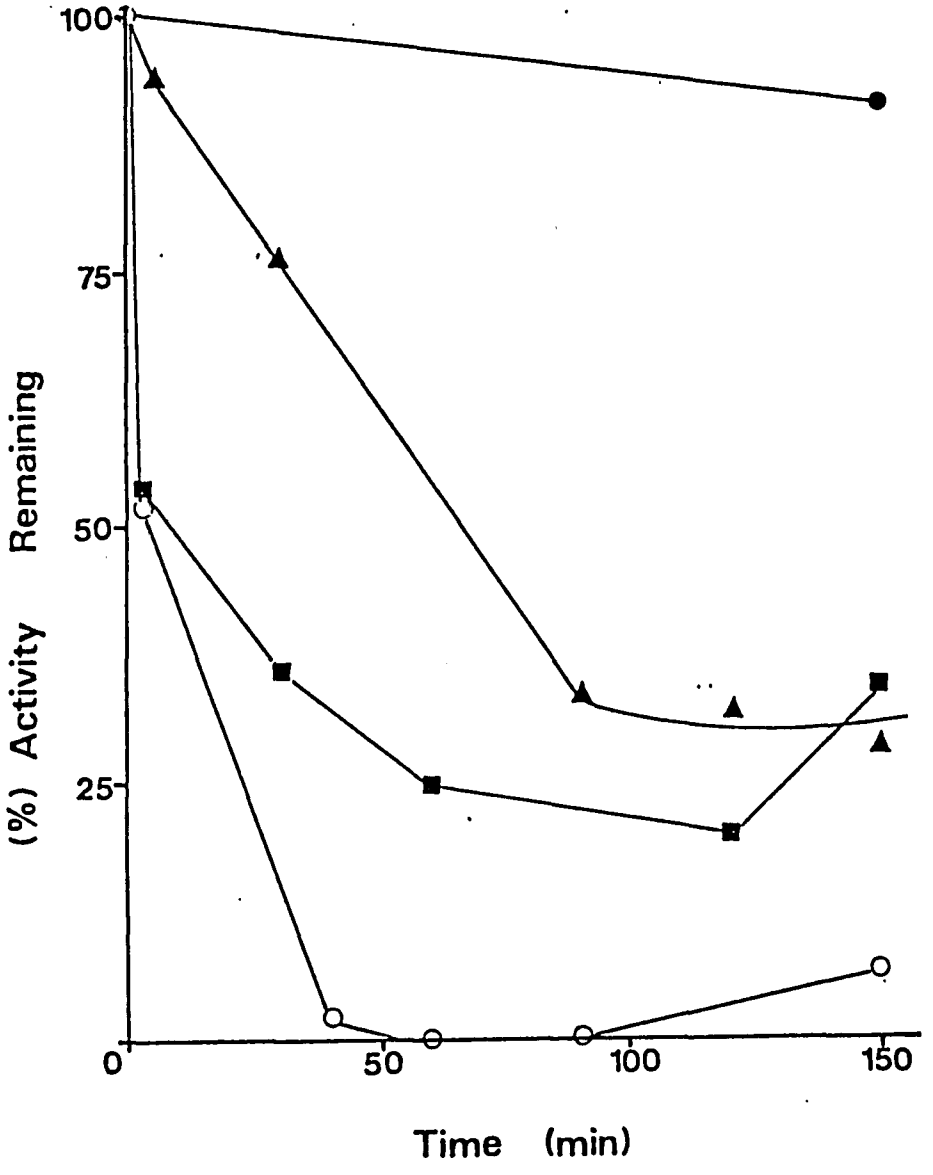

100
75
50
25
0
50
100
150
(%) Activity Remaining
Time (min)

Figure 26: Inactivation of urogen I synthase by BD in borate buffer. The enzyme was incubated with various concentrations of BD. Aliquots were removed at the indicated times dialyzed against borate buffer (50 mM, pH 8.2) and assayed for urogen formation. The incubations included BD in the following concentrations:

●, 0 mM;

▲, 10 mM;

■, 50 mM;

0, 100 mM.

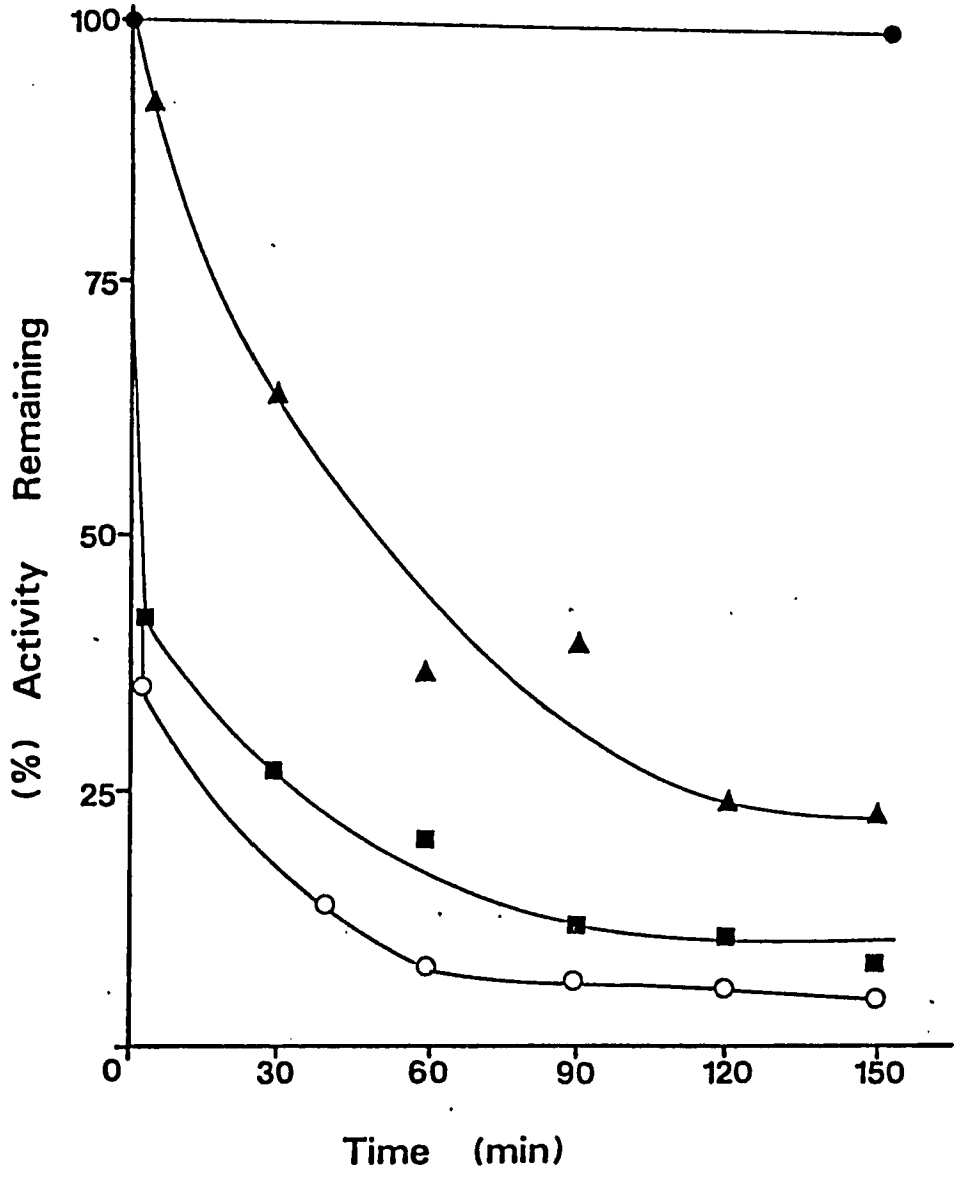

100
75
50
25
0
30
60
90
120
150
(%) Activity Remaining
Time (min)

Figure 27: Protection by PBG of urogen I synthase inactivation by BD. The enzyme was incubated with 10 mM BD with and without 80 μM PBG at 25°C. Aliquots were removed at indicated times and dialyzed overnight against 50 mM borate, pH 8.2 at 4°C, and then assayed. Activity was nanomoles of PBG consumed in 4 hours at 37°C. The incubation mixtures included BD and PBG in the following final concentrations:

●, 0 mM BD and 0 μM PBG;

▲, 10 mM BD and 80 μM PBG;

■, 10 m BD and 0 μM PBG.

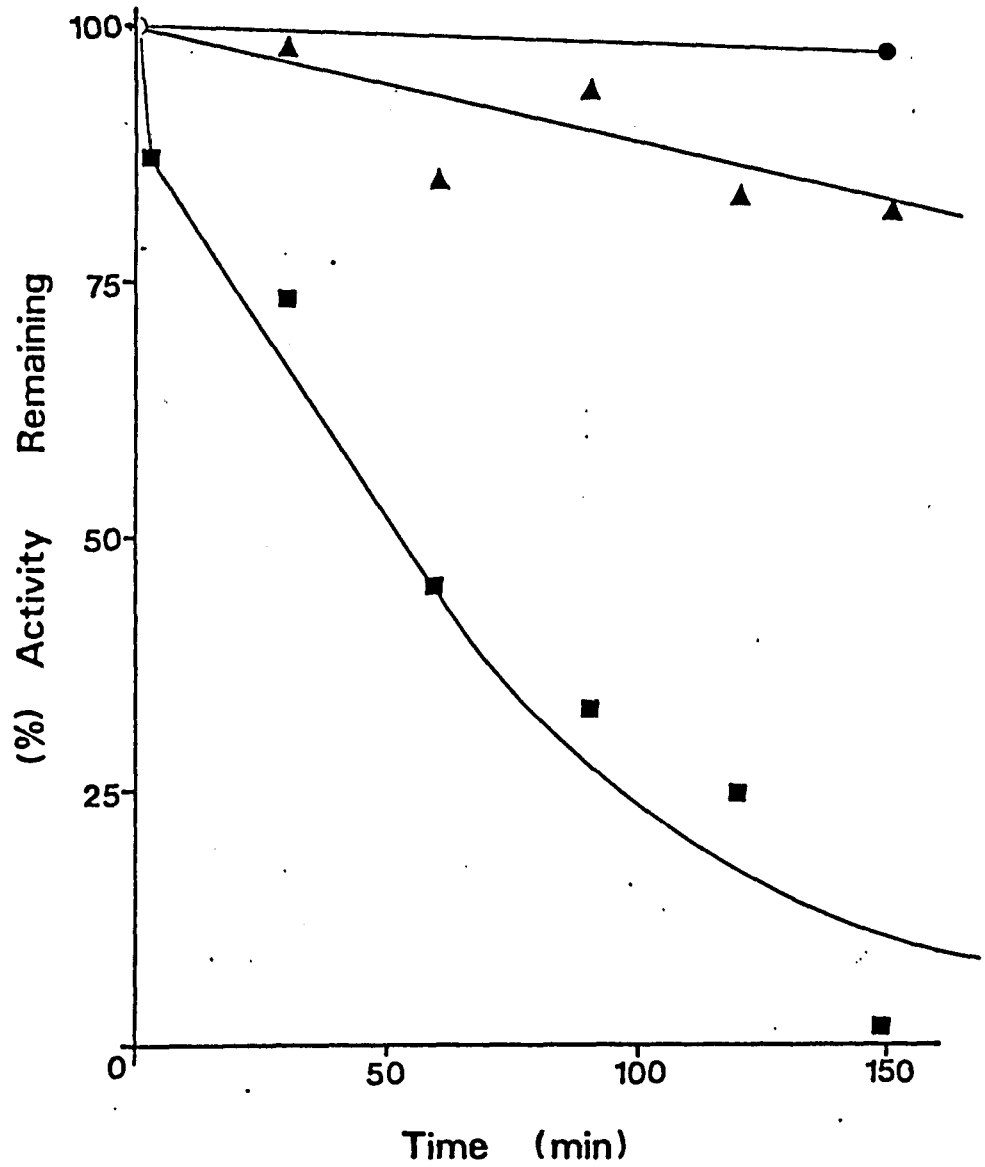
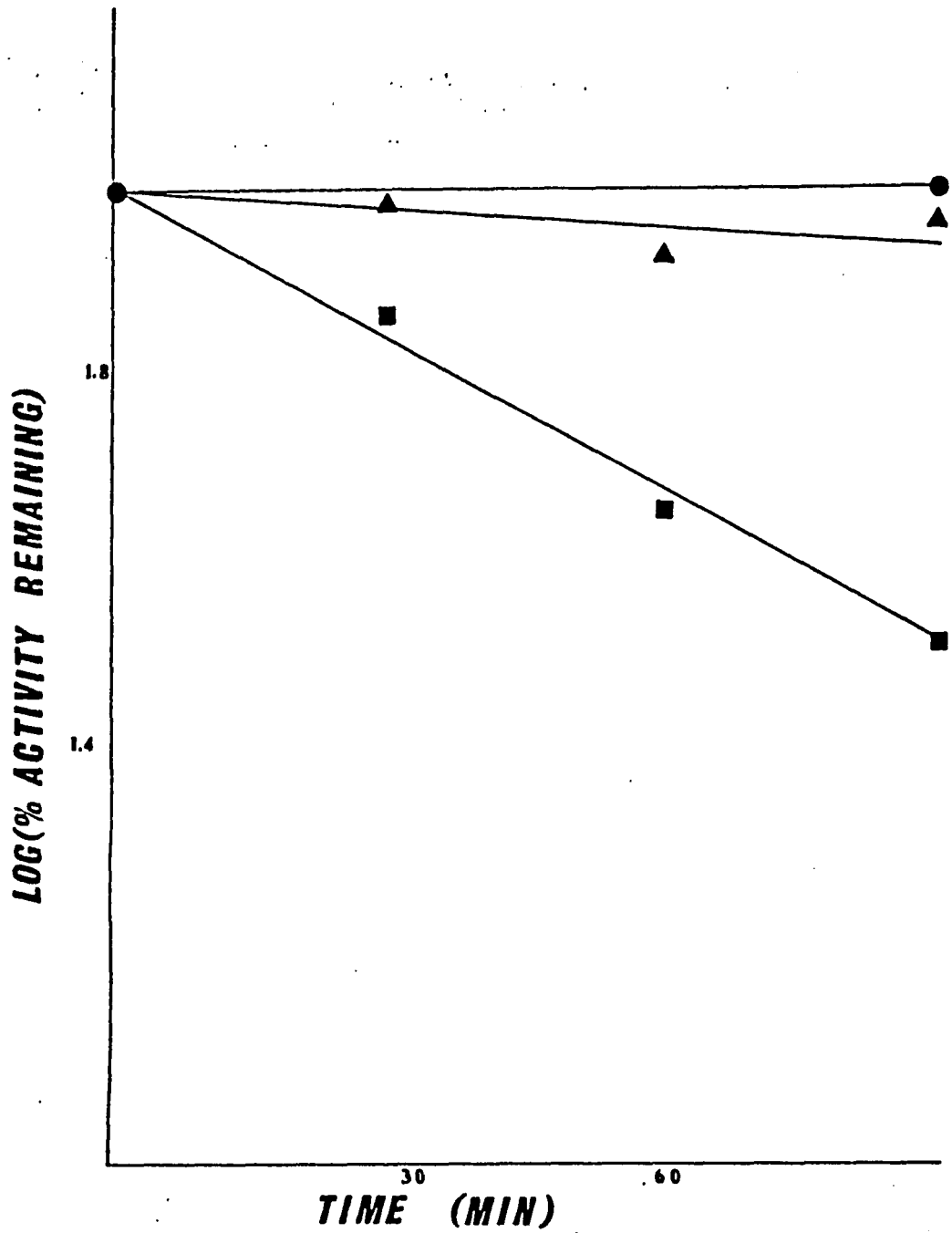

100
75
50
25
0
50
100
150
(%) Activity Remaining
Time (min)

Figure 28: The data of Figure 26 expressed as log % activity remaining versus time.


LOG(% ACTIVITY REMAINING)
1.8
1.4
30
60
TIME (MIN)

inhibition by BD; urogen I synthase was mixed with OPDC (0.56 mM) 5 minutes before the addition of BD (10 mM) in borate buffer. Aliquots were removed at various time intervals dialyzed against borate at 4°C before being assayed. The activity was expressed as nanomoles of urogen formed at 37°C in 4 hours. The competitive inhibitor, OPDC was able to protect urogen I synthase from BD inactivation. This can be seen in Figure 29 . The $t_{0.5}$ for the inactivation in the absence of OPDC was 8.5 minutes and in the presence of OPDC at $t_{0.5}$ of 32 minutes was obtained.

This inactivation of BD suggests that there is probably an arginyl residue in the enzyme's active site. The fact that BD inactivation is relieved by PBG and OPDC shows that BD is active site-directed and that the arginyl residue which is modified lies at the PBG and OPDC binding locus.

K_m and V_{max} were determined for modified and unmodified urogen I synthase in 50 mM borate buffer pH 8.2 at 25°C. Unmodified enzyme had a K_m of 26 $\pm$ 3.0 μM. When urogen I synthase was modified for 1 hour in 25 mM butanedione, then dialyzed against borate and assayed with varying concentrations of PBG for 1 hour and 2 hours incubation times, the kinetic parameters in Table 7 and Figure 30 were obtained. K_m for porphyrin formation was slightly higher or the same for modified enzyme and V_{max} was lower by about a factor of 2. The results suggest that the modified enzyme mixture consists of roughly 50% totally inactivated enzyme and 50% native enzyme. This data also

Figure 29: Protection by OPDC of urogen I synthase inactivation by BD, in 50 mM borate pH 8.2 at 37°C. Aliquots were removed at the indicated times and dialyzed overnight against 50 mM borate pH 8.2 at 4°C and assayed the next day. Activity is expressed as nanomoles of urogen formed at 37°C in 4 hours. The incubation mixtures included BD and OPDC in the following final concentrations:

■, 0 mM BD and 0 mM OPDC;

▲, 10 mM BD and 0.56 mM ODPC;

●, 10 mM BD and 0 mM OPDC.

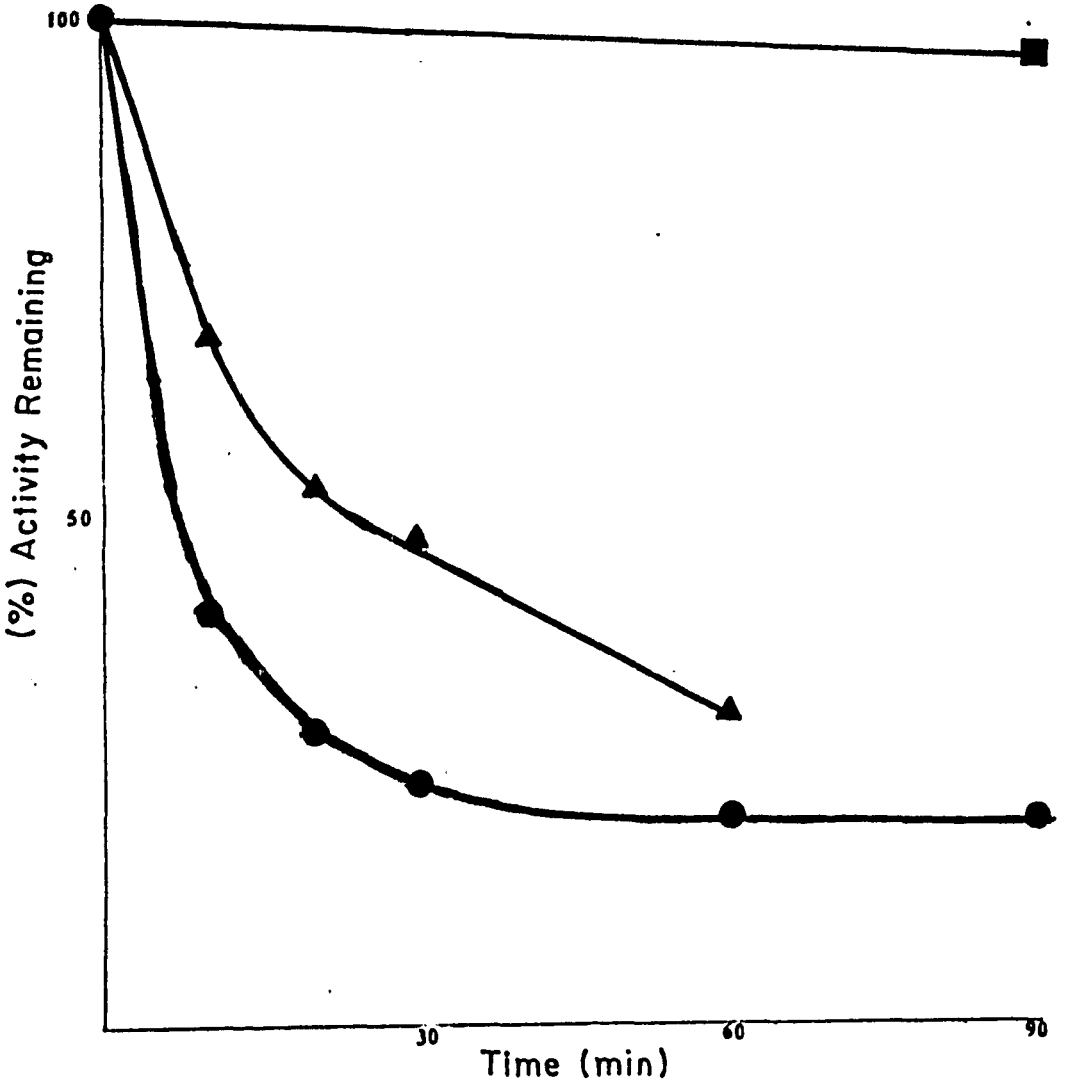

100
50
(%) Activity Remaining
30
60
90
Time (min)

Figure 30: Double reciprocal plot of urogen formation by unmodified (●) and BD modified urogen I synthase (▲). Urogen I synthase was incubated with 25 mM BD for 60 minutes at 25°C. Velocity is expressed as nanomoles of urogen formed per ml per hour.

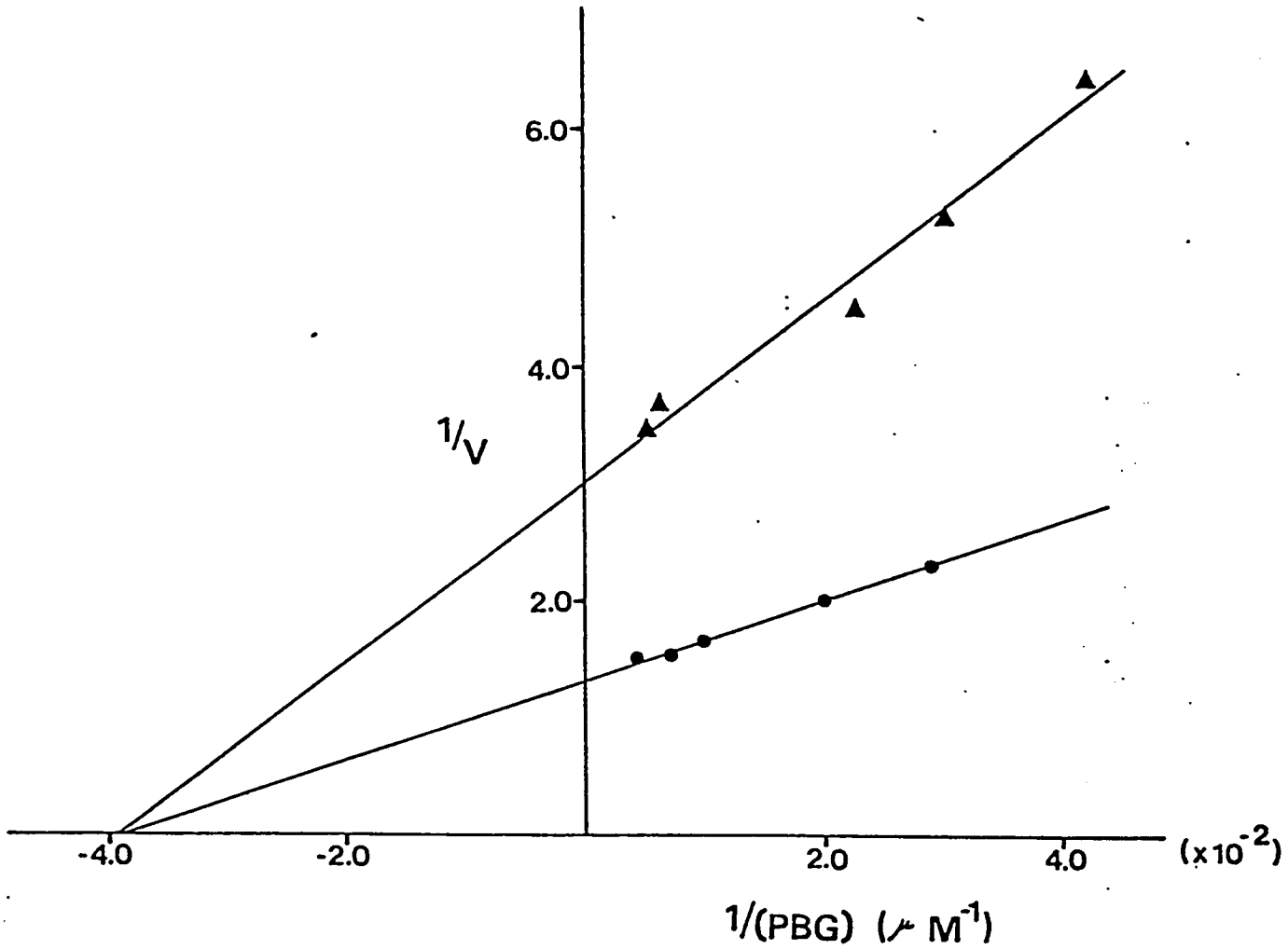

6.0
4.0
2.0
1/V
-4.0
-2.0
2.0
4.0
(x10^-2)
1/(PBG) (μ M^-1)

Table 7: Kinetic Parameters for Urogen I Synthetase Before and After Inhibition by 25 mM Butanedione Measured After One Hour and Two Hour Incubation Times. Activity was Measured as Porphyrin Formation.

	1 Hour		2 Hours	
Incubation Time	K_M (μM)	V_{Max} (NMoles/Ml/Hr)	K_M (μM)	V_{Max} (NMoles/Ml/Hr)
Unmodified	26	7.5	22	7.3
Modified Run #1	36	2.9	42	3.4
Modified Run #2	24	3.3	27	3.1
Modified Run #3	30	2.6	24	2.2

suggests that modification does not occur at a locus outside of the PBG binding domain and exerts its inhibitory effects through a conformational change--as this mechanism of inactivation would result in a changed K_m for the BD modified enzyme (31, 48, 101, 103, 105, 109).

For data in the first hour of reaction when log $1 / t_{0.5}$ was plotted against log BD concentration (Figure 31), according to equation (2.1)

$$\log (1 / t_{0.5}) = n \log [I] + K' \qquad (2.1)$$

which was derived in the introduction. The slope n, the order of the reaction, was 0.89 for PBG and 0.65 for porphyrin formation. The results suggest that the order of the reaction of arginine with BD is one.

A graph of the reciprocals of the first order rate constants versus the reciprocals of the BD concentrations, where activity is expressed as nanomoles of urogen formed in 4 hours at 37°C, is presented in Figure 32. The physical significance of the linear fit of the data to equation (2.2) and the fact that there is a

$$1 / k \text{ inact.} = \frac{K_I T}{0.693} \frac{1}{[I]} + \frac{T}{0.693} \qquad (2.2)$$

finite vertical intercept is that inactivation follows saturation kinetics and validates the idea that inactivation occurs from preformed inactivator. All data of this form are plotted according to the Lineweaver-Burk type plot shown in equation (2.2). The parameters K_I and T are obtained from equation (2.2) except where it is stated that the parameters K_I and T were

Figure 31: Logarithmic plot of urogen I synthase activity after treatment with various concentrations of BD. Half-times of inactivation ($t_{0.5}$) values were calculated from semilogarithmic plots of log urogen I synthase activity vs. time in minutes for initial reaction times up to sixty minutes.

●, PBG consumption;

▲, uroporphyrinogen formation.

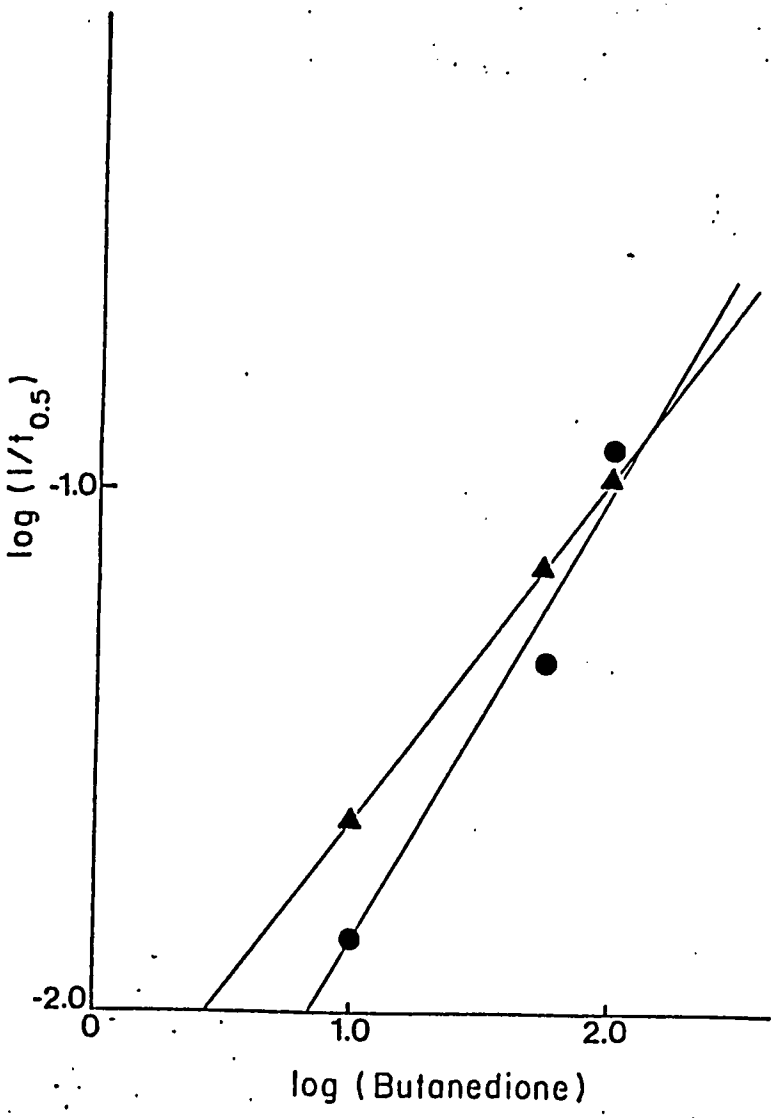
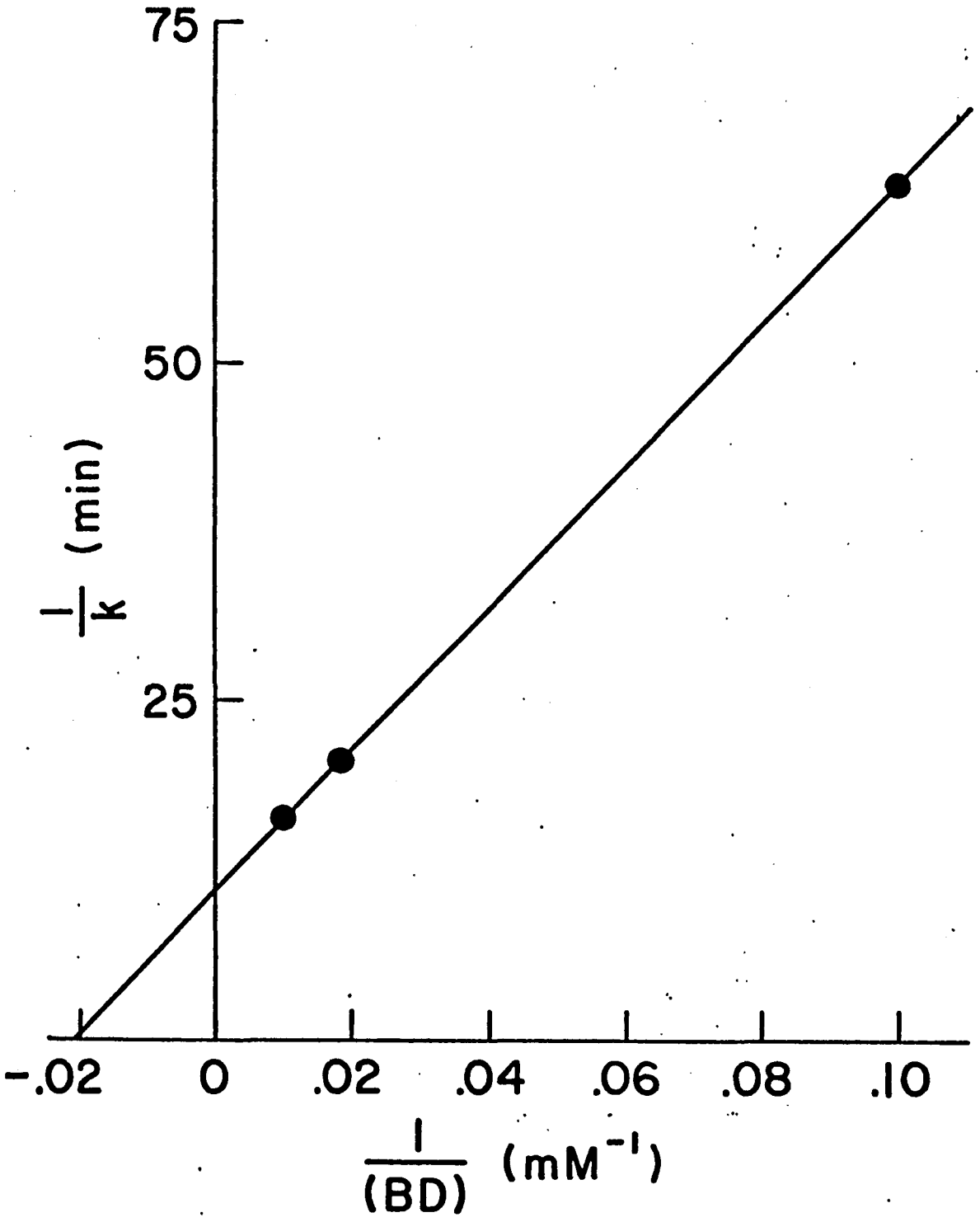

log (1/t0.5)
-1.0
-2.0
0
1.0
2.0
log (Butanedione)

Figure 32: A plot of the reciprocals of the first order rate constants for inactivation versus the reciprocals of the BD concentration. Urogen I synthase was modified with BD in 50 mM borate pH 8.2 at 25°C. Activity is expressed as nanomoles of urogen formed in 4 hours at 37°C.


75
50
25
$-\frac{1}{k}$ (min)
-.02
0
.02
.04
.06
.08
.10
$\frac{1}{(BD)}$ (mM^{-1})

obtained from equation (2.3)

$$k \text{ inact.} = - K_I \left(\frac{k \text{ inact.}}{[I]} \right) + \frac{0.693}{T} \qquad (2.3)$$

the Eadie-Hofstee type equation. In most cases the values obtained by using equation (2.2) agreed well with those of equation (2.3). In those few cases where there was a difference the Eadie-Hofstee treatment was considered less-biased (108).

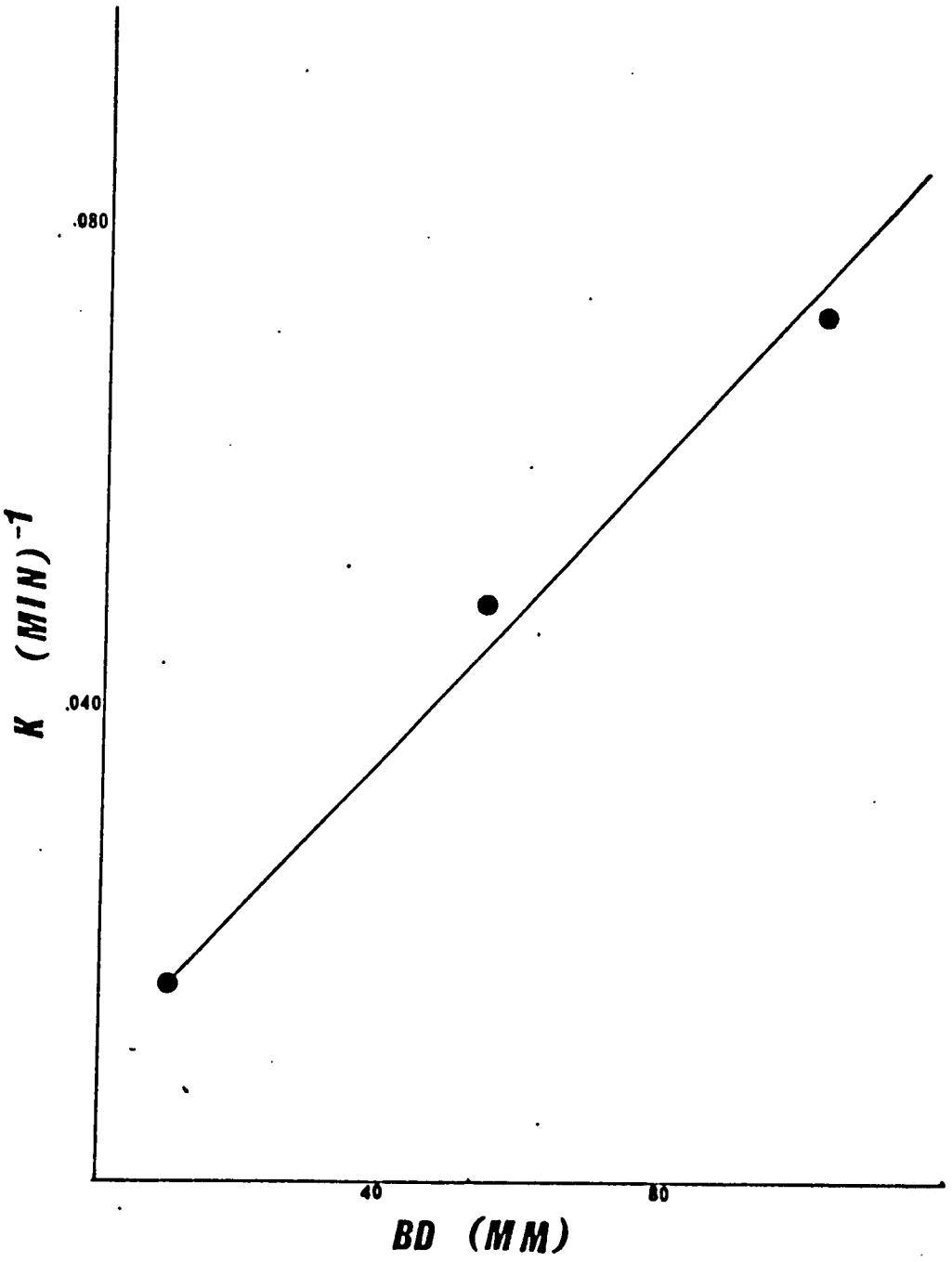
For the BD modification data shown in Figure 32, activity is expressed as nanomoles of urogen formed in 4 hours at 37°C. A K_I value of 49.0 $\pm$ 1.3 mM was obtained. This dissociation constant for BD from the E · BD complex provides some measure of affinity of BD for the enzyme. T, the half time for inactivation at infinite concentration of BD, when all the enzyme is in the E · BD complex, was found to be 7.00 minutes. This value is a measure of the maximal rate of inactivation. Both the K_I and T were obtained from equation (2.3) the Eadie-Hofstee type of equation. The value of K_I was 50.0 $\pm$ 0.1 mM and the value of T was 8.50 minutes, where activity was expressed as nanomoles of PBG consumed in 4 hours at 37°C.

A plot of the first order rate constant for inactivation versus the concentration of BD, has a slope equal to the overall second order rate constant for the reaction, as can be seen from equation (2.4), where k inact. is the observed

$$K \text{ inact.} = k_2 [I] \qquad (2.4)$$

first order rate constant and k_2 is the overall second order rate constant and I is the concentration of inhibitor.

Figure 33: A plot of the first order rate constant for the inactivation versus the concentration of BD. Urogen I synthase was modified with BD in 50 mM borate pH 8.2 at 25°C. Activity is expressed as nanomoles of urogen formed in 4 hours at 37°C.


.080
.040
K (MIN)-1
40
80
BD (MM)

A plot of this nature where the activity is expressed as nanomoles of urogen formed in 4 hours at 37°C is shown in Figure 33. A second order rate constant of 0.607 $(M)^{-1}$ $(minutes)^{-1}$ was obtained. A second order rate constant calculated from PBG consumption data in nanomoles consumed in 4 hours at 37°C was equal to 0.800 $(M)^{-1}$ $(minutes)^{-1}$.

Phenylglyoxal inhibition:

Urogen I synthase was incubated with phenylglyoxal (PGO) another irreversible inhibitor specific for arginine residues. Urogen I synthase was incubated with various concentrations of PGO in borate buffer at 37°C for a series of time intervals. Aliquots of enzyme were removed at various times and applied to a G-25 column equilibrated and eluted with borate pH 8.2, in order to remove excess PGO. The activity of the enzyme measured as urogen formation was severely and rapidly reduced by PGO, as shown in Figure 34.

It was important to test if any PGO was left after the G-25 column chromatography and also to see if PGO reacted with PBG. If the inhibitor consumes substrate and some excess is present this could also be interpreted as inactivation of the enzyme. As can be seen from Table 8, the most concentrated modification mixture contained 50 mM PGO and after passage through the G-25

Figure 34: Inactivation of urogen I synthase by PGO in 50 mM borate pH 8.2 at 37°C. Aliquots were removed at the indicated times and passed through a G-25 column equilibrated with 50 mM borate pH 8.2 and eluted with the same buffer at 4°C. Activity is expressed as nanomoles of urogen formed at 37°C in 4 hours: The concentrations of PGO in the incubation mixtures were:

X, 0 mM;

●, 1 mM;

□, 10 mM;

Δ, 25 mM;

o, 50 mM.

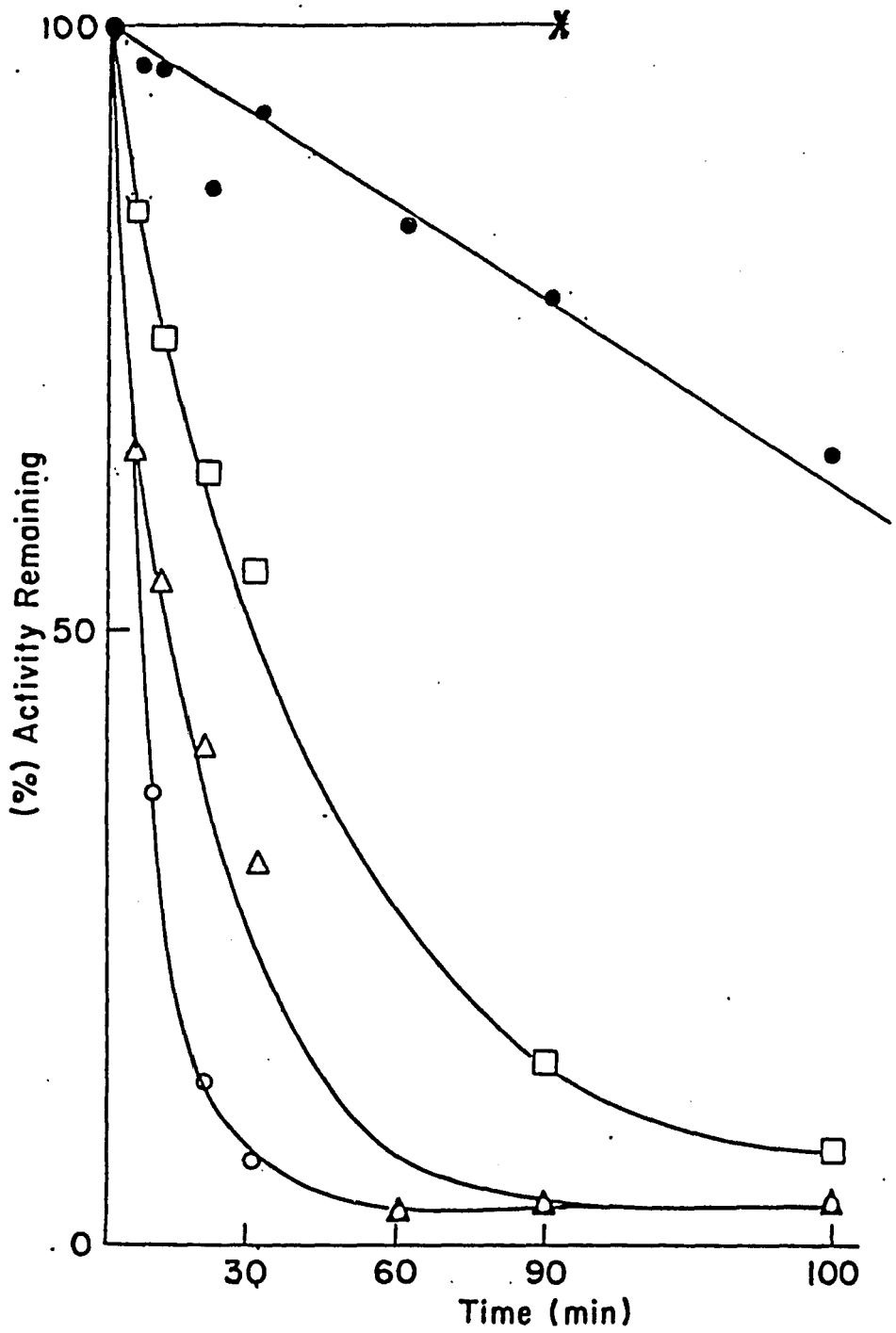

100
50
0
(%) Activity Remaining
30
60
90
100
Time (min)

Table 8: PGO Remaining in a Modification Mixture of PGO and Urogen I Synthase After Passage Through a G-25 Column

mM PGO in Modification Mixture	mM PGO Remaining after G-25	% PBG Consumed Nonenzymatically in 4 hours at 37°C in Borate by the Remaining PGO
1.0	0.00	0.00
10.0	0.13	0.68
25.0	0.32	1.70
50.0	0.71	3.70

column 0.71 mM free PGO remained, as judged from the OD at 250 nm for free PGO. This amount of PGO will cause the nonenzymatic loss of 3.7% of the initial concentration of PBG, used in these PGO inactivation assays, in 4 hours at 37°C in 50 mM borate pH 8.2. This was determined by incubation of various concentrations of PGO with PBG in borate buffer (50 mM, pH 8.2) for four hours at 37°C. PBG concentration at time zero and after four hours was measured with Ehrlich's reagent, and the % PBG lost was calculated.

The initial concentration of PBG used to assay the enzyme after passage through the G-25 column was 23 times the K_m for substrate. Hence, the enzyme was always saturated with substrate.

For data in the first hour of reaction when log 1 / $t_{0.5}$, was plotted against log PGO concentration (Figure 36) a slope of 0.89 was found for urogen formation in 4 hours at 37°C and a slope of 0.95 was found for urogen formation in 6 hours at 37°C. These results suggest that the order of the inactivation reaction with respect to PGO is one.

Although Takahashi's organic model showed that two molecules of PGO condense with one of arginine, Werber et al. (68) have shown that in borate buffer only one PGO molecule is incorporated. The initial adduct which is formed may be stabilized by a second molecule of borate as shown in Figure 15.

A plot of the reciprocals of the first order rate constants versus the reciprocals of the PGO concentrations is presented in

Figure 35 : Logarithmic plot of urogen I synthase activity after treatment with various concentrations of PGO in 50 mM borate pH 8.2 at 37°C. Activity was expressed as nanomoles of urogen formed in 4 hours at 37°C.

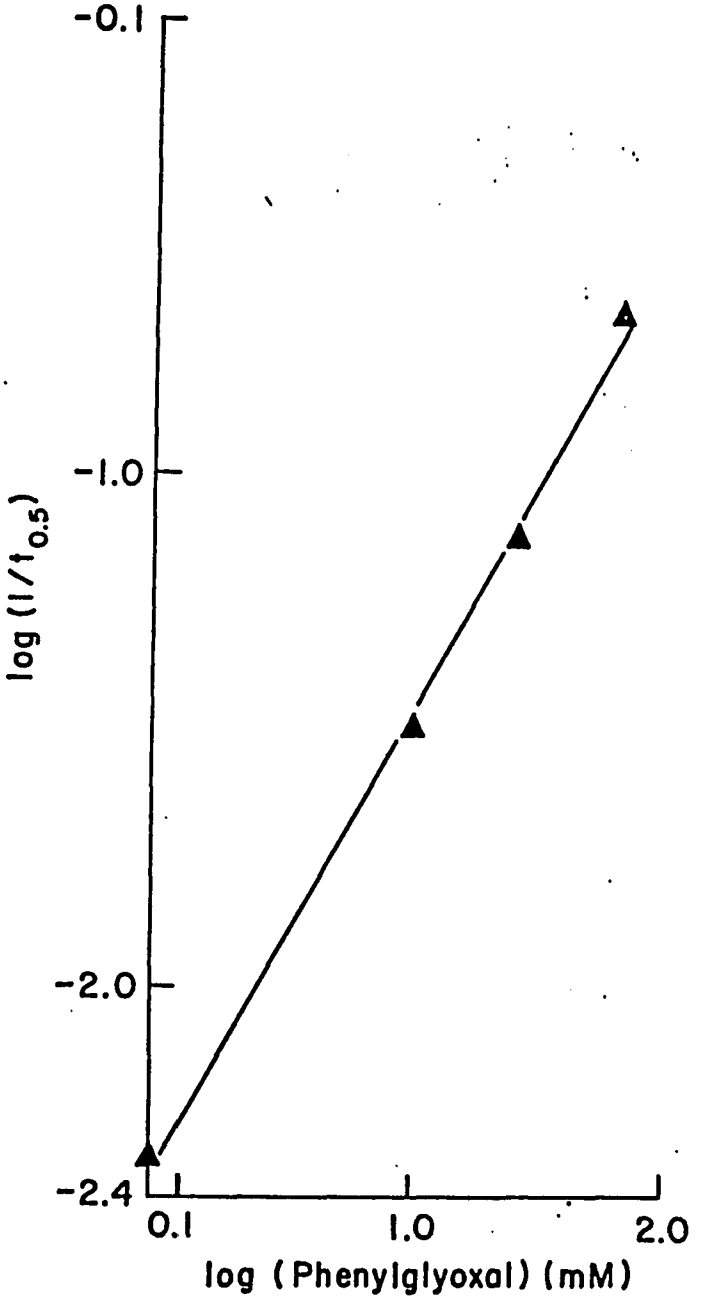

-0.1
-1.0
-2.0
-2.4
log (1/t0.5)
0.1
1.0
2.0
log (Phenylglyoxal) (mM)

Figure 36. The activity is expressed as nanomoles of urogen formed at 37°C in 4 hours. A K_I value of 45.9 ± 4.0 mM and a T of 4.20 minutes were obtained. For the urogen formation after 6 hours at 37°C; a K_I of 51.3 ± 0.1 mM and a T of 4.28 minutes were obtained. The six hour K_I and T values were obtained from equation (2.3). PGO seems to show the same affinity for enzyme as does BD. The rate of maximal inactivation of urogen I synthase by BD at 25°C is one half the rate of maximal inactivation of urogen I synthase by PGO at 37°C, as can be seen in Table 9.

As was mentioned in the introduction, Chung and Fonda (66, 67, 124) presented evidence that bicarbonate buffer might be a superior buffer for the modification of enzymes by PGO. The reaction is first order in bicarbonate buffer and the rate of modification of lysine or cysteine is low or zero in this buffer. A comparison of the free concentration of PGO, as measured by its optical density at 250 nm was undertaken for PGO in 50 mM borate pH 8.2, in 0.2 M bicarbonate -0.1 M carbonate pH 8.2 and H_2O. This data is shown in Figure 37. It was assumed that PGO in H_2O existed as 100% uncomplexed PGO monohydrate. The OD at 250 nm for a given concentration of PGO in borate is greatly reduced as compared to H_2O, with no change in λ_{max}. This is presumably due to a PGO borate complex which reduces the free concentration of PGO. In 50 mM borate buffer, at PGO concentrations ranging from 0.16 mM to 0.62 mM PGO, 95% of the PGO appears to exist as a complex with borate. Relative to water the OD at 250 nm at a given concentration of PGO in bicarbonate buffer is

Figure 36 : A plot of the reciprocals of the first order rate constants for inactivation versus the reciprocal of the PGO concentrations. Urogen I synthase was modified by PGO in 50 mM borate pH 8.2 at 37°C. Activity is expressed as nanomoles of urogen formed in 4 hours at 37°C.

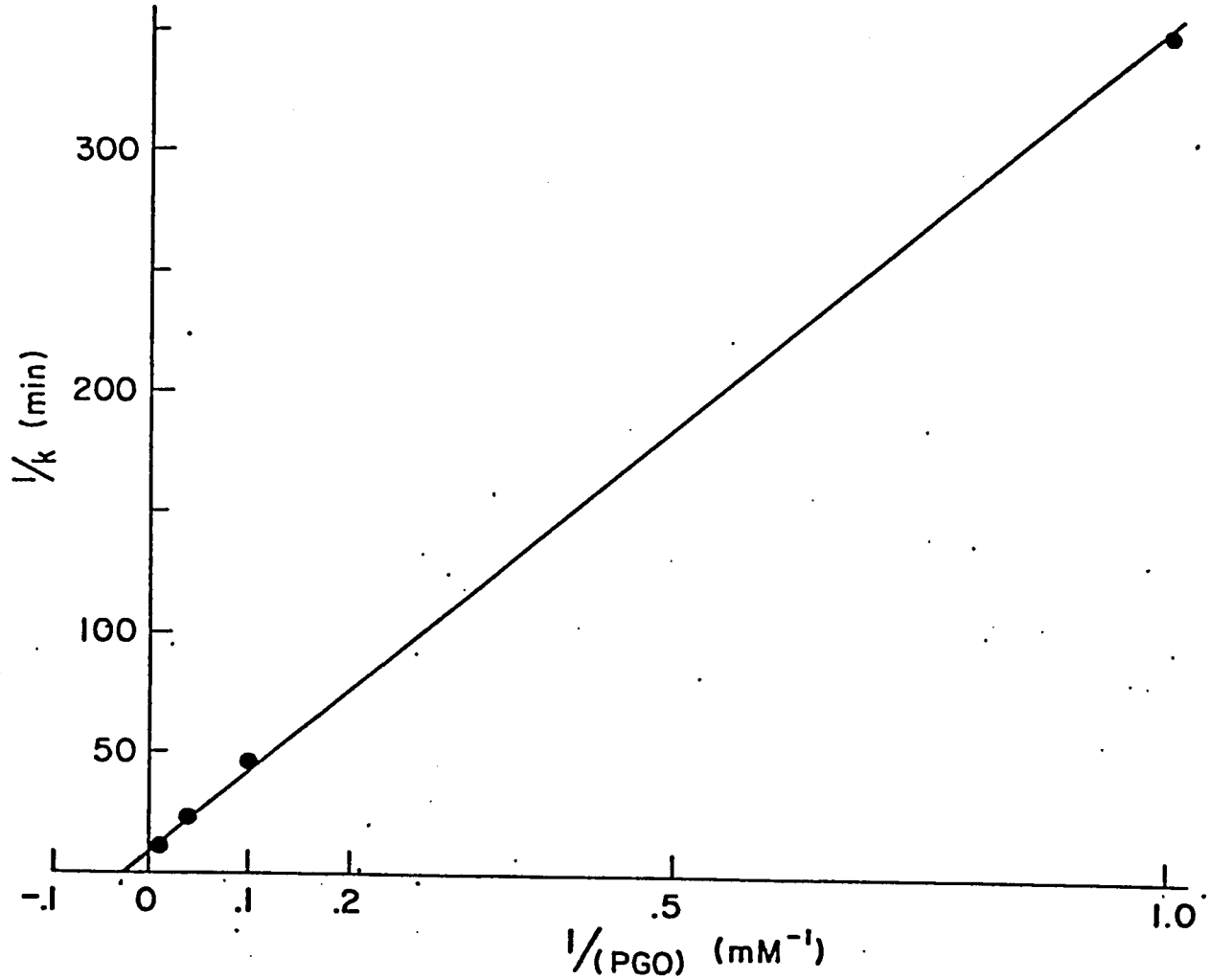

300
200
100
50
1/k (min)
-.1
0
.1
.2
.5
1.0
1/(PGO) (mM⁻¹)

Table 9: Summary of BD and PGO Inactivation of Urogen I Synthase in Borate Buffer

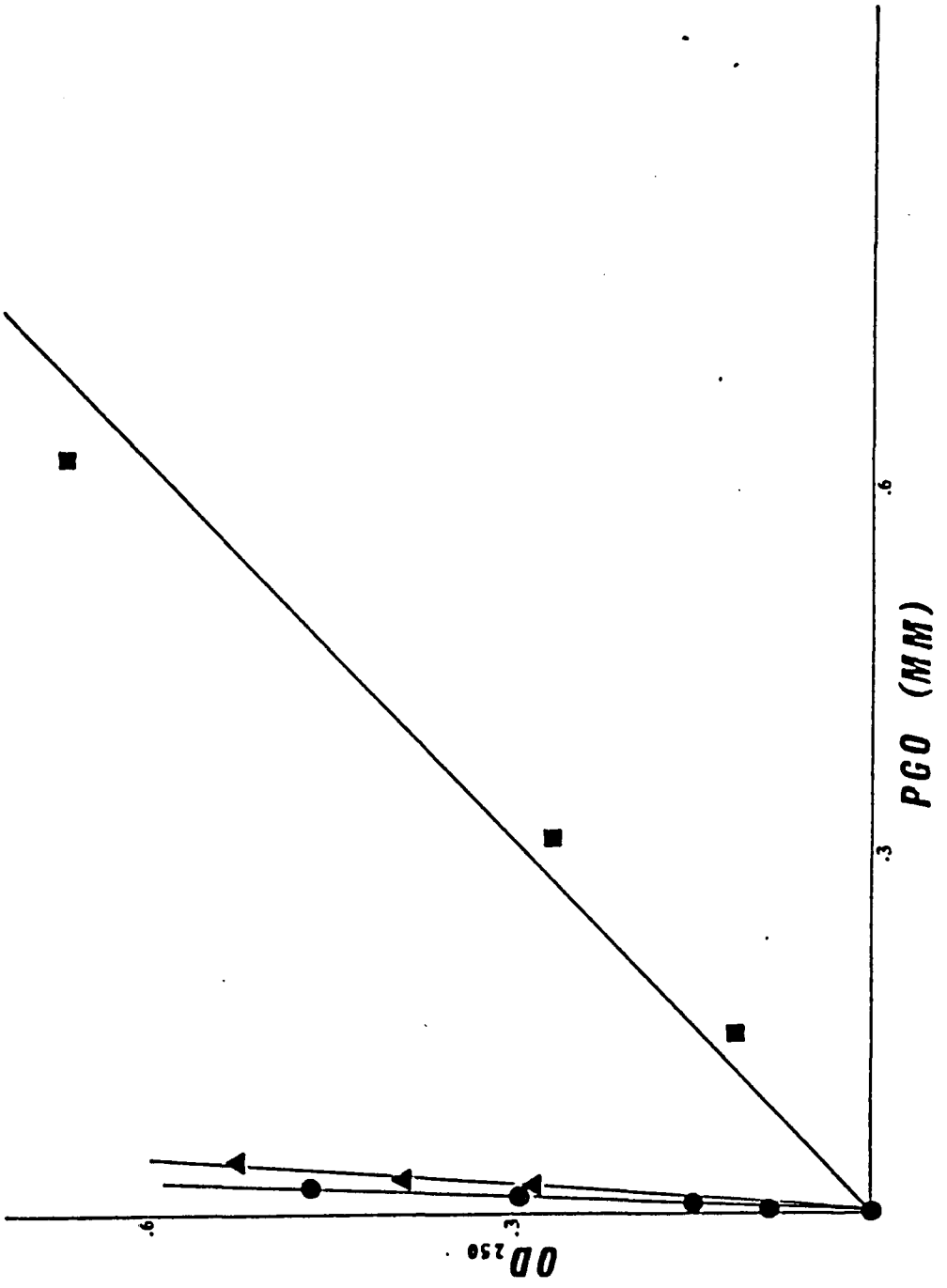
BD in Borate	BD (mM)	$t_{0.5}$ (min.)	K $(min.)^{-1}$	Reaction Order	K_I (mM)	T (min.)	$k_2(M)^{-1}$ (
Activity measured as PBG utilization after 4 hours	10	72	0.0097				
	54	21	0.033	0.89	50.0 ± 0.1	8.50	0.800
	102	8.3	0.083				
Urogen formation after 4 hours	10	42	0.017				
	54	14	0.049	0.64	49.0 ± 1.3	6.98	0.607
	102	9.5	0.073				
PGO in Borate							
Activity measured as Urogen formation after 4 hours	1	244	0.0028	0.89	45.9 ± 4.0	4.20	1.97
	10	32	0.0220				
	25	16	0.0430				
	50	6.9	0.100				
Urogen formation after 6 hours	1	278	0.0025				
	10	29	0.0240				
	25	15	0.0460	0.95	51.3 ± 0.1	4.28	2.30
	50	6.1	0.1140				

Figure 37: A plot of the optical density at 250 nm of the concentration of PGO (mM) for three different buffer systems:

■, 50 mM borate pH 8.2;

▲, 0.2 M bicarbonate -0.1 M carbonate pH 8.2; and

●, water.


PGO (MM)
.6
.3
OD_{250}
.6
.3

only slightly reduced, and again there is no change in λ_{max}. In bicarbonate 22% of the PGO appears to be complexed relative to water. These structures are presented in Figure 38. Chung and Fonda (66, 67) also pointed out that bicarbonate lowers the pk_a of the guanidinium group of N-acetylarginine. It was concluded by the authors that bicarbonate buffer catalyzes a nucleophilic attack by the quanidinium group on the carbonyl groups of PGO.

Urogen I synthase was then incubated with various concentrations of PGO in bicarbonate buffer for a series of time intervals. Aliquots were removed at various times and added to about 10 mg of sodium borohydride, pH adjusted to 8.2 as necessary and assayed for activity. A blank was treated in the same way without the addition of PGO. The activity of the enzyme measured by urogen formation was severely and rapidly reduced by PGO in bicarbonate buffer as shown in Figure 39.

For data in the first hour of reaction when log $1 / t_{0.5}$ was plotted against log PGO concentration (Figure 40) a slope of 1.15 was found for urogen formation in 4 hours at 37°C and a slope of 1.17 was found for urogen formation in 6 hours at 37°C. These results suggest that the order of the inactivation reaction with respect to PGO is one.

Again these results differ from Takahashi's (65) which indicates that two molecules of PGO would be expected to bind to one arginine molecule. However, in his study Takahashi used

Figure 38: Complex formation of PGO with borate and carbonate.

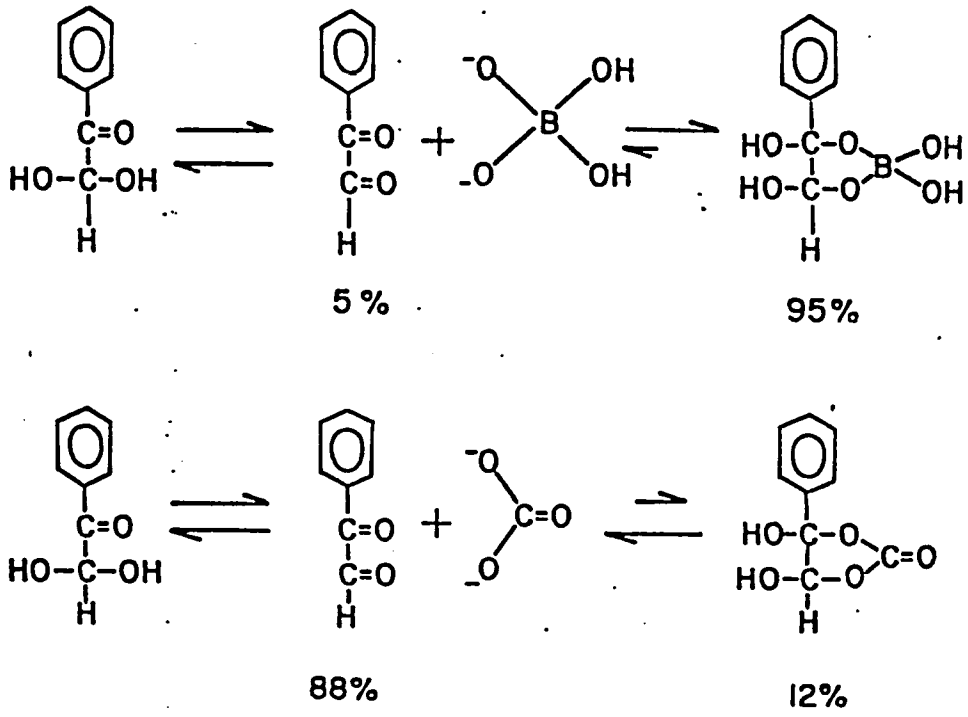

5 %
95%
88%
12%

Figure 39: Inactivation of urogen I synthase by PGO in 0.2 M bicarbonate - 0.1 M carbonate pH 8.2 at 37°C. Activity is expressed as nanomoles of urogen formed in 4 hours at 37°C. The incubations included PGO in the following concentrations:

■, 0 mM;

▲, 1.3 mM;

◐, 1.9 mM:

●, 4mM

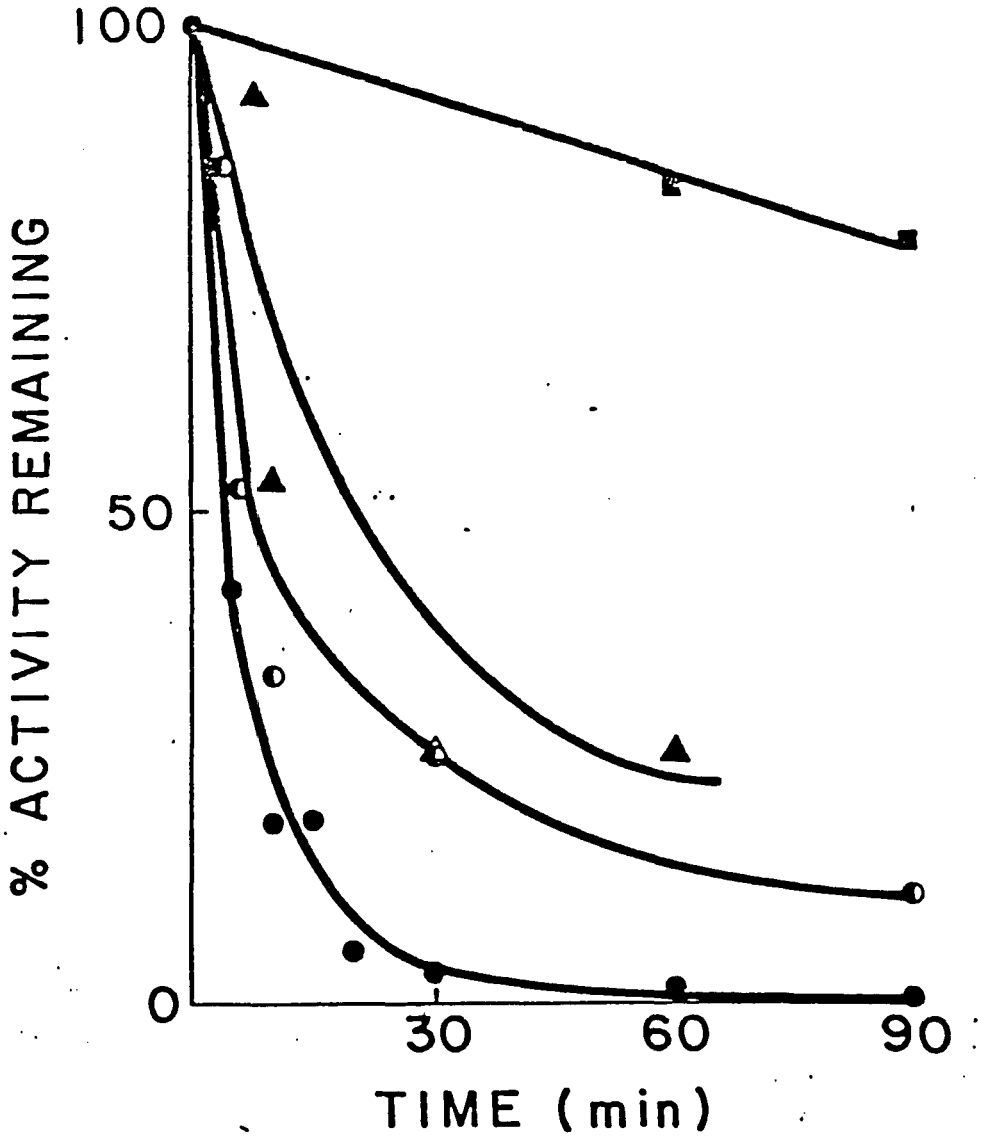
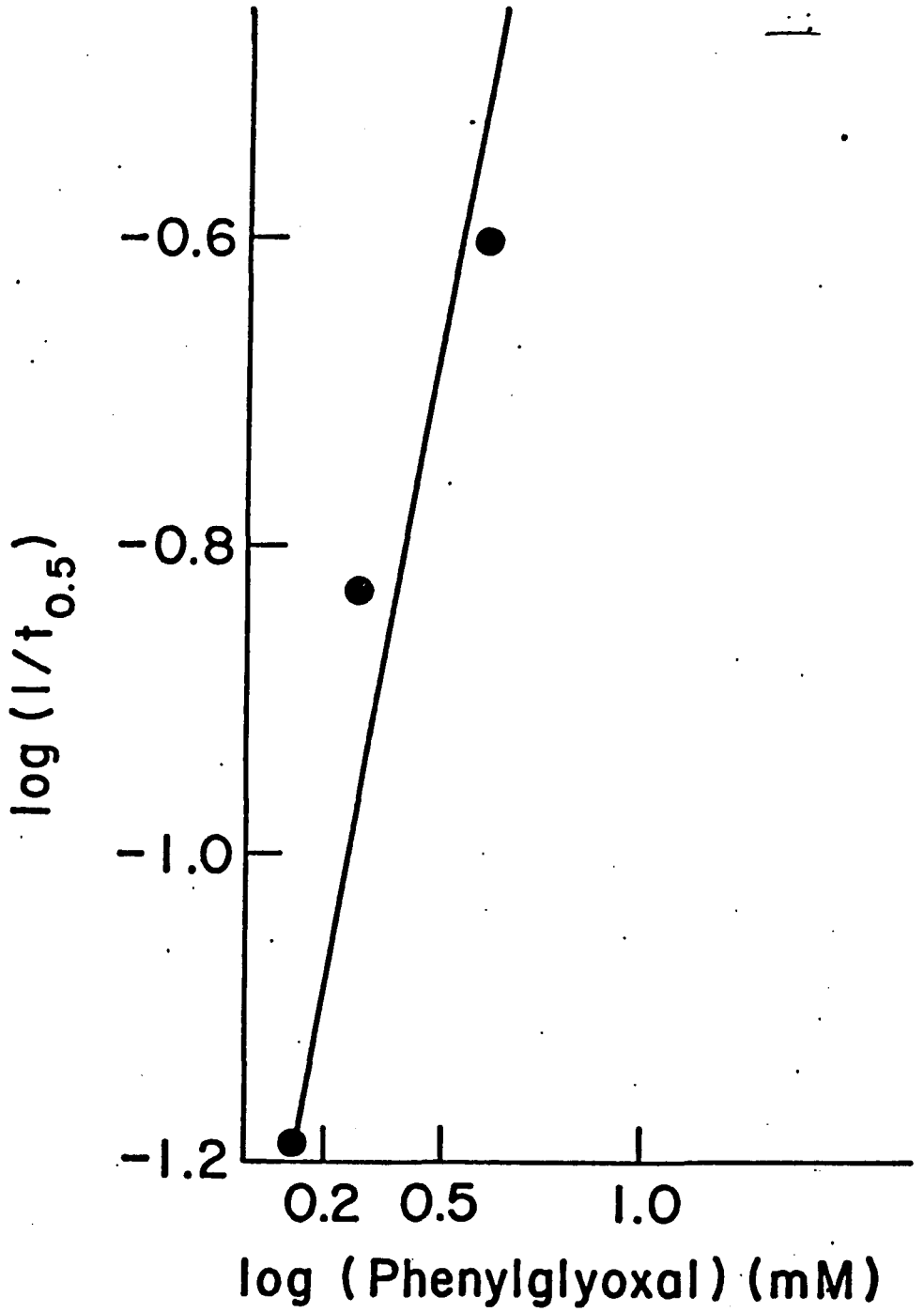

100
50
0
% ACTIVITY REMAINING
30
60
90
TIME (min)

Figure 40 : Logarithmic plot of urogen I synthase activity after treatment with various concentrations of PGO in 0.2 M bicarbonate - 0.1 M carbonate buffer pH 8.2 at 37°C. Activity is expressed as nanomoles of urogen formed in 4 hours at 37°C.


-0.6
-0.8
-1.0
-1.2
log (1/t0.5)
0.2
0.5
1.0
log (Phenylglyoxal) (mM)

a high concentration of PGO. At low concentrations of PGO 1.3 - 4 mM is used in this study, it may be that the first PGO molecule inactivates the enzyme by forming a glyoxaline adduct with the quanidino group of an arginyl residue as Takahashi proposed. However, at the low PGO concentration used the reaction of a second molecule to form the proposed cyclic acetal (shown in Figure 15) proceeds only to a small degree.

Borders and Riordan (54) found this to be true in the modification of creatine kinase with 0.20 mM PGO in 0.1 M bicarbonate pH 8.2. Loss of activity coincided with the incorporation of 1.2 [^{14}C] - PGO molecules, and the modification of 0.9 arginine residues per subunit as judged by amino acid analysis.

Philips and coworkers (77) also deduced a 1:1 stoichiometry for the PGO-arginine complex. They modified 3-phosphoglycerate kinase with from 0.5 mM PGO - 4.0 mM PGO in Veronal buffer pH 7.5. A plot of log ($1 / t_{0.5}$) versus log concentration of PGO resulted in a straight line with a slope of 1.05 indicating that the order of the reaction with respect to PGO is one. Incorporation of [^{14}C] PGO showed that 2.3 molecules of PGO are incorporated per enzyme molecule. It might be the result of a successive reaction of first one and subsequently a second molecule of PGO with a singly arginyl residue. Alternatively, the latter result might suggest that two arginyl residues which are both essential for activity are each modified by one molecule of PGO. This is confirmed by amino acid analyses which show that

the number of arginyl residues lost correspond to the number of moles of PGO incorporated.

A plot of the first order rate constant for inactivation versus the concentration of PGO where the activity is expressed as nanomoles of urogen found in 4 hours at 37°C gave a second order rate constant of 43.8 $(M)^{-1}$ $(min.)^{-1}$. The data where the activity is expressed as nanomoles of urogen formed in 6 hours at 37°C gave a second order rate constant of 44.1 $(M)^{-1}$ $(min.)^{-1}$.

A graph of the reciprocals of the first order rate constants versus the reciprocals of the PGO concentrations is presented in Figure 41. The activity was expressed as the nanomoles of urogen formed at 37°C in 6 hours. A K_I value of 36 $\pm$ 2.1 mM and a T of 0.42 minutes were obtained. For the urogen formation after 4 hours a K_I value of 26 $\pm$ 2.8 mM and a T of 0.87 minutes were obtained.

A summary of inhibition study results are presented in Table 10.

Based on the second order rate constants the rate of the inactivation process by PGO has increased some 21 fold in bicarbonate buffer relative to borate buffer, as shown in Table 10. One reason for the increased rate is because the effective free concentration of PGO is greater in bicarbonate buffer as compared to borate.

Figure 41: A plot of the reciprocals of the first order rate constant versus the reciprocals of the PGO concentration. Urogen I synthase is modified by PGO in 0.2 M bicarbonate - 0.1 M carbonate buffer pH 8.2 a5 37°C. Activity was expressed as nanomoles of urogen formed in 6 hours at 37°C.

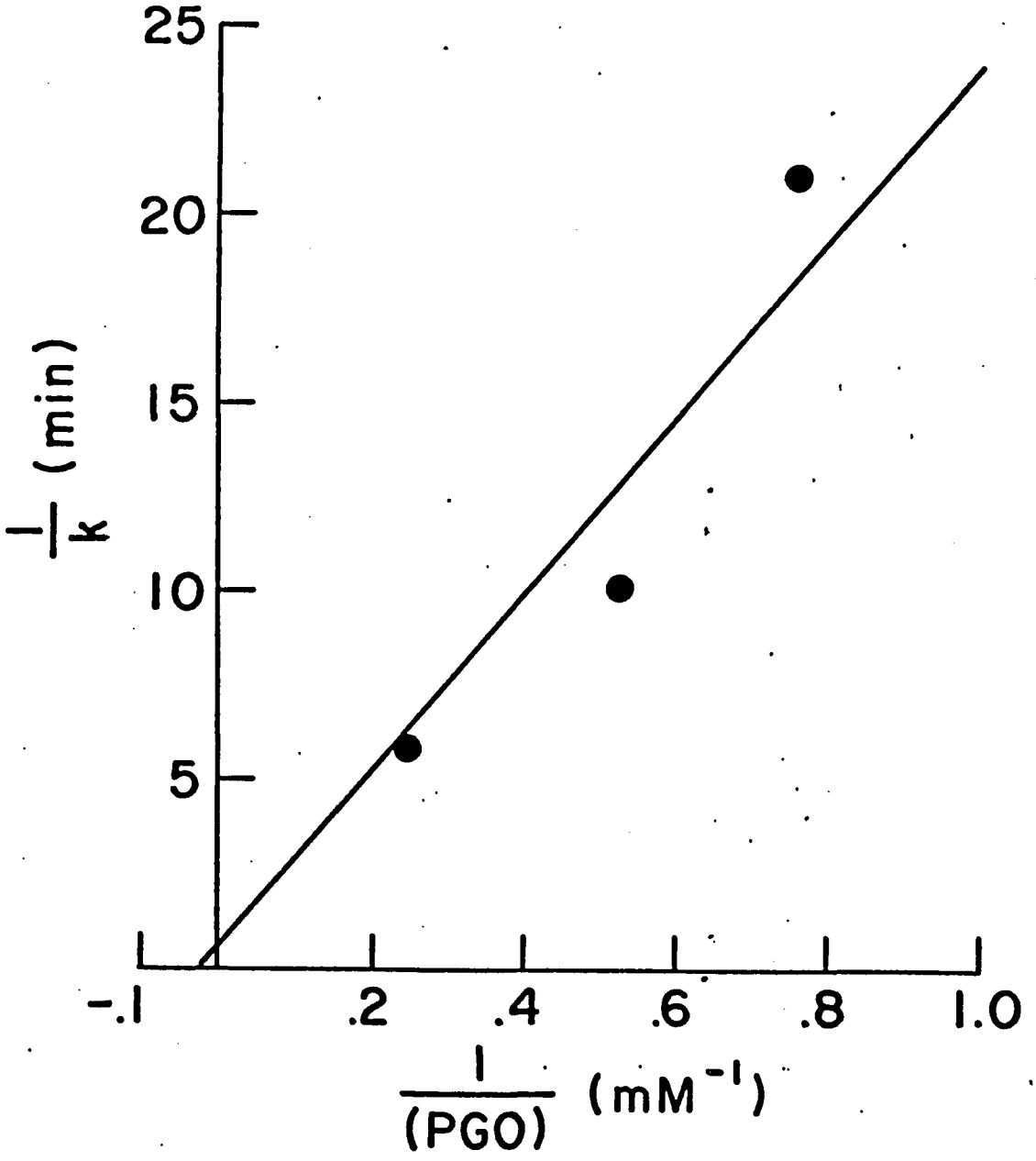

25
20
15
10
5
$-\frac{1}{k}$ (min)
-.1
.2
.4
.6
.8
1.0
$\frac{1}{(PGO)}$ (mM^{-1})

Table 10: Summary of BB and PGO Inactivation of Urogen I Synthase in Borate and Bicarbonate Buffers

BD in Borate	BD (mM)	$t_{0.5}$ (min.)	k $(min.)^{-1}$	Reaction order	K_I (mM)	T (min.)	k_2 $(m)^{-1}$
activity measured as;	10	72	0.0097				
PBG utilization	54	21	0.033	0.89	50.0 ± 0.1	8.50	0.800
after 4 hours	102	8.3	0.083				
urogen formation	10	42	0.017				
after 4 hours	54	14	0.049	0.64	49.0 ± 1.3	6.98	0.607
	102	9.5	0.073				

PGO in Borate	PGO (mM)	$t_{0.5}$(min.)	k $(min.)^{-1}$	Reaction order	K_I (mM)	T (min.)	$k_2(m)^{-1}$
activity measured as;							
urogen formation	1	244	0.0028				
after 4 hours	10	32	0.0220	0.89	45.9 ± 4.0	4.20	1.97
	50	16	0.0430				
		6.9	0.1000				
urogen formation	1	278	0.0025				
after 6 hours	10	29	0.0240	0.95	51.3 ± 4.28	4.28	2.32
	25	15	0.0460				
	50	6.1	0.1140				

Table 10 (continued): Summary of BB and PGO Inactivation of Urogen I Synthase in Borate and Bicarbonate Buffers

PGO in Bicarbonate Buffer	PGO (mM)	$t_{0.5}$ (min.)	k $(min.)^{-1}$	Reaction order	K_I (mM)	T (min.)	$k_2(m)^{-1}$
activity measured as;	1.32	16	0.045				
urogen formation	1.90	6.8	0.101	1.15	26.0 ± 2.8	0.87	43.8
after 4 hours	4.00	4.0	0.172				
urogen formation	1.32	16	0.045				
after 6 hours	1.90	7.8	0.089	1.17	36.0 ± 2.1	0.42	44.1
	4.00	4.1	0.170				

In addition Chung and Fonda (65) indicated that bicarbonate carbonate buffer forms a complex with N-acetylarginine based on gel chromatography and kinetic studies. Bicarbonate also lowers the pk_a of the guanidinium group of N-acetylarginine. They concluded that bicarbonate catalyzes a nucleophilic attack by the guanidinium group on the carbonyl groups of PGO. These effects may account for the rate enhancement which was observed.

Having evidence (to be shown later) that an essential lysyl residue is also at the active site of urogen I synthase, it was considered that reducing the excess PGO with borohydride might also reduce a Schiff base formed between lysine and a carbonyl group in the PGO molecule. In order to test this hypothesis, urogen I synthase was modified with various concentrations of PGO in bicarbonate buffer, the excess PGO was removed by dialysis for 15 hours at 4°C against 200 mM bicarbonate buffer.

The activity of the enzyme, measured as urogen formation in 4 hours at 37°C, was severely and rapidly reduced. One would expect a Schiff base formed between PGO and lysine to be reversible by dialysis for 15 hours at 4°C.

Kantrowitz and Lipscomb (71) tested the reversibility of PGO by dialysis for the enzyme aspartate transcarbamylase. The enzyme was modified with 1.0 mM [^{14}C] - PGO at 25°C in 125 mM bicarbonate buffer, pH 8.3. Excess reagent was removed, after 60 minutes, by gel filtration on Sephadex G-25. The sample was divided into 0.5 ml portions placed into dialysis bags and dialyzed against 50 mM bicarbonate buffer, pH 8.2 at 4°C. Bags were removed from dialysis, and the protein concentration and radioactivity were determined at various times. After 20 hours at 4°C 10% of the modified arginine residues were restored. After 200 hours at 4°C 45% of the modified arginine residues were restored. The activity of the enzyme at the beginning of the reversibility was 20% of the native enzyme and changed very little over the whole course of dialysis. It seems then that in 15 hours of dialysis under these conditions less than 10% of the arginine - PGO derivative of urogen I synthase would break down.

A comparison of the data for the PGO inactivation of urogen I synthase in bicarbonate buffer where excess PGO is removed by borohydride reduction or by dialysis is presented in Table 11.

Comparing the four-hour urogen formation data, one sees a decrease in the reaction order by 38% relative to the reaction order when borohydride reduction was used. This indicates less PGO incorporation when dialysis is used to remove excess PGO. A 21% reduction in the speed of the reaction relative to the

Table 11: Comparison of PGO Inactivation of Urogen I Synthase in Bicarbonate Buffer where Excess PGO is Removed by Borohydride Reduction or by Dialysis

PGO in bicarbonate buffer with borohydride reduction	PGO (mM)	$t_{0.5}$ (min.)	k $(min.)^{-1}$	Reaction order	K_I (mM)	T (min.)	$k_2(M)^{-1}$ (
activity is measured as, urogen formation after 4 hours	1.32	16	0.045				
	1.90	6.8	0.101	1.15	26.0 ± 2.8	0.87	43.8
	4.00	4.0	0.172				
urogen formation after 6 hours	1.32	16	0.045				
	1.90	7.8	0.089	1.17	36.0 ± 2.1	0.42	44.1
	4.00	4.1	0.170				

PGO in bicarbonate buffer with dialysis	PGO (mM)	$t_{0.5}$ (min.)	k $(min.)^{-1}$	Reaction order	K_I (mM)	T (min.)	$k_2(M)^{-1}$ (
activity is measured as, urogen formation after 4 hours	2.90	4.36	0.159				
	3.20	4.19	0.165	0.71	10.0 ± 2.7	1.05	34.8
	4.00	3.66	0.189				

reaction in bicarbonate followed by borohydride is seen, as judged by the second order rate constants for the reaction. 10% of the decrease in rate can be accounted for by the reversibility of 10% of the arginine-PGO complex (71). This leaves 11% reduction in rate which may have been due to lysine modification by PGO with borohydride reduction. Since PGO reacted with PBG protection experiments were conducted with the substrate analogue, OPDC. Urogen I synthase was challenged with PGO (2.9 mM to 4.0 mM) in the presence and absence of various concentrations of OPDC (0.11 to 8.4 mM) in bicarbonate buffer. The excess PGO and OPDC was removed by dialysis for 48 hours at 4°C against 200 mM bicarbonate pH 8.2, instead of borohydride reduction. OPDC seemed to bind the enzyme tightly and was difficult to remove by dialysis, as judged by the positive Ehrlich test the enzyme plus OPDC gave. However, the positive Ehrlich could have been due to tryptophan on the enzyme which could also give a positive Ehrlich test. The data are summarized in Table 12. A typical protection experiment is shown plotted on semilogarithmic form in Figure 43. From this data is is evident that OPDC protects the enzyme well from inactivation by PGO, and that the protection effect is concentration-dependent with respect to OPDC.

The dissociation constant of the urogen I synthase - OPDC complex was calculated from the data presented in Table 12, using equation (2.3)

$$K_{\text{inact.}} - k_{\text{min.}} = \frac{K_o - k_{\text{min.}}}{1 + \frac{[S]}{K_s}} \qquad (2.3)$$

Table 12: Protection by OPDC of Inactivation of Urogen I Synthase by PGO

PGO (mM)	$t_{0.5}$ (min.)	k $(min.)^{-1}$	OPDC (mM)	[OPDC] / K_I*
3.2	4.19	0.1654	0	-
3.2	9.10	0.0762	0.11	0.39
4.0	3.66	0.189	0	-
4.0	72.4	0.0096	0.84	3
2.9	4.63	0.149	0	-
2.9	90.9	0.0076	8.4	30

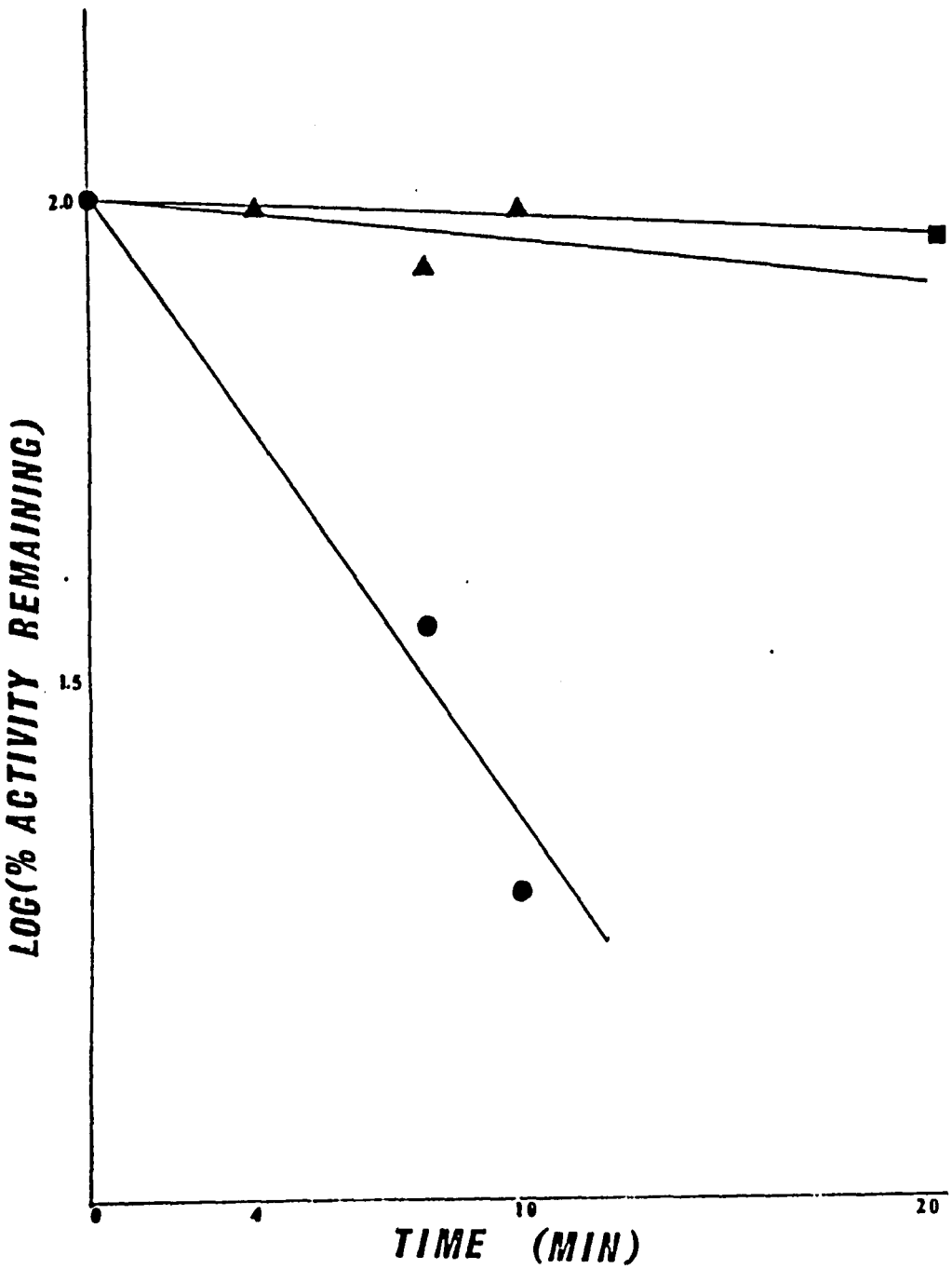
*K_I for OPDC is 0.28 mM (26).

Figure 42 : A graph of log % activity remaining versus time for protection by OPDC against inactivation of urogen I synthase by PGO in 0.2 M bicarbonate - 0.1 M carbonate buffer pH 8.2 at 37°C. Excess PGO and OPDC were removed by dialysis against the same buffer for 48 hours at 4°C.

■, 0 mM PGO and 0 mM OPDC;

▲, 2.9 mM PGO and 8.4 mM OPDC;

●, 2.9 mM PGO and 0 mM OPDC.


2.0
1.5
LOG(% ACTIVITY REMAINING)
0
4
10
20
TIME (MIN)

where; k inact. is the observed first order rate constant, k min. is the rate constant at saturating levels of protecting ligand, ko is the rate constant in the absence of ligands, [S] is the concentration of protecting ligand and K_S is the dissociation constant for the enzyme-ligand complex (57, 102).

From the 2.9 mM PGO data, presented in Table 12, a value for ko of 0.149 and a value of k min. of 0.0076 were obtained. From the 3.2 mM PGO data, of the same table a k inact. of 0.0762 at an OPDC concentration of 0.11 mM was obtained. Equation (2.3) assumes the concentration of PGO to be held constant. The variation in concentration of PGO from 2.9 to 3.2 introduces a 10% error into the measurement of the dissociation constant of the urogen I synthase - OPDC complex. The dissocation constant calculated from this data using equation 2.3 was 102 $\pm$ 10.2 μM in 0.2 M bicarbonate - 0.1 M carbonate buffer at pH 8.2 at 37°C, as compared to a value of 280 μM obtained by Carpenter and Scott (26) in 0.1 M Tris pH 8.1 at 37°C.

Formaldehyde inhibition:

Bogorad reported that formaldehyde accelerates the non-enzymatic synthesis of porphyrins from PBG although it appears to inhibit the enzymatic conversion of PBG to porphyrins (3).

Bogorad did his enzymatic assays in the presence of formaldehyde making it difficult to separate inhibition from nonenzymatic converstion of PBG to porphyrin. Bogorad and Marks (28) presented evidence that formaldehyde was neither a stoichiometric by-product nor a reactant in this process although it was readily incorporated into urogen in the non-enzymatic condensation of PBG.

Urogen I synthase was treated with 66 mM formaldehyde in 0.033 M phosphate buffer pH 8.2, for 30 minutes and reduced with borohydride. The enzyme was then dialysed to remove excess formaldehyde. The % inhibition of PBG was 65% and the % inhibition of urogen was 36%. To further investigate this phenomenon, urogen I synthase was treated with 96 mM formaldehyde in 0.033 M phosphate buffer pH 8.2, and reduced with borohydride. Aliquots were dialyzed overnight at 5°, to remove the excess formaldehyde, and assayed the following day. As can be seen from Figure 43, the urogen-forming ability of the enzyme was markedly reduced. The PBG consuming ability of the enzyme, however, was not as severely decreased as shown in Figure 44. A $t_{0.5}$ of 105 minutes was calculated for the PBG data and a $t_{0.5}$ of 3.6 minutes was calculated from the urogen data.

Figure 43 : Formaldehyde inhibition (96 mM) in 0.033 M phosphate buffer pH 8.2 at 37°C. Samples were incubated with formaldehyde for the indicated times, reduced with borohydride, dialyzed overnight at 4°C against 0.033 M phosphate buffer pH 8.2, and assayed the following day. Controls were treated in the same way, for the indicated times, except that formaldehyde was omitted from the incubation mixture. Activity was expressed as nanomoles of urogen formed in 4 hours at 32°C.

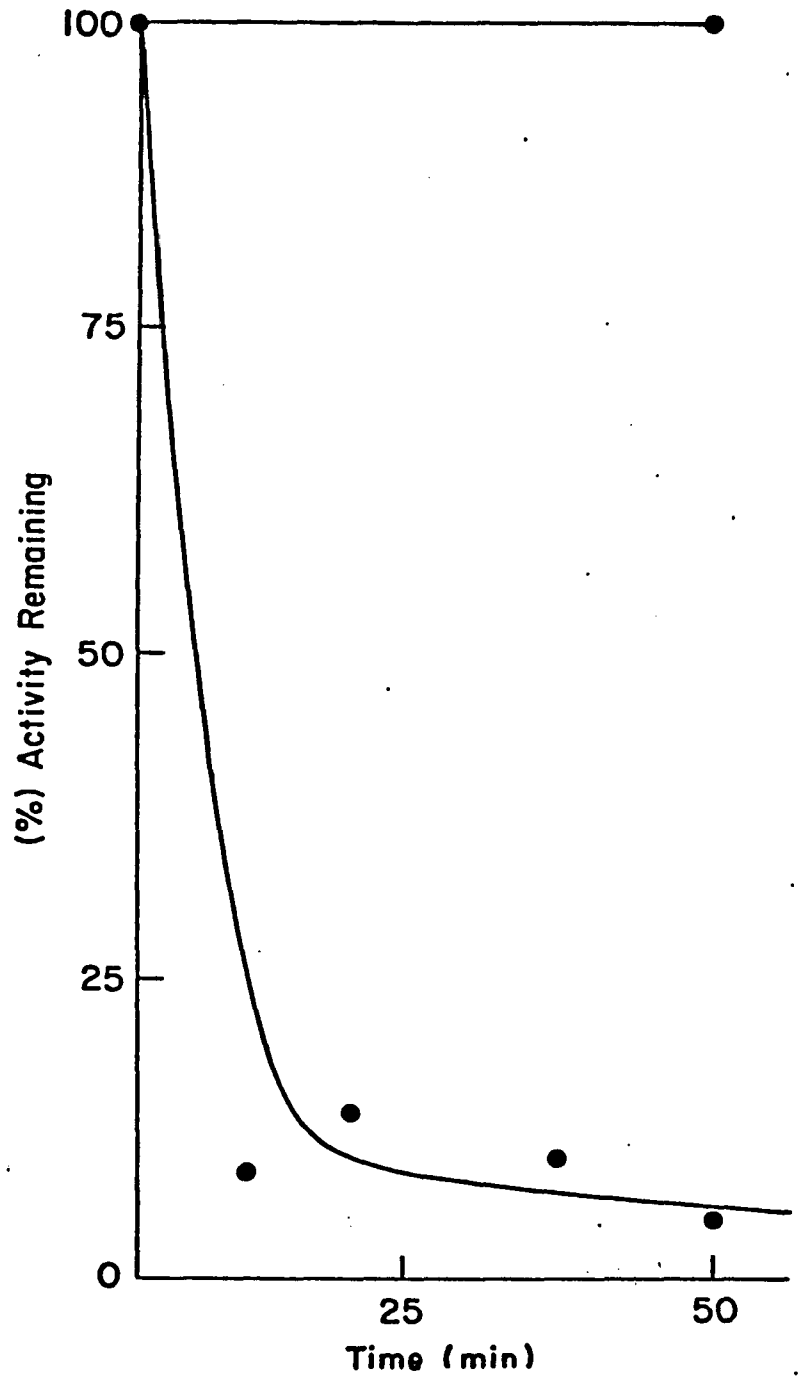
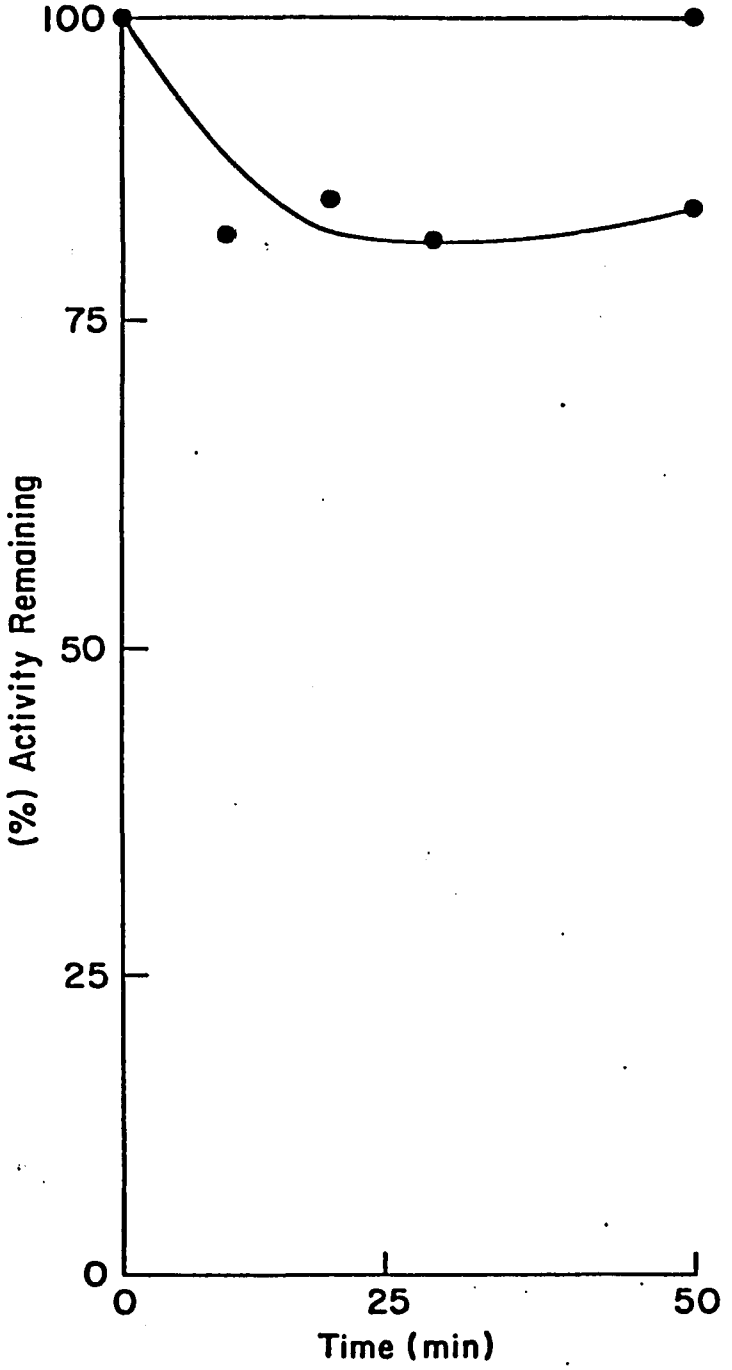

100
75
50
25
0
(%) Activity Remaining
25
50
Time (min)

Figure 44: Formaldehyde inhibition (96 mM) in 0.033 M phosphate buffer pH 8.2 at 37°C. Samples were incubated with formaldehyde for the indicated times, reduced with borohydride, dialyzed overnight at 4°C against 0.033 M phosphate buffer pH 8.2, and assayed the following day. Controls were treated in the same way, for the indicated times, except that formaldehyde was omitted from the incubation mixture. Activity was expressed as nanomoles of PBG consumed in 4 hours at 37°C.


100
75
50
25
0
(%) Activity Remaining
0
25
50
Time (min)

Pyridoxal-5'-phosphate inhibition:

Urogen I synthase was challenged by another lysine-specific modifier, pyridoxal-5'-phosphate (PLP). The enzyme was modified by 9.0 mM PLP at 37°C in 0.075 M phosphate buffer pH 8.2 and assayed or added to 10 mg or borohydride and then assayed. The data presented in Table 13, for two duplicate experiments, shows that the enzyme without borohydride reduction has on the average 82% activity remaining. The enzyme with borohydride reduction has only 25% activity remaining. This behavior characteristic of PLP modification is due to reduction of the Schiff base formed between PLP and lysine as shown in Figure 17.

Urogen I synthase was modified by various concentrations of PLP in phosphate at pH 8.2 at 37°C. Aliquots were removed at various times and added to 10 mg of borohydride, and assayed. As can be seen from Figure 45, the enzyme was rapidly inhibited by PLP.

In order to determine whether the substrate can protect urogen I synthase from inactivation by PLP, the enzyme was mixed with PBG (80 μM) before the addition of PLP (10 mM). The substrate showed a pronounced ability to protect urogen I synthase from inactivation, as can be seen in Figure 46.

This time and concentration-dependent loss of activity on incubation with PLP and the fact that PBG protects against

Table 13: Modification of Urogen I Synthase by PLP in the Presence and Absence of Sodium Borohydride

	% Activity Remaining*	
Enzyme	100	100
Enzyme + 9.0 mM PLP	70	94
Enzyme + 9.0 mM PLP + 5 MM $NaBH_4$	25	24

*Activity was urogen formed in 3.5 hrs. at 37°C.

Figure 45: The inactivation of urogen I synthase by PLP in 0.075 M phosphate buffer pH 8.2 at 37°C. Aliquots (2.75 ml) were removed at various times and added to 10 mg quantities of borohydride. Activity is expressed as urogen formation after 4 hours at 37°C. The concentrations of PLP employed were as follows:

●, 0 mM;

□, 5 mM;

Δ, 10 mM;

O, 25 mM.

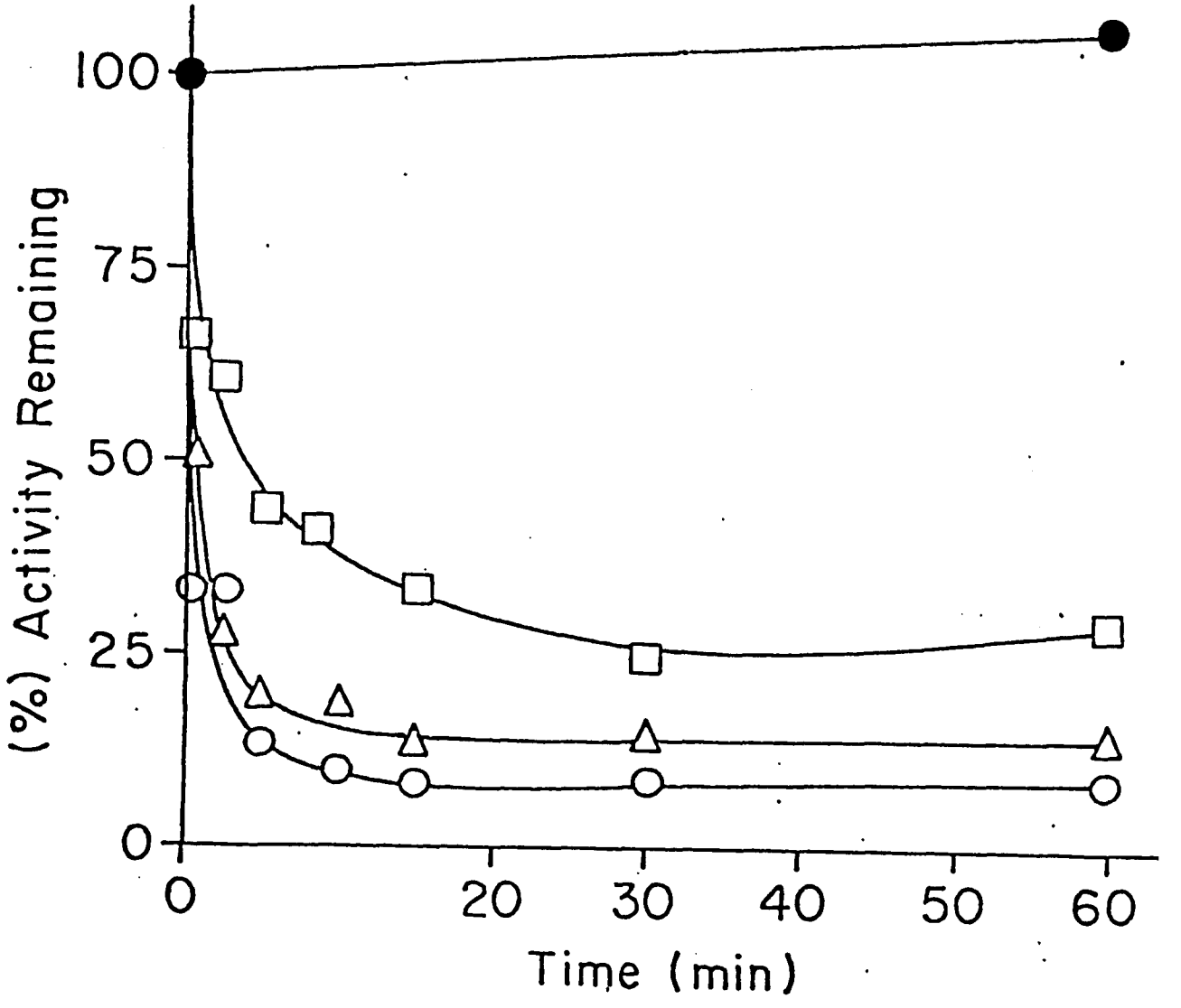
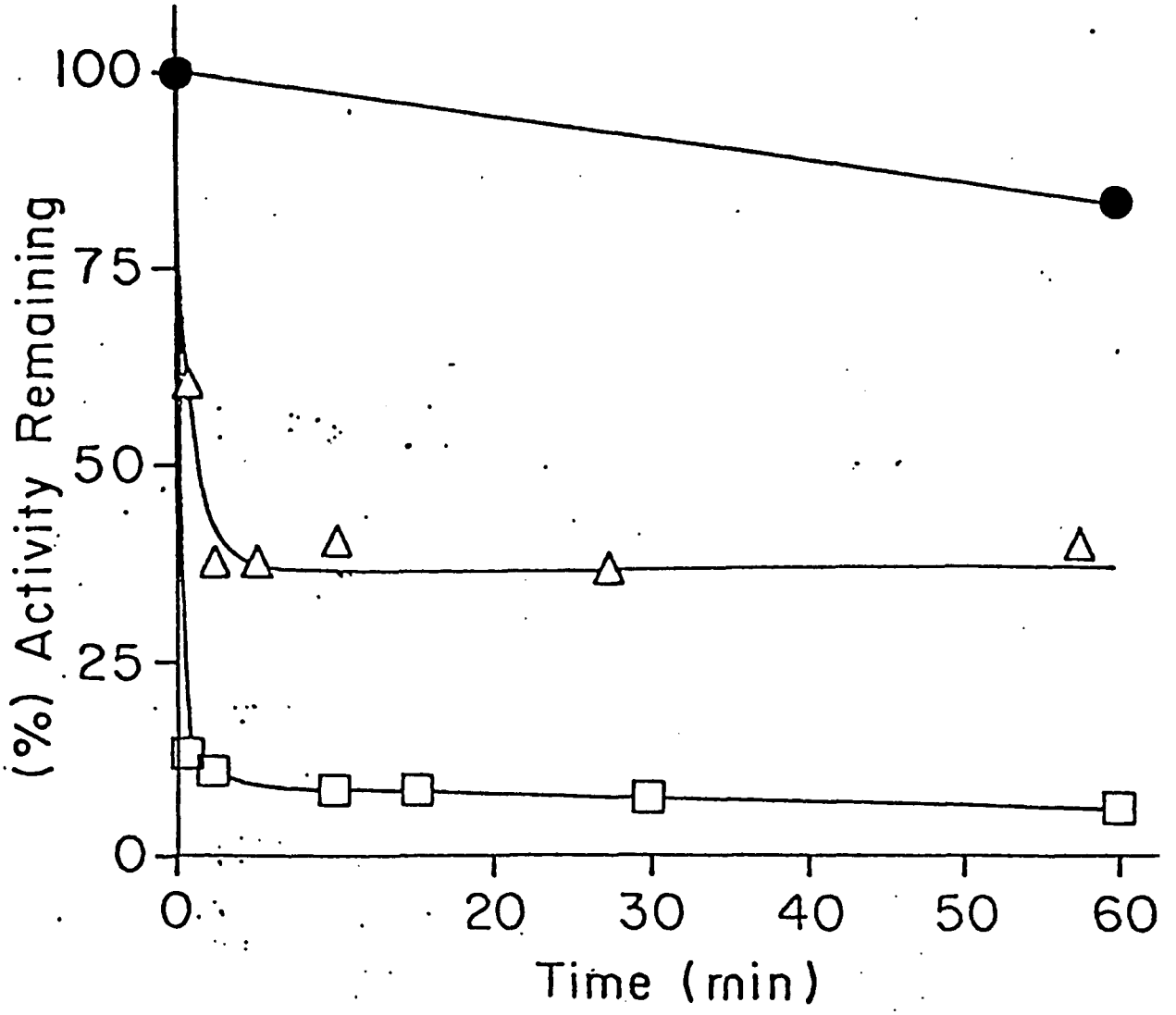

100
75
50
25
0
(%) Activity Remaining
0
20
30
40
50
60
Time (min)

Figure 46: Protection of PLP inactivation by substrate PBG (80 μM) in 0.075 M phosphate buffer pH 8.2 at 37°C. Aliquots (2.75 ml) were removed at various times and added to tubes containing 10 mg of borohydride. The samples were then dialyzed against 0.075 M phosphate buffer pH 8.2 at 4°C for 24 hours and then assayed. Activity is expressed as urogen formed after 4 hours at 37°C. The concentrations of PLP and PBG were as follows:

●, 0 mM PLP and 0 μM PBG;

Δ, 10 mM PLP and 80 μM PBG;

□, 10 mM PLP and 0 μM PBG.


100
75
50
25
0
(%) Activity Remaining
0
20
30
40
50
60
Time (min)

this inactivation suggests that an essential lysine residue is at the active site of urogen I synthase.

A reaction order of 0.79 was calculated from a plot of log 1 / $t_{0.5}$ as log PLP concentration. This indicates that one PLP molecule reacts with one lysine residue forming an inactive enzyme-PLP complex.

DISCUSSION

The two different molecular weight forms of the enzyme urogen I synthase of roughly 30,000 and 40,000 daltons may represent two enzymes, one of which is removable by either reprecipitation by ammonium sulfate or by agarose chromatography.

Inhibition by BD and PGO and protection against inhibition by substrate PBG and the competitive inhibitor OPDC suggest that an active site arginine residue of urogen I synthase is essential for catalysis. Reaction orders in the range of 0.64 to 0.95 for modification in borate buffer indicate that one arginine residue in the active site binds one molecule of either BD or PGO in the rate determining step. The BD and PGO complexes are stabilized by borate buffer.

When PGO inactivation was carried out in bicarbonate buffer reaction orders of 1.15 to 1.17 were obtained. This was again indicative of a 1:1 stoichiometry between PGO and arginine. Takahashi's (65) study, however, shows that two molecules of PGO bind to one arginine residue. In this study a high concentration of PGO was used. At low concentrations Borders and Riordan (54) and Philips and coworkers (97) found that only one PGO molecule was incorporated per arginine.

Alternatively, steric restriction in the reaction of the second PGO molecule by the localized environment of the specific arginine residue of urogen I synthase could account for the observed 1:1 stoichiometry. The adduct formed after the reaction of the first PGO molecule with arginine may by stabilized by reaction with another group at the active site, perhaps the nearby lysine residue.

In addition, first one PGO molecule might be incorporated in the rate-determining step and that is reflected in n, the order of the reaction, even if another PGO molecule is incorporated in a faster step.

BD modified enzyme had the same K_m as unmodified enzyme, however, the V_{max} was 50% decreased. This implies that modification does not occur at an arginine outside of the PBG binding domain and exert its inhibitory effect through a conformational change at the active site.

Binding of BD in borate or PGO in borate to this arginine residue as measured by the K_I is 50 mM.

The rate of the modification reaction is fastest for PGO in bicarbonate buffer as judged from the second order rate constant for the overall reaction.

Inhibition by formaldehyde and PLP and the lifting of PLP incubation by PBG suggest that there is also an essential lysyl

residue at the active site of urogen I synthase.

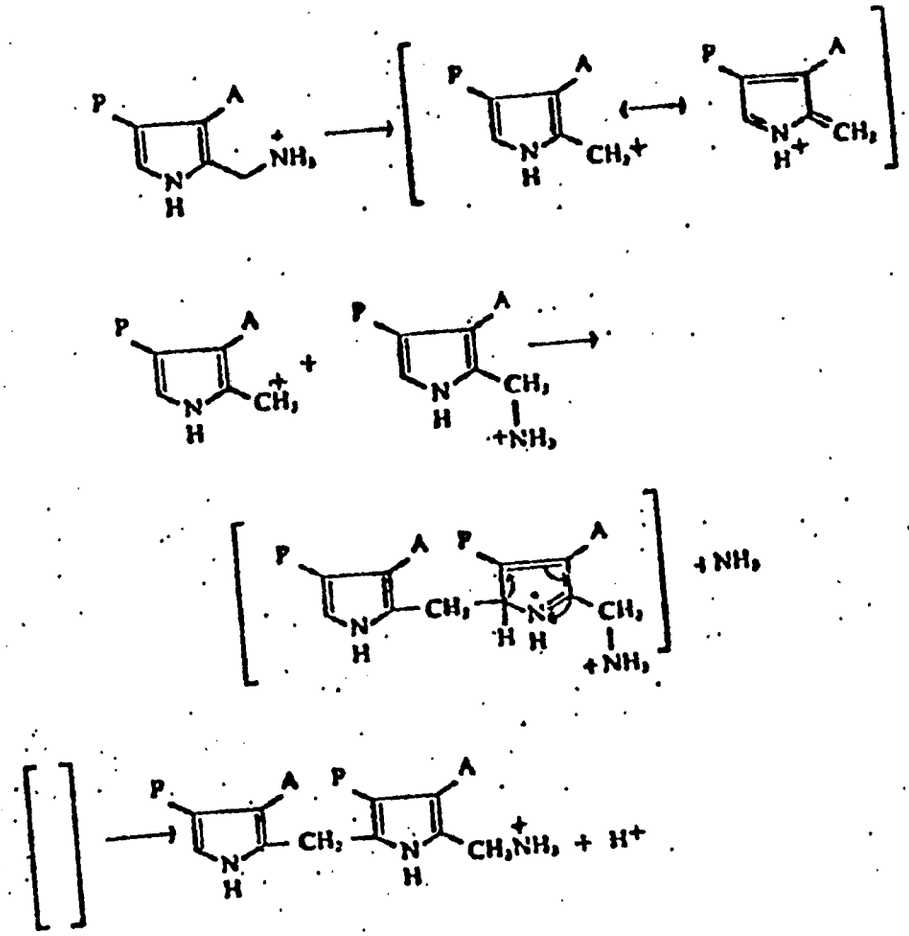
A reaction order of 0.79 indicates that one molecule of PLP combines with one lysyl residue in the rate determining step.

The rapid inhibition of urogen I synthase by PLP at low concentrations, could be due to the fact that PLP is an active site directed modifier of urogen I synthase. The PLP's phosphate group is recognized by the enzyme's arginine group before the aldehyde portion of the PLP molecule reacts with the ε-amino group of lysine.

The structure of PBG has several features which could explain its unique reactivity. It is an α-Mannich base of a pyrrole, and as such it reacts by releasing ammonia and giving rise to a reactive positive carbon (a carbonium ion or the equivalent diene) which initiates the polymerization by an electrophilic attack on the C-5 of a second PBG molecule (Figure 47). The head-to-tail condensation would lead to the unrearranged dipyrrolmethane shown in Figure 5.

The enzyme can be thought of as having two sites (see Figure 48). The first incoming PBG molecule could interact with lysine to form a covalent enzyme substrate complex with the displacement of an ammonium ion. This can be followed by translocation of the PBG molecule to site II, by interaction of the arginyl group in site II and the PBG molecule's propionic acid residue. The second molecule of PBG now enters site I and

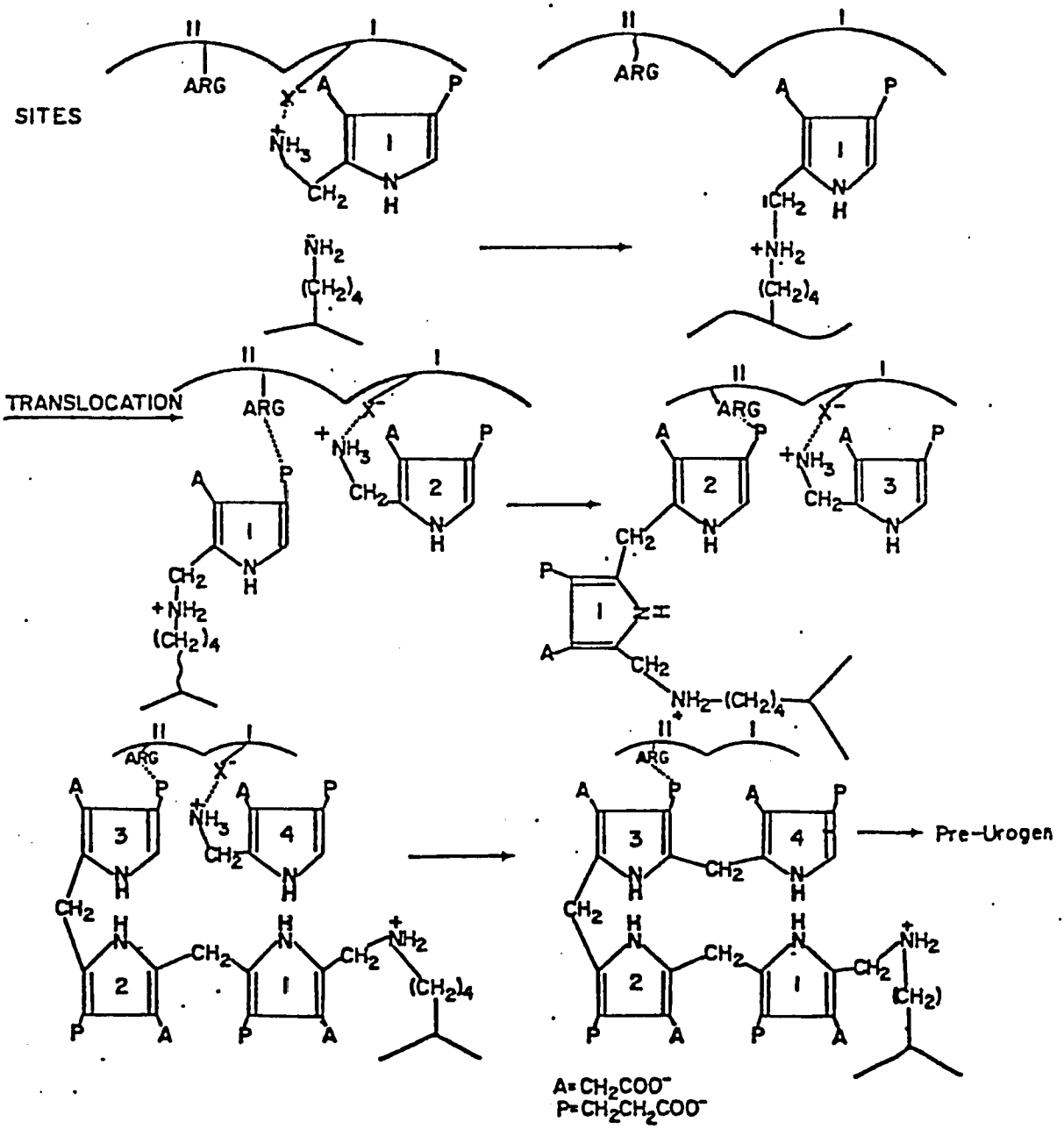
Figure 47 : PBG as an α-Mannich base which polymerizes by attack on C2 or C5 of a second PBG molecule.



A = CH_2COO^-

P = $CH_2CH_2COO^-$

Figure 48 : Polymerization of PBG by urogen I synthase.


SITES
ARG
TRANSLOCATION
Pre-Urogen
A=CH2COO⁻
P=CH2CH2COO⁻

an anionic recognition group could bind the C2 NH_3^+ portion of the incoming PBG molecule. Since lysine is not available the CH_2^+ group binds to the free α-position of PBG 1 with displacement of an ammonium ion, in a head to tail fashion. The second PBG molecule is now translocated to site II, as the third PBG molecule enters site I. The pocket changes shape so that the chain with lysine is further away from site I, but is coming around in a spiral. The third PBG molecule binds to the second and after translocation and binding of the fourth PGB molecule the unrearranged tetrapyrrole enzyme bound intermediate is formed. This then becomes pre-urogen which either relaxes to urogen I or in the presence of cosynthase becomes urogen III.

It seems likely that the arginine binds to the carboxylate group of the propionic acid residue of PBG, since Frydman and Feinstein (33) have demonstrated the need for effective inhibitors of urogen I synthase to have a free propionic residue at C-4.

Radmer and Bogorad (120) have reported on the isolation from a spinach urogen I synthase - PBG system in the presence of an ammonium ion, a tetrapyrrylmethane which could be converted to urogen I.

Davies and Neuberger (121) have reported the accumulation of polypyrroles when PBG was incubated with urogen I synthase from Rhodopseudomonas spheroides in the presence of ammonium ion, hydroxylamine or methoxyamine. It is reasonable to conclude

that the amines are removing the polypyrrole from the point of attachment to the enzyme by nucleophilic displacement on the methylene carbon which is the point of attachment. This point of attachment in urogen I synthase may be nitrogen with unshared electrons, such as the ε-amino group of lysine.

One arginine group involved in binding substrates through a carboxyl group to the enzyme's active site have also been reported for carboxypeptidase A (32), isocitrate dehydrogenase (44), and aconitase (38).

Glucose-6-phosphate dehydrogenase (42, 97) and aspartate transcarbamylase (70, 71) both have one arginine and one lysine each at their active site.

Appendix 1: Inhibition of Wheat Germ Porphobilinogen Deaminase Activity by Butanedione

INHIBITION OF WHEAT GERM PORPHOBILINOGEN DEAMINASE ACTIVITY BY BUTANEDIONE

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1. Introduction

Porphobilinogen (PBG) deaminase or uroporphyrinogen I synthetase is the enzyme which catalyzes the tetramerization of porphobilinogen. The structural requirements for competitive inhibitors of the enzyme [1,2] suggest that there is at the substrate binding site at least one amino acid residue which binds carboxylate ion.

Arginyl residues can serve as recognition sites on a variety of enzymes when the substrates contain carboxylate or phosphate groups [3,4]. Butanedione in borate buffer inhibits such enzymes by binding to the guanidino groups of arginine residues in the binding site. This paper reports the results of studies of the effect of butanedione on PBG deaminase.

2. Experimental

PBG was purchased from Porphyrin Products (Logan, Utah) and Sigma (St. Louis, MO). Butanedione was purchased from Aldrich Chemical (Milwaukee, WI). All other chemicals were reagent grade or better. Wheat germ was obtained from Sigma. Spectrophotometric measurements were made with the Cary 15 and Zeiss M4QIII spectrophotometers. Wheat germ PBG deaminase was prepared as in [5] and carried through the heat-denaturation step. Protein was determined as in [6] with bovine serum albumin as standard. Butanedione stock solutions were made up in borate buffer and the pH adjusted to pH 8.2 with solid sodium hydroxide.

Deaminase activity was measured in final reaction mixture vol. 5.0 ml containing: 50 mM borate buffer, pH 8.2, 0.001 M EDTA, 1 ml enzyme preparation (1.5 mg protein/ml assay mixture, spec. act. 2.97 nmol PBG consumed/h/mg protein) and PBG (60–70 nmol). After removing aliquots for zero time assays, the reaction mixture was evacuated and flushed with nitrogen repeatedly and then incubated for 4 h at 37°C. Aliquots were assayed for PBG with modified Ehrlich reagent [7]. Uroporphyrinogen was oxidized as in [8] and determined as uroporphyrin at 405 nm.

Modification of deaminase with butanedione was carried out at 25°C in final vol. 8.0 ml containing: 5 ml enzyme preparation (62 mg protein, spec. act. 2.87 nmol PBG consumed/h/mg protein), 50 mM borate buffer, pH 8.2 and concentrations of butanedione ranging from 0–100 mM. Aliquots (1.4 ml) were removed after incubation from 0–150 min, chilled to 4°C and dialyzed overnight at 4°C against 50 mM borate buffer, pH 8.2. Dialyzate, 1 ml, was assayed as described above. As a control, enzyme without butanedione was treated in the same way for the longest incubation time and then assayed.

When the ability of substrate to prevent inhibition was determined, PBG (80 μM) was added to the modification reaction mixture before the addition of butanedione (10 mM). PBG, uroporphyrinogen, uroporphyrin and butanedione were removed by extensive dialysis (24 h) at 4°C against 50 mM borate buffer before the enzyme was assayed.

The kinetic parameters were obtained using unmodified enzyme and enzyme which had been treated with 25 mM butanedione for 1 h at 25°C, dialyzed overnight at 4°C against 50 mM borate buffer, pH 8.2. PBG concentrations of 25 μM through

145 μM were used in separate incubation mixtures. Least squares analysis of data gave kinetic parameters with correlation coefficients greater than 0.97.

Reactivation of modified enzyme was attempted by treating deaminase at 25°C with 50 mM butanedione and dialyzing the mixture against either 50 mM borate buffer, pH 8.2 or against water for 23 h at 4°C.

3. Results and discussion

PBG deaminase was incubated with various concentrations of butanedione in borate buffer for a series of time intervals. In some experiments, PBG, the substrate, was added before the inhibitor. The enzyme aliquots were dialyzed individually against borate buffer before they were assayed for activity. The activity of deaminase, measured as PBG consumption and as porphyrin formation, was severely and rapidly reduced by butanedione in borate (fig.1). Borate alone had no effect on deaminase activity. Inhibition was not reversed by dialysis against borate or against water. The rate of inhibition was a function of butanedione concentration and the level of inhibition increased with time of incubation. For 100 mM butanedione, PBG uptake and porphyrin formation were almost completely inhibited before 60 min.

When deaminase was modified for 1 h in 25 mM butanedione, then dialyzed against borate and assayed with varying concentrations of PBG for 1 h and 2 h incubation times, the kinetic parameters in table 1 and fig.2 were obtained. K_m for porphyrin formation was slightly higher or the same for modified enzyme and V_{max} was lower by about a factor of 2. The results suggest that the modified enzyme mixture consists of totally inactivated enzyme and some native enzyme.

In order to determine whether the substrate can

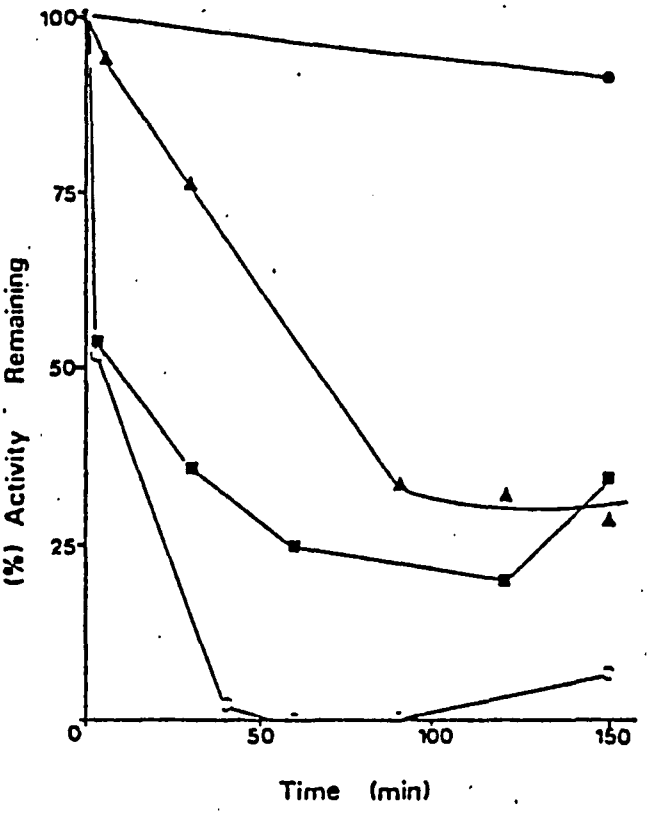


Fig.1. Inactivation of PBG deaminase by butanedione in borate buffer. The enzyme (7.8 mg/ml modification mixture; spec. act. 2.87 nmol PBG consumed/h/mg protein) was incubated with various concentrations of butanedione. Aliquots (1.4 ml) were removed at indicated times and dialyzed against 50 mM borate buffer, pH 8.2 at 4°C and 1 ml was assayed for PBG-consuming activity. The incubations included butanedione in the following concentrations: (•) 0 mM; (▲) 10 mM; (■) 50 mM; (▼) 100 mM. Uroporphyrinogen formation gave similar curves.

Table 1
Kinetic parameters for wheat germ PBG deaminase before and after inhibition by 25 mM butanedione measured after one hour and two hour incubation times

Incubation time	K_m (μM) 1 hr	V_{max} (nmol/ml/h)	K_m (μM) 2 hr	V_{max} (nmol/ml/h)
Unmodified	26	7.5	22	7.3
Modified run 1	36	2.9	42	3.4
Modified run 2	24	3.3	27	3.1
Modified run 3	30	2.6	24	2.2

Activity was measured as porphyrin formation

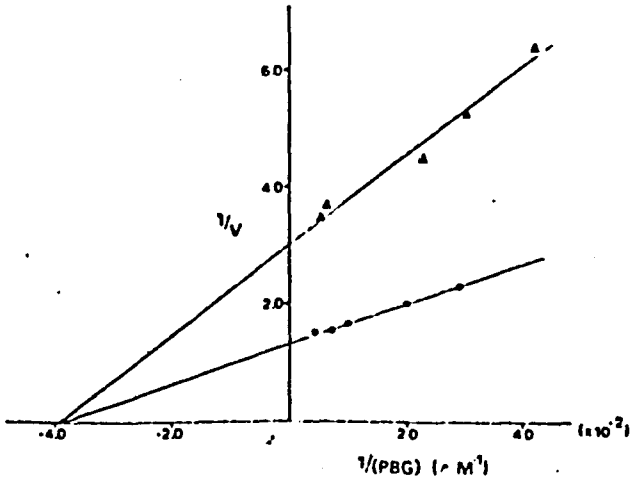
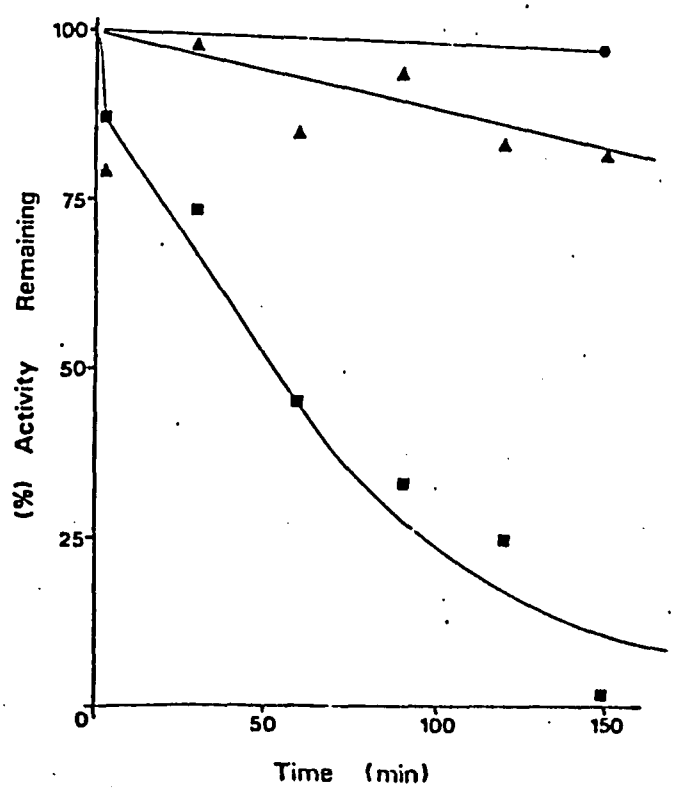


Fig.2. Double reciprocal plot for uroporphyrinogen formation by unmodified (•) and 2,3-butanedione modified PBG deaminase (▲). PBG deaminase was incubated with 25 mM 2,3-butanedione for 60 min at 25°C. Velocity is expressed as nmol uroporphyrinogen formed/ml/h.

protect the enzyme from inactivation by butanedione, deaminase was mixed with PBG (80 μM) before addition of butanedione (10 mM) and borate buffer. Aliquots were removed at various time intervals and dialyzed against borate buffer at 4°C before assay.



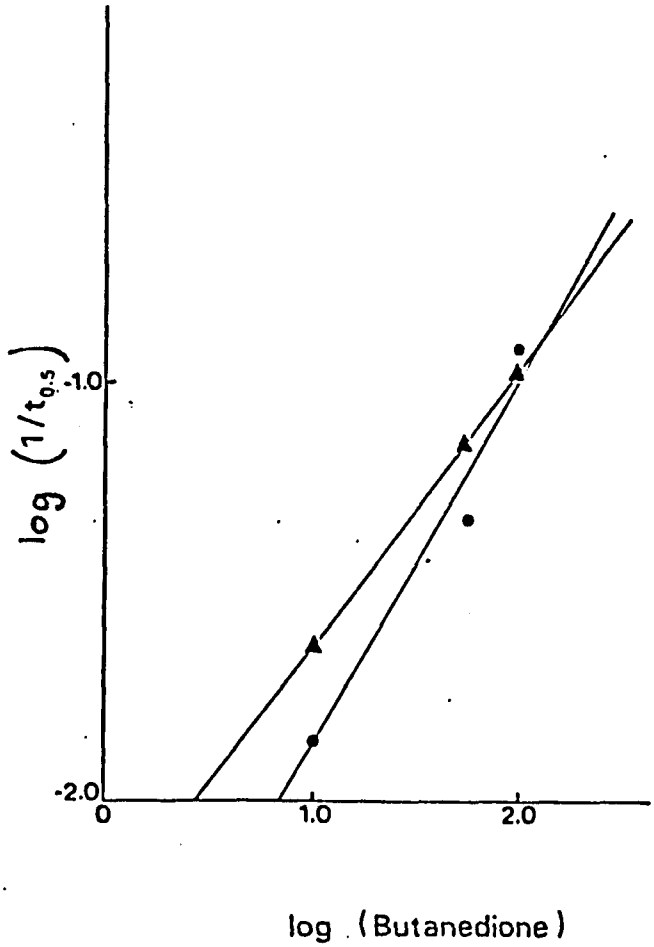


Fig.4. Logarithmic plot of PBG deaminase activity after treatment with various concentrations of butanedione. Half-times of inactivation ($t_{0.5}$) values were calculated from semilogarithmic plots of log PBG deaminase activity versus time (min) for initial reaction times up to 60 min. (•) PBG consumption; (▲) uroporphyrinogen formation.

Fig.3. Protection by substrate of PBG deaminase against inactivation by butanedione. The enzyme (7.9 mg/ml modification mixture with spec. act. 1.7 nmol/h/mg protein) was incubated with various concentrations of butanedione with and without PBG. Aliquots (1.4 ml) were removed at indicated times and dialyzed overnight against 50 mM borate buffer, pH 8.2 at 4°C and 1 ml was assayed for PBG-consuming and uroporphyrinogen-forming activities. The incubation mixtures included butanedione and PBG in the following concentrations: PBG consumption: (•) 0 mM butanedione and 0 μM PBG; (▲) 10 mM butanedione and 80 μM PBG; (•) 10 mM butanedione and 0 μM PBG.

The substrate showed a pronounced ability to protect deaminase activity from butanedione inhibition. Both PBG-uptake (fig.3) and porphyrinogen formation (results not shown) were appreciably protected by addition of PBG before inhibitor.

For data in the first hour of reaction when log $1/t_{0.5}$, where $t_{0.5}$ is the time required for 50% inhibition, was plotted against log butanedione concentration the slope was 0.89 for PBG and 0.65 for porphyrin formation (fig.4). These preliminary results suggest that one arginine is being inactivated and is required for deaminase activity [9].

Carboxypeptidase A [3], aspartate amino transferase [10], and isocitrate dehydrogenase [11] are enzymes which have substrates with carboxylate groups and which have been shown to require one or more arginine residues to bind these substrates. PBG deaminase may be another enzyme of this kind because butanedione inhibits its activity and the substrate lifts this inhibition. It is probable that PBG deaminase catalyzes at least two different types of reactions: condensation of PBG units and cyclization of a linear tetrapyrrole. How the PBG-binding site, the intermediate(s) binding site and the cyclization site relate to each other is not known.

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Appendix II: Application of Affinity Chromatography to the Purification of Wheat Germ Porphobilinogen Deaminase

CHROM. 11,386

Note

Application of affinity chromatography to the purification of wheat germ porphobilinogen deaminase

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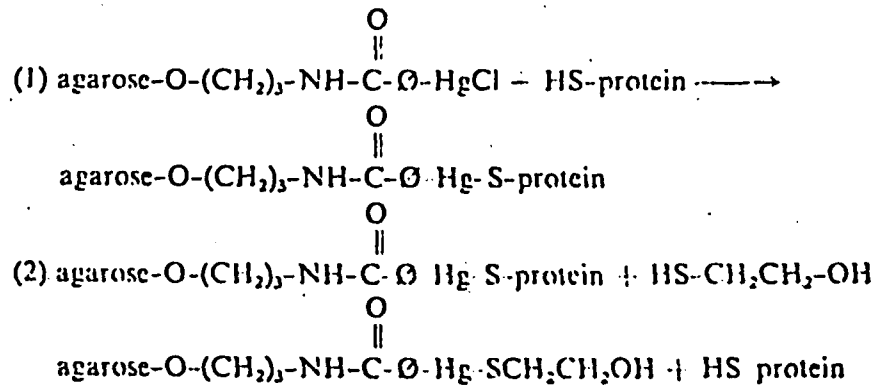
Porphobilinogen deaminase (uroporphyrinogen I synthetase) was first isolated from spinach[1] and wheat germ[2] by Bogorad. This enzyme catalyzes the head-to-tail condensation and cyclization of four moles of porphobilinogen (PBG) to form uroporphyrinogen I. Deaminase has been prepared from a variety of sources including avian[3] and mammalian erythrocytes[4], *Euglena gracilis*[5] and *Rhodopseudomonas spheroides*[6]. The latter was purified by Jordan and Shemin[7] by ammonium sulfate precipitation, column chromatography and preparative acrylamide gel electrophoresis. Frydman and Frydman[8] have reported on the purification and properties of wheat germ deaminase. Deaminase from various sources appears to be a single protein of molecular weight about $3.6 \cdot 10^4$ with a pH optimum near 8.0 and K_m values in the range of 20 μM.

Wheat germ deaminase is inhibited by sulfhydryl reagents: Ag^+, Hg^{++} and *p*-chloromercuribenzoate (PCMB)[2]. Inhibition by PCMB is reversed by cysteine. It was concluded that a cysteine residue is essential for enzymatic activity.

In connection with other studies on wheat germ deaminase, the need arose for a large-scale, homogeneous preparation of the enzyme. A form of affinity chromatography has been successful in removing many impurities from deaminase in one rapid step. Chromatography on organomercurial agarose has been used to purify plasma factor XIII, fraction 4 (ref. 9). The reaction of a sulfhydryl protein with organomercurial agarose removes it from a crude mixture [reaction (1)]. After washing the column with buffer, a concentration gradient of mercaptoethanol in the same buffer is used to elute the sulfhydryl protein from the organomercurial bond (2).



The success of this approach depends on the removal of deaminase from a crude preparation because the sulfhydryl groups of the former will bind to mercury on the agarose support. Elution with a mercaptoethanol concentration gradient should release the enzyme from the solid support.

MATERIALS AND METHODS

PBG was purchased from Porphyrin Products (Logan, Utah, U.S.A.) and from Sigma (St. Louis, Mo., U.S.A.). Affi-gel 501 was purchased from Bio-Rad Labs. (Richmond, Calif., U.S.A.). All other chemicals were reagent grade or better. Wheat germ was obtained from Sigma.

Spectrophotometric measurements were made with the Cary 15 spectrophotometer. Fluorimetric measurements were made with the Perkin-Elmer MPF-2A spectrofluorometer.

Protein was determined by measuring absorbance at 280 nm or according to Lowry *et al.*[10] with bovine serum albumin as standard. PBG was determined using the modified Ehrlich reagent of Mauzerall and Granick[11]. Uroporphyrinogen I was oxidized by the method of Jordan and Shemin[7] and determined as uroporphyrin spectrophotometrically or fluorimetrically. Polyacrylamide disc gel electrophoresis was performed using the method of Davis[12]. Wheat germ deaminase was prepared according to Bogorad[2] and carried through the heat-treatment step.

Enzyme assay

The fractions eluted from agarose columns were assayed in the following way. In a 400-μl tube were placed 100 μl of the contents of the fraction and 25 μl of a PBG solution (0.2 mg/ml). The tube was stoppered and incubated at 37°. The assay tubes were evaluated when fluorescence appeared under ultraviolet (UV) light in the control prepared from crude sample. The elution profile was obtained by adding one drop of the iodine reagent of Jordan and Shemin[7] to 50 μl from each tube and 350 μl of buffer and reading the intensity of fluorescence at 598 nm in the spectrofluorimeter with excitation at 410 nm.

Specific activities were determined by assaying for PBG consumption and uroporphyrinogen formation [incubation mixtures containing 1.0 ml of enzyme preparation, 4.0 ml of 0.1 *M* Tris buffer, pH 8.2, 0.50 ml 0.001 *M* EDTA and 0.20 ml of PBG (1.0 mg/10 ml)] at zero time and at 4 to 5 h.

Enzyme purification with Affi-gel 501

In a typical run, a preparation of wheat germ deaminase prepared by the method of Bogorad[2] and carried through heat-treatment contained 9 mg protein/ml and had a specific activity of 0.322 nm uroporphyrinogen/h/mg. A 10-ml volume was mixed with 15 ml of Affi-gel 501 (mercuriphenylagarose) which had been washed three times with three volumes each of 0.075 *M* Tris buffer, pH 8.0. The mixture was stirred at room temperature for 10 min with a glass rod and then poured into a small chromatography column and washed with the same buffer until the UV monitor (254 nm) indicated that no more protein was being eluted from the column (fraction 9). A large amount of protein and the yellow-brown color of the crude mixture was removed in this step. A linear gradient of Tris buffer 0.075 *M*, pH 8.0, containing 0.001

M EDTA and the same buffer with 0.02 *M* mercaptoethanol was started and fracti of 3.3 ml collected.

Affi-gel 501 can be regenerated by washing first with 10 m*M* $HgCl_2$, 20 n EDTA in 50 m*M* sodium acetate pH 4.8. Excess $HgCl_2$ is removed by washing the with 0.2 *M* NaCl, 1 m*M* EDTA in 50 m*M* sodium acetate at pH 5.0. It is import to avoid introducing phosphate ions during regeneration of the gel.

Chromatography on DEAE-cellulose

In a typical run, a sample of enzyme (6 mg protein/5 ml) was dialyzed agai 25% glycerol, 10 m*M* mercaptoethanol in 0.02 *M* phosphate buffer pH 7.9 at overnight. The sample was put on a column 1 × 7 cm and eluted with a linear gradi of 0–0.4 *M* KCl in the same buffer. Activity eluted in one peak at 0.13 *M* KCl.

Analytical polyacrylamide disc gels of the active tubes from the chroma graphy runs were cut into 0.5-cm slices, and each slice incubated with 25 μl PBG (mg/ml 0.75 *M* Tris, pH 8.0) at 37° and evaluated for fluorescence under UV lig Parallel gels were stained whole for protein with Coomassie Blue.

RESULTS AND DISCUSSION

Because deaminase activity is vulnerable to sulfhydryl reagents, it was reas able to expect rapid immobilization by mercuriphenylagarose if the support arm v long enough to reach an accessible sulfhydryl group. It was hoped that the extranec proteins in the crude preparations would be far less susceptible to this inhibitor. yellow-brown enzyme preparation which was the result of carrying Bogorad's wh germ preparation "B" through the heat treatment step, and the mercurated chron tography support were mixed batch-wise and loaded onto a column. A yello fluorescent colored impurity and a large amount of protein were removed by sim elution with buffer (Table I). Elution with a mercaptoethanol gradient showed t protein peaks, one associated with enzymatic activity as determined by spectropho metric and fluorimetric assays of aliquots of the individual fractions which had be incubated with PBG. The activity and elution profiles appear in Fig. 1. Table I prese the results of enzyme assays. A large amount of non-enzyme protein is removed in o step by this method. Recovery of activity was over 50%. The method is fool-pro rapid and reproducible. Frydman and Frydman[8] have noted that purification of t enzyme lowers its stability considerably. We found the purified enzyme was fai stable in 0.1 *M* Tris buffer and 20 m*M* mercaptoethanol, but lost activity with freezi and thawing and by removal or oxidation of mercaptoethanol.

TABLE I

PURIFICATION OF WHEAT GERM DEAMINASE BY CHROMATOGRAPHY ON ME CURIPHENYLAGAROSE

	Total protein (mg)	*Units*	*Specific activity*[a]
Heat-treated enzyme	89.6	28.0	0.311
Fractions 21–30	2.12	9.79	4.62
Fractions 31–40	1.72	4.21	2.45

[a] Specific activity is expressed as nmoles of uroporphyrinogen produced per h per mg prote

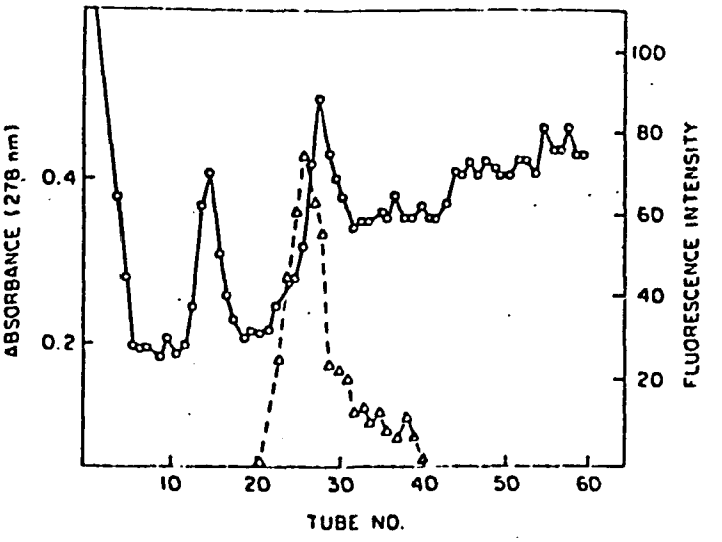


Fig. 1. Elution profile of PBG deaminase from mercuriphenylagarose column. Conditions of absorption and elution are described in the text. Enzyme activity is described in experimental methods. ○, Absorbance at 278 nm; △, uroporphyrinogen-forming activity. Fractions of 3.3 ml were collected after tube 9 when gradient was started.

Analytical polyacrylamide gels of the active fractions off mercuriphenylagarose chromatography showed a pronounced protein band at R_F 0.43 (using mobility of bromphenol blue as reference) and a faint band at R_F 0.87. The latter band was the only one with enzymatic activity. DEAE-cellulose chromatography in 25% glycerol and 10 mM mercaptoethanol of the active fractions off mercuriphenylagarose, removed most but not all of the band with R_F 0.43. While much protein was removed by DEAE-cellulose chromatography the specific activity of the enzyme did not increase, purification probably being offset by destabilization of the enzyme[6]. Preparative gel electrophoresis[7] preferably in buffer containing mercaptoethanol after mercuriphenylagarose chromatography would be the method of choice for preparing homogeneous wheat germ deaminase.

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