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STUDIES ON SENSITIVITY AND RESISTANCE TO PROFLAVINE IN *ESCHERICHIA COLI* B

by

Robin C. McKellar

Thesis submitted to the School of Graduate Studies as partial fulfillment of the requirements for the degree of Ph.D. in Biology.

UNIVERSITY OF OTTAWA

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TO MY WIFE,

LOUISE

Thank you

for being patient.

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LIST OF ABBREVIATIONS

PF	-	proflavine
AF	-	acriflavine
9AA	-	aminacrine
AO	-	acridine orange
EDTA	-	ethylenediamine tetraacetic acid
αCT	-	αchymotrypsin
NA	-	Nitroakridin 3582
TMG	-	methyl-β-D-thioglactopyranoside
EB	-	ethidium bromide
PEA	-	phenethyl alcohol
SDS	-	sodium dodecyl sulfate
TSB	-	trypticase soy broth
TSA	-	trypticase soy agar
Tris	-	tris(hydroxymethyl)aminomethane
IPTG	-	isopropyl-β-D-thiogalactopyranoside
PKI	-	FDP-regulated pyruvate kinase
PKII	-	AMP-regulated pyruvate kinase
NADH	-	nicotinamide adenine dinucleotide
LDH	-	lactic dehydrogenase
PEP	-	phosphoenol pyruvate
MOPS	-	morpholinopropane sulfonic acid
FDP	-	fructose 1,6-diphosphate

TCA - trichloracetic acid

DNP - dinitrophenol

CCCP - carbonylcyanide m-chlorophenylhydrazone

DCCD - dicyclohexyl carbodiimide

αMG - α methyl glucoside

αMGP - α methyl glucoside phosphate

F6P - fructose 6-phosphate

DTT - dithiothreitol

ANS - 1,8-anilino naphthalene sulfonate

LPS - lipopolysaccharide

ABSTRACT

Proflavine (PF) inhibited glucose breakdown by sensitive cells of E. coli B. The only glycolytic enzyme susceptible to PF was the FDP-regulated pyruvate kinase (PKI). PF was an allosteric inhibitor of the purified enzyme. PF increased the PEP concentration required for half maximal saturation and decreased the V_{max}; however, it had no effect on the Hill number for PEP. The inhibition produced by up to 0.3 mM PF was completely reversible as indicated by dilution studies. FDP, but not cyclic AMP, AMP, ATP, fructose 6-phosphate or dithiothreitol partially reversed PF action. Inhibition studies with a variety of acridines indicated that substitution at the 3 position on the acridine ring was required for inhibition and also that inhibition may involve hydrophobic interactions. PKI purified from PF-resistant cells was also sensitive to PF.

Cells of E. coli B growing in acetate and succinate mineral salts were significantly more sensitive to PF than cells growing in glycerol, glucose and pyruvate. Sensitivity could be correlated with generation time in the various media; cells grew slowly in acetate and succinate and more rapidly in glycerol, glucose and pyruvate. Five times more bound PF was required to

inhibit glucose-grown than acetate-grown cells by 50 %.

DNA replication was more sensitive to PF than glucose utilization in PF-sensitive cells growing logarithmically in glucose mineral salts. Inhibition of growth was more closely correlated with inhibition of DNA replication than with that of glucose utilization.

PF stimulated the rapid and complete loss of ATP in sensitive cells in the presence and absence of glucose. PF lowered ATP levels by 50 % in resting resistant cells but had no effect on ATP levels of these cells in the presence of glucose. Inhibition of glucose utilization was closely correlated with lowered ATP levels in sensitive cells. Iodoacetate had significantly less effect than PF on ATP levels in sensitive cells. These results were taken to indicate that PF directly affected ATP levels to some extent, though it may have also affected these levels indirectly by inhibiting ATP formation.

PF stimulated membrane-bound ATPase activity by 40-50 % in membrane fragments from sensitive and resistant cells but had no effect on the solubilized enzyme from sensitive cells. PF was an uncompetitive stimulator for the membrane-bound enzyme.

PF stimulated respiration by 50-75 % in sensitive cells. The classical uncouplers DNP and CCCP had a similar effect. PF also inhibited tyrosine, aspartate

and TMG transport by approximately 60 % in membrane vesicles prepared from sensitive cells. PF had a similar effect on amino acid transport in vesicles from resistant cells. Aspartate transport was inhibited by 70-80 % in sensitive and resistant whole cells.

Several PF-resistant strains of E. coli B were cross-resistant to penicillin and cephalothin. PF-resistant cells were 5-10 times more resistant to penicillin than PF-sensitive cells. Replica plating studies showed that when mutants were selected for either PF or penicillin resistance, cross-resistance to the other inhibitor occured with a frequency of greater than 80 %. None of the PF-resistant strains possessed β-lactamase activity. These results suggested that a change in "permeability" of cell membranes was responsible for both kinds of resistance.

Chapter 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Chemical Properties

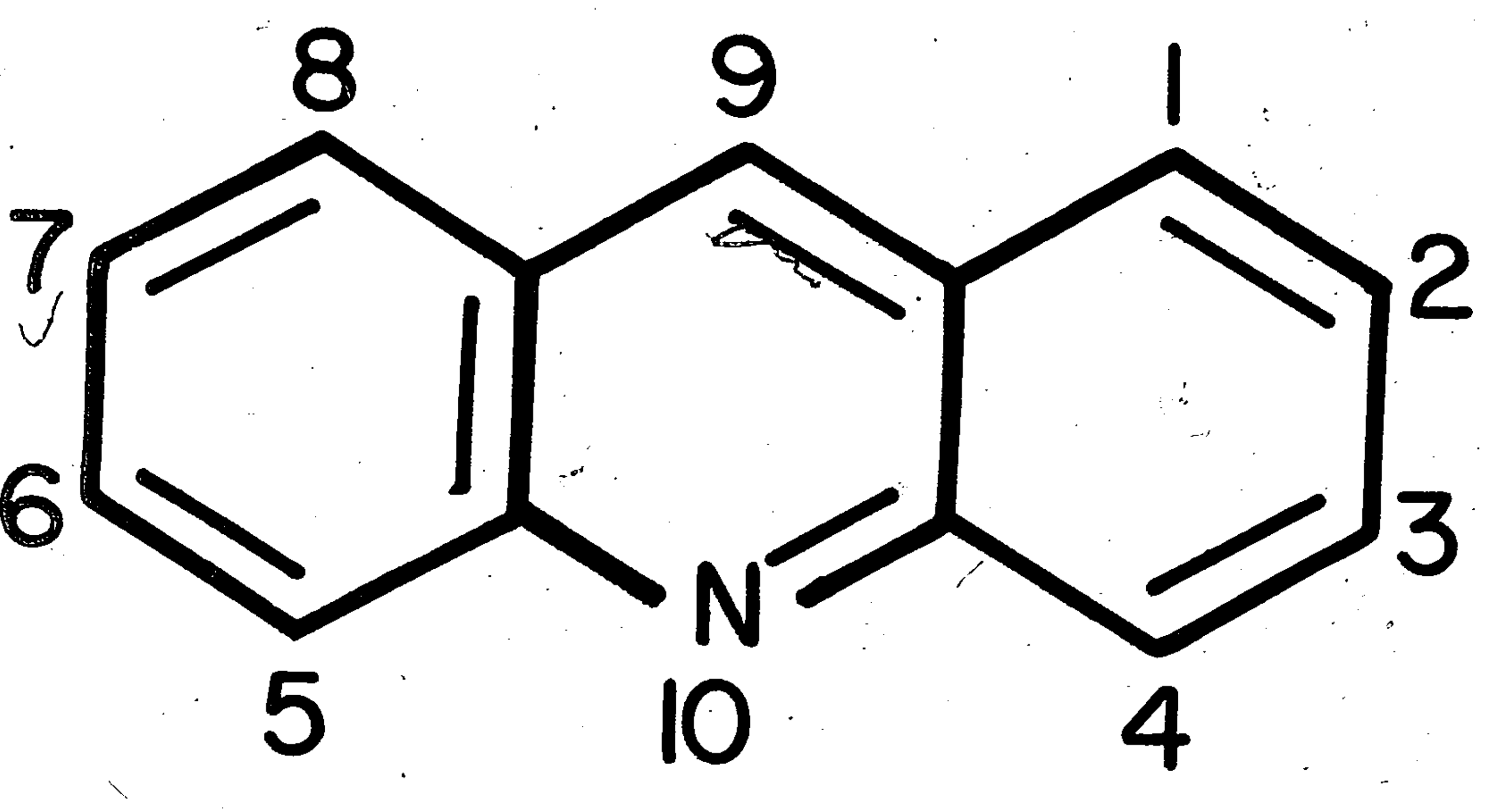
In 1870, Graebe and Caro isolated a substance from the high-boiling fraction of coal tar which they named acridine because of its irritating effects on mucus membranes (ie. acrid substance)(93). Since then, a wide variety of substituted acridines have been synthesized by a number of methods including ring closure of diphenylamine-2-carboxylic acids, -2-aldehydes or -2-ketones as well as the reaction between m-phenylenediamines and formic acid (3). Acridine is a fused six-membered heterocyclic compound posessing seven conjugated double bonds and is represented, along with several important acridine derivatives, in Figs 1a and b.

Various methods of numbering the acridine ring have been proposed at different times. The one illustrated in Fig 1a was proposed by Graebe and Lagodzinski in 1893 (94) and is now universally used.

Chemotherapeutic Action

A number of mono and diaminoacridines have bacteriocidal properties and several of these, including proflavine (PF), acriflavine (AF), mepacrine and aminacrine (9AA) have been used in the treatment of various diseases. Due to its strong bacteriocidal action and low toxicity, PF was

Fig. 1a. Numbering for the Acridine Ring.


8
9
1
7
2
6
N
3
5
10
4

4

Fig. 1b. Some Important Acridine Derivatives.

Proflavine (3,6-diaminoacridine monohydrogen sulfate)

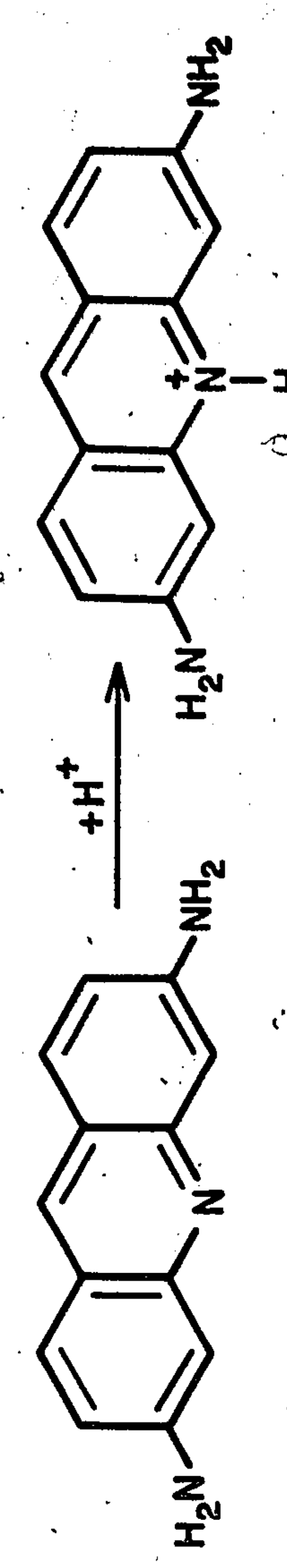
Acridine Orange (3,6-Bis(dimethylamino)-acridine hydrochloride)

Mepacrine, Quinacrine, Atebrin (3-chloro-9-(4-diethylamino-1-methylbutylamino)-7-methoxy-acridine dihydrochloride)

Aminacrine (9-aminoacridine hydrochloride)

Euflavine, Trypaflavin, Purified Acriflavine (3,6-diamino-10-methylacridinium chloride)

Fig. 1c. Ionization of Proflavine.



H2N
NH2
N
+H+
H2N
NH2
+N—H

employed extensively during World War II to prevent infection of gunshot wounds (3). Acridines have been used to control a number of protozoal diseases including those caused by *Giardia intestinalis* (88), *Theileria aunalata* (219), and *Eimeria tenella* (270) and to inhibit viruses such as herpes keratitis (256), vaccinia (89) and polio (207).

Mepacrine was probably the single most important chemotherapeutic acridine developed. It was effective against *Plasmodium sp.* and was subsequently used to control malaria during World War II (3). This drug still enjoys widespread use today despite the developement of a wide variety of other effective antimalarials (33,191). Mepacrine has also been used to treat tapeworm infection in man (164), autoimmune disease (184) and pneumothorax resulting from cystic fibrosis (127). Antagonism of morphine side effects and the prevention of physical addiction to various opiates are properties attributable to mepacrine (6) suggesting an important role for this compound in the treatment of drug addiction. Mepacrine has also been used synergistically with other antibiotics to prevent the emergence of antibiotic-resistant strains of bacteria (76,102,215).

Several acridines were used effectively to control various tumors including hamster melanoma (30,16), ovarian carcinoma (133) and murine leukemia (15). Acridine orange (AO) was shown to bind selectively to and to increase light

sensitivity of mouse epithelial tumors (251) and it was suggested that selective binding of acridines to tumors may be used to increase X-ray killing of these cells (85).

Acridines have been used effectively to cure R factors which control antibiotic resistance (55,70,101,114,159) and synthesis (131) in a number of bacteria. In 1963, Watanabe suggested that acridines may be used clinically to prevent the emergence and spread of antibiotic resistance (107). Recent studies, however, have shown that curing may be of little clinical value (34,101,114). Curing frequencies were low, on the order of 1-5 % in strains of Pseudomonas and Staphylococcus (114,101) and uncured R factors were easily transfered (34). Curing frequencies also varied depending on the strain of host bacteria and its location in the body (34).

Curing of R factors by acridines plays an important role in the study of bacterial genetics (55,70,101,114,159); however, the biochemical mechanism involved is poorly understood. Aminoacridines may preferentially bind to small circular DNA molecules thus affecting R factor DNA more strongly than chromosomal DNA (87). It was also proposed that R factor initiation, polymerization or attachment to segregation sites may be blocked (34) resulting in loss of plasmid due to subsequent dilution during cell division (281). On the other hand, it has recently

been suggested that acridines act on the membrane rather than the plasmid DNA during the curing process and affect plasmid-membrane association (170).

Physiochemical Propeties

It was shown earlier that the presence of a complete acridine ring (41) as well as cationic ionization of at least 50 % were required for strong antibacterial activity (5). Acridines possessing amino, chloro, methyl or nitro groups were strongly ionized under physiological conditions and were effective antibacterial agents (5) while acridines having a pKa of less than 7.0 were poorly ionized and were consequently ineffective (5 cited in 3). Acridines ionizing as anions or zwitterions were also ineffective antibacterial agents (5). PF ionization is shown in Fig 1c.

Poor antibacterial activity may be associated with an increase in bulk of the molecule; for example, mepacrine was relatively weaker than nor-mepacrine as an antibacterial as a result of steric hindrance due to a side chain methyl group (3).

Albert (4) showed that lipophilic properties played no part in determining the antibacterial effectiveness of an acridine and suggested that these drugs exert their action on the outside of the membrane and do not penetrate into the cell (3). On the other hand, Nakamura showed that AF

entered Escherichia coli by a process controlled by a membrane protein (171).

Acridines and Nucleic Acids

Acridine dyes were shown to bind to DNA by two distinct mechanisms (27,249). The first involved intercalation of the dye molecule between adjacent base pairs of the double stranded DNA (56,137,259) and has been studied by a variety of methods including viscosity, sedimentation (137) and fluorescence (211). These dyes were shown to be associated with AT and GC base pairs resulting in a change in the dye environment from a hydrophilic to a hydrophobic one as determined by fluorescence changes (211). The strength of the interaction was thought to be related to the proportion of AT base pairs in the DNA (254). Binding occured in two stages; an initial external binding followed by a transition to an internal site (186). The most recent model of intercalation has visualized the acridine molecule lying between successive nucleotide bases on the same polynucleotide chain in a plane approximately parallel to the base planes, but at an angle such that the positive ring nitrogen was close to the polynucleotide phosphate group (186).

The second mechanism was found to involve a relatively weak cooperative binding due to electrostatic forces (186).

It was suggested that the dye molecules were bound externally and edgewise with the positive ring nitrogen close to the phosphate group of the polynucleotide chain (186). At high dye concentrations, interaction between bound dye molecules may take place resulting in dye stacking (186).

It was also shown that acridines bind to RNA (80,186,210), synthetic RNA (71) and tRNA (71,80,266,209,274) by mechanisms similar to those described above, but binding is somewhat weaker (80,210).

Acridine binding to ribosomes involved intercalation with rRNA and resulted in accelerated ribosome breakdown (209,274). Ethylenediamine tetraacetic acid (EDTA) increased acridine binding due to unfolding of the RNA and exposure of new sites (156).

Consequences of Acridine Dye-Nucleic Acid Complex Formation

A/. Photodynamic Inactivation

E. coli treated with AO showed increased sensitivity to light (246,113). DNA polymerase I was required for recovery, due to its ability to repair single strand gaps in DNA that were produced either directly by AO or as a result of excision of AO-modified bases (113). Similarly, AO treatment of mouse embryo and L cells resulted in photodynamic inactivation of thymidine incorporation(140). Viruses

such as polio (207) and polyoma (140) as well as several coliphages (38,83,197) have also been inactivated by light in the presence of acridine dyes.

B/. Virus Inhibition

9AA has been shown to affect time and rate of lysis after T4D phage infection of E. coli, as well as to stimulate recombination, increase mutation frequency, delay maturation and interfere with head finishing steps (197). PF inhibited phage multiplication by blocking subunit assembly (241) and it was also able to prevent the formation of fully infective particles of vaccinia (250), fowl plague (82), polio (135) and foot and mouth disease viruses (40).

C/. Mutagenesis

PF has been used as a mutagenic agent for viruses (68, 121,188,234), bacteria (68,130,271), yeast (28,29,75), Clamydomonas (7) and silk worms (163). Due to permeability barriers, frequencies of PF-induced mutations are low in bacteria. These barriers may, however, by breached by phage attachment or EDTA treatment (130).

Acridine dyes produce frame shift mutations by intercalation into a single strand of DNA causing either insertion or deletion of a single base pair (39,182,247). This interaction may also involve double stranded DNA (138,235).

More recently it has been suggested that frame shift mutations cannot be explained in terms of simple intercalation since AO intercalated but was not mutagenic for E. coli in the dark (211,264). The formation of a specific charge-transfer complex between intercalated acridine molecules and GC base pairs may be required for mutagenesis (211).

In some cases, acridines may act as antimutagenic agents (53). Mepacrine was capable of blocking both induction of streptomycin resistance (118) and emergence of spontaneous azide resistance in E. coli (283). Other acridines were able to prevent T5 phage resistance in E. coli (264) and canavanine resistance in yeast (146). The biochemical mechanism involved in antimutagenesis is not well understood. It was proposed that antimutagenesis resulted from acridine binding to the DNA polymerase or replicating fork complex rather than from simple intercalation (53).

Acridines also increased killing and mutational yield in E. coli exposed to UV (54) or X-rays (85), due to their ability to inhibit excision repair (54,122). Under certain conditions, acridines were shown to bind to DNA and protect bacteria from the lethal effects of UV by preventing pyrimidine dimer formation (242).

D/. Staining

AO was shown to bind to the DNA and RNA of living cells without causing cell death (3). This process, known as vital staining, has been used to demonstrate the presence of trypanosomes in blood (237), to study infection by psittacosis virus (194) and to diagnose uterine (200,217) and lung cancer (2). When AO was bound to DNA and RNA it gave rise to green and red fluorescence respectively (9,10). This phenomenon has been used to determine the cellular location of nucleic acids (232).

Use of Acridines as Probes

A/. Enzyme Probes

PF has been used as a probe to study structure-function relationships in alpha-chymotrypsin (αCT). After the initial discovery that PF and several other acridines inhibited αCT (258), it was found that PF was bound to substrate binding sites (25) in a stoichiometric fashion (26,90,265). PF was a competetive inhibitor of αCT (258) and was bound to the active form of the enzyme only (79).

PF has also been used as a probe for the proteolytic enzymes papain and ficin. PF was a non-competetive activator of both papain (226) and ficin(108) and was bound stoichiometrically to both enzymes (99). The dye did not affect the rate constant (226) or substrate binding (100) for

either enzyme, but did influence the ratio of non-productive to productive substrate binding (100).

Acridines have also been used as probes of a number of other enzymes including horse serum cholinesterase (46) and electric eel acetylcholinesterase (162,218,268).

B/. Permeability Probes

Acridine dyes may be useful in determining cell permeability. It has been demonstrated that permeability barriers to acridines exist in *E. coli* (222,130). The frequence of acridine-induced mutation in *E. coli* may be used to determine the effectiveness of the cell envelope as a permeability barrier to these dyes (130). This procedure has been recently employed to determine variations in yeast cell permeability at different stages in the life cycle of yeast (116,214). However, other workers have suggested that it might be difficult to distinguish between increased permeability and the opening up of new acridine binding sites (96).

C/. Membrane Probes

In recent years, acridine dyes have been used extensively as membrane probes. In 1970, Kraayenhof showed that mepacrine fluorescence was quenched when the dye was bound to energized chloroplasts and suggested that mepacrine

could be used as a probe of the energized membrane (132). Similar results were obtained with mepacrine and phosphorylating particles from Azotobacter vinlandii (24) and membranes of Rhodopseudomonas capsulata (154) as well as with 9-amino-6-chloro-2-methoxyacridine and sonicated E. coli membranes (177). The ability of mepacrine to interact with energized membrane fragments has recently been employed to elucidate the role of ATPase in energy transduction in E. coli (253a).

It has been proposed, on the other hand, that mepacrine was distributed across the membrane in response to a pH gradient (64,97,212). It was suggested that this dye could be used to determine the internal pH of chloroplasts (213) and submitochondrial particles (136).

The nature of the interaction between acridine dyes and the membrane is still unclear. It was suggested that these dyes may be attached to nucleophilic sites situated in environments of low polarity which are close enough to permit dye-dye interactions (151) leading to stacking and subsequent quenching of fluorescence (13). It was also shown that membrane energization enhanced dye binding by increasing the nucleophilicity of the sites and the hydrophobicity of the environment (66). Dye binding was also affected by probe hydrophobicity (13) as well as by the presence of electrolytes (67).

Mechanism of Acridine Inhibition of Bacterial Growth

A/. Nucleic Acids

Acridine dyes have been shown to inhibit *in vitro* DNA replication (111,120,176,210,228,276) and, to a lesser extent, RNA (111,176,210,273,276) and protein synthesis (228,276). It has been suggested that these dyes block DNA replication and RNA synthesis by interfering with the DNA template (120,176); however, this may not be the only site. PF inhibited RNA synthesis in *E. coli* by acting as a competetive inhibitor of RNA polymerase, occupying nucleoside triphosphate sites on the enzyme (176).

It has been demonstrated that acridines strongly inhibit DNA replication (19,44,50,229,275) and slightly affect RNA (19,50,112,275) and protein synthesis (1,19,189,225, 275) *in vivo*. Inhibition of DNA replication has, on occasion, been associated with degradation of DNA (19,229) as well as with the formation of filamentous bacterial forms at subinhibitory concentrations of the dye (44,50,275). Inhibition of RNA synthesis by acridines has been implicated in the inhibition of enzyme induction by these dyes (1,112,185, 189,225). It was suggested that inhibition of protein synthesis took place at the level of transcription (112,273) since in *E. coli* PF had no affect on translation of the lac-operon (112). It was also shown that decreased levels of mRNA were responsible for the inhibition of protein

synthesis by mepacrine in E. coli (50).

On the other hand, synthesis of specific proteins was inhibited by acridines in Aspergillus oryzae (225). These dyes may also affect protein synthesis by preventing attachment of aminoacyl tRNA to ribosomes as has been demonstrated in E. coli (276) and rat liver (266).

The bactericidal action of acridines has been attributed to their ability to inhibit DNA replication. In E. coli, inhibition of growth by Nitroakridin 3582 (NA) (275) and mepacrine (50,149) was correlated with inhibition of DNA replication. Inhibition of growth and DNA replication in E. coli by AO were affected simultaneously by pH changes (19). AO also stimulated degradation of E. coli DNA (229). Mutants of E. coli lacking DNA polymerase I showed increased sensitivity to this dye due to their inability to repair damaged DNA (229).

It has been suggested that the inhibition of protozoal growth by acridines is due to the effects of these dyes on DNA. In Plasmodium sp., mepacrine was found to inhibit adenosine uptake and incorporation into RNA (270), orthophosphate incorporation into DNA and RNA (208) as well as purine phosphorylation and polymerization (45). Acridines also inhibit replication of (252) and stimulate the loss of (224) kinetoplast DNA in Trypanosome sp..

It has been suggested that in vivo DNA replication may not be directly affected by acridines. Wolfe et al (275) showed that concentrations of NA and mepacrine in excess of those required to completely inhibit DNA replication increased the killing rate of *E. coli*. They suggested that these dyes may affect the association of DNA with the cell membrane (275). Nakamura et al (170) have also proposed that AF cured plasmid DNA indirectly through its effect on the cell membrane.

B/. Metabolism

Acridines have been shown to bind to a wide variety of macromolecules in addition to nucleic acids, including proteins, phospholipids and mucopolysaccharides (221,222,223). Not surprisingly, therefore, these dyes were found to inhibit diverse metabolic processes including those associated with energy metabolism. In 1946, it was shown that mepacrine blocked acid production in *E. coli* (63) and *Lactobacillus casei* (84). This dye also inhibited pyruvate oxidation (230) as well as cytochrome oxidase, cytochrome reductase, glucose-6-phosphate dehydrogenase (98) and phosphofructokinase activities (36). Mepacrine also blocked oxygen consumption in *Streptococci sp.* (157) and *Crithedia fasiculata* (105), electron transport in *L. casei* (236) and glucose-6-phosphate dehydrogenase activity in yeast (284).

PF inhibited yeast glyceraldehyde-3-phosphate dehydrogenase (272) while AF affected yeast cytochrome function(165) and inhibited growth and oxygen uptake in Leptomonas sp. (91) and C. fasiculata (14,224).

Acridine dyes also interfered with membrane function. As early as 1955, it was shown that mepacrine uncoupled mitochondrial oxidative phosphorylation (109). This dye stimulated electron flow and prevented phosphorylation in chloroplasts (12) and was also able to stimulate the light-induced proton efflux in photosynthetic bacteria (129). Accumulation of methyl-beta-D-thiogalactopyranoside (TMG) in E. coli was also inhibited by PF (112). Mitochondrial Na^+, K^+-ATPase was stimulated at low and inhibited at high concentrations of mepacrine and AF (142). Ethidium bromide (EB), an inhibitor similar to acridines in structure and site of action, acted as an uncoupler of mitochondrial function (156).

It has been suggested that inhibition of DNA replication by acridines is an indirect affect due to inhibition of energy production. For example, inhibition of DNA replication by mepacrine in Trypanosome pyriformis was thought due to lowered energy levels (48). In E. coli, on the other hand, DNA replication was more sensitive than RNA synthesis to the inhibitory action of acridines (50,275); however, both processes were equally affected by reduced

intracellular ATP levels (193). Inhibition of RNA synthesis in *E. coli* by mepacrine was also thought to be unrelated to inhibition of energy production since this dye had no effect on the formation of uridine phosphate from uracil (210).

Most of the available evidence supports the hypothesis that the primary inhibitory effect of acridines is on DNA replication. A variety of sites involved in the generation and utilization of energy are sensitive to acridines; however, Albert has reported that these inhibitions require high concentrations of dye and he has proposed that they are not likely important sites of inhibition (3). Mehta *et al* (153) have suggested that glucose metabolism may be the primary site of PF inhibition of *E. coli* B; however, as yet, no serious attempt has been made to implicate inhibition of metabolism in the bacteriocidal and/or bacteriostatic action of acridine dyes.

Mechanism of Acridine Resistance

Acridine resistance has been demonstrated in a wide variety of microorganisms including *Escherichia coli* (167,134), *Serratia marscescens* (277), *Neisseria gonorrhoeae*(150), *Klebsiella aerogenes* (95), *Dictostelium discoidium* (269), *Plasmodium berghi* (192), *Leishmania tarentolae* (233), *Aspergillus nidulans* (202), *Saccharo-*

myces cerevisiae (92), Bacillus subtilis (17) and the coliphage T4D (197), and T2H (103). Levels of resistance may vary considerably depending on the organism and the particular acridine involved.

A/. Evidence for Permeability Barriers in E. coli

A number of workers have demonstrated that the cell envelopes of microorganisms present permeability barriers to the entry of acridines. It has been suggested that these barriers may provide the basis for acridine resistance; however, the evidence presented could easily be interpreted in terms of modification of dye binding sites rather than reduced permeability.

Silver (220) has shown that the adsorption of T even phage to the cell surface of E. coli promoted uptake of AF by the bacterial cell. This may have been due to induction of either specific changes in the dye binding capacity of the cell envelope or an acridine-specific pump permitting entry of the dye into the cell. Cells infected with phage carrying AF-resistant mutations (ac or pr) took up less dye than cells infected with the wild-type phage. These results suggest that AF resistance may be associated with reduced dye binding. Other workers (103) have observed decreased PF uptake in cells of E. coli infected with AF-resistant phage and have interpreted this as reduced cell permeability. It has also been observed that the

mutation frequency of PF for E. coli was increased by treatment of the cells with P22 phage and EDTA (130) and it was proposed that the cell wall may prevent entry of the dye into the cell.

Silver (223) has shown that AF binding by E. coli increased and became essentially irreversible after treatment with alkaline pH, phenethyl alcohol (PEA), heat or phage. He thought that this phenomenon was due to destruction of cell permeability barriers in either the cell wall or membrane. He also showed that the greater AF-sensitivity of E. coli B than of E. coli K12 was due to increased dye uptake and suggested that permeability differences, possibly related to differences in membrane fatty acid composition, were involved. Silver also showed that the relative potency of a series of acridines was directly related to their ability to bind to the cells.

Nakamura (167) has also proposed that AF resistance was related to dye uptake. He showed that AF-resistant cells of E. coli bound less dye than AF-sensitive cells in growth medium but not in buffer and that carbon starvation, metabolic inhibitors and reduced temperature promoted increased dye uptake. He postulated that resistance may be due to a modification of the dye binding capacity of the cells. He also reported that treatment of resistant cells with chloramphenicol, puromycin, 2-thiouracil and

8-azaguanine permitted increased dye uptake and he suggested that a basic protein may be involved in controlling dye binding. More recently, Nakamura (171) has demonstrated that resistance was a property of the cytoplasmic membrane and that AF binding was controlled by a specific membrane protein. It was thought that AF exerted its bacteriocidal action by interacting with specific membrane sites.

Kushner and Kahn (134) have also shown that PF-resistant cells of *E. coli* took up less dye than sensitive cells in growth media but similar amounts in buffer. They have attributed this to the ability of resistant cells to release PF by an energy-dependent process and have implicated this phenomenon in PF resistance. More recent studies (96) have shown that conversion of cells to spheroplasts did not significantly alter the PF binding capacity of either strain. It was also shown that cells bound considerable amounts of PF without the addition of permeabilizing agents. Also, since alcohol treatment did not lower the binding ability of heated cells, membrane lipids may not constitute quantitatively important binding sites. It was therefore suggested that PF binding was not confined to the outer surface of the cell and, in fact, that the cell envelope posed no barrier to the entry of the dye. It is quite possible that the cell wall does not affect dye binding in this strain of *E. coli*; however, the role of membrane proteins has not yet been evaluated. These workers have

demonstrated that cell proteins may make a significant contribution to dye binding in E. coli B (96).

B/. Other Mechanisms in E. coli

Other possible mechanisms for PF resistance may exist in E. coli. Kushner and Kahn (134) observed that PF-resistant cells of E. coli B grew while binding levels of dye capable of inhibiting sensitive cells suggesting that release of bound dye may not be the only line of defense. Mehta et al (153) also postulated that since glucose utilization was inhibited in PF-sensitive but not -resistant cells, drug resistance may lie at the metabolic level. Nakamura (167) has suggested that AF sensitivity in E. coli K12 cannot be due solely to modified binding sites. It was also shown (197) that cells of E. coli treated with T4 phage carrying 9AA-resistant mutations took up as much dye as cells infected with the wild-type phage. This mechanism of resistance is not clearly understood, but appears to be related to resistant intracellular processes (197).

C/. Resistance in Other Organisms

Acridine resistance has been studied in other organisms and found, in several cases, to differ from that found in E. coli. Woods et al (278) found that AF-sensitive

cells of S. marcescens bound more dye than AF-resistant ones and that carbon starvation, metabolic inhibition and reduced temperature had no effect on dye binding by either strain. They also showed that conversion of sensitive cells to spheroplasts or treatment of cells with $MgSO_4$ and sucrose reduced AF binding and rendered the cells resistant to the dye. These results suggest that resistance may be due to modified receptor sites on the membrane. Since AF binding in S. marcescens, unlike that in E. coli, was not readily reversible, it is unlikely that resistance was due to a modified membrane protein as suggested by Nakamura (171) for E. coli. Barabás et al (17) showed that PF-sensitive and -resistant cells of B. subtilis bound similar amounts of the dye in buffer and in growth media. Resistant cells growing in PF gradually released the dye, indicating a reduction in dye binding capacity. It was also found that various salts but not glucose caused release of dye from cells of both strains. These results suggest that differences in potential binding sites rather than permeability barriers may be involved in resistance.

The spoilage molds Trichoderma viride, Aspergillus niger, Penicillium notatum and P. roquefortii are normally resistant to the action of 9AA. Treatment of these organisms with PEA or EDTA increased the inhibitory effectiveness of the dye and it is therefore possible that the cell

envelopes of these organisms act as permeability barriers to 9AA (216). AF and EB resistance in yeast was also ascribed to reduced dye uptake into the mitochondria (92). AF sensitivity in yeast (8) and EB resistance in B. subtilis (31) have been associated with modified cell membranes. In L. tarentolae, on the other hand, AF-sensitive and -resistant strains bound similar amounts of the dye in growth media suggesting that permeability was not involved in resistance (233).

D/. Cross Resistance

Bacteria resistant to one drug may also be cross-resistant to drugs having similar structures and modes of action (95). Cross-resistance has been observed between different acridines (134,170,197) as well as between acridines and EB (8,92,95,233) and basic dyes (166a,196,202,240).

Cross resistance has also been observed between inhibitors having different chemical compositions and sites of action. Woody-Karrer and Greenberg (279) showed that PF-resistant mutants of E. coli S were also resistant to structurally unrelated alkylating agents and both X- and UV-radiation. Although all these inhibitors affect DNA, they are presumed to act by different mechanisms. The precise mechanism of resistance has not been determined, but is believed to be due to mutation

at several sites on the chromosome.

In other cases, cross-resistance between unrelated inhibitors has been attributed to reduced permeability. AF-resistance in D. discoidium was found to be associated with resistance to methanol and thiabendazole due to a mutation in the Acr A gene (269). Mutants of N. gonorrhoeae posessing low-level resistance to penicillin, erythromycin, chloramphenicol, rifampicin, EB and AO could be lost as well as restored by a single mutational step (150). It was proposed that multiple resistance was not exclusively due to selection of independent mutants but could involve instead a common mechanism of resistance, possibly permeability changes in the cell envelope (150). Nakamura (169) has shown that AF-resistance in E. coli K12 was associated with PEA and sodium dodecyl sulfate (SDS) resistance. He ascribed this resistance to the presence of a membrane protein that protected DNA from the inhibitory effects of these compounds (169). On the other hand, Rosenkranz et al (204) showed that there was no cross-resistance relationship between PEA and PF in E. coli B and B/r.

The evidence presented above suggests that the most likely mechanism of acridine resistance in E. coli involves modification of dye binding capacity. This could arise as a result of reduced cell wall permeability which may be overcome by phage adsorption (130,220) or

EDTA treatment (130); however, most authors have proposed that reduced binding capacity is a property of the cell membrane (17,171,223,278). Nakamura (170,171) has postulated that acridines exert their inhibitory action by interacting with specific membrane sites, in which case the availability of these sites could determine the dye sensitivity of an organism. The energy-dependent release of bound dye observed by several authors (134,167,223) might then be interpreted as a masking of sensitive membrane sites. On the other hand, glucose utilization was shown to be inhibited in PF-sensitive but not resistant cells of _E. coli_ B (153) suggesting that some inhibitory effects may result after passage of the dye through the membrane. At the present state of knowledge, it is difficult to differentiate between increased permeability and increased dye binding capacity.

E/. Resistance and Pigment

Acridine resistance has been associated with loss or modification of cell pigment. The isolation of acridine-resistant strains of _Pl. berghei_ was accompanied by the loss of a haemozoin pigment (190,192). AF-resistant mutants of _S. marcescens_ appeared orange in colour rather

than red because the pigment was no longer bound to the cell envelope (277).

F. Genetics of Resistance

A number of acridine-resistant mutants from several microorganisms have been studied genetically. Working with E. coli K12, Nakamura showed that AF resistance was controlled by a chromosomal gene, designated Acr A, coding for a structural protein in the cell membrane (171). More recently, he demonstrated that Acr A was located near and interacted with pla A, a gene controlling phospholipid biosynthesis (172). Nakamura also showed that methylene blue sensitivity in mutants isolated by Sugio (240) was linked to Acr A (73). AF resistance in several mutants of A. nidulans (202) as well as resistance to AO and other drugs in mutants of N. gonorrhoeae (150) were shown to be due to mutations on a single gene. In D. discoidium, AF resistance was recessive and was located in two unlinked genes Acr A and Acr B (269). Cross-resistance to methanol and thiobendazol was found only in mutants with an altered Acr A gene (269).

The genetics of acridine resistance has been extensively studied in the coliphage T4 and T2. Mutants of T4 resistant to AF (ac)(73) and 9AA (rs, rc and ama)(197) were isolated; these mutations were associated with the

rII cistron of the phage and confered resistance on the infected cells. Cells infected with mutant ac and the wild type phage were AF-sensitive suggesting that sensitivity was dominant (73). The mutants rs and rc showed similar patterns of resistance but mapped on opposite sides of rII(197) while ama mapped close to rs. These mutants have been used to demonstrate that the minimum number of acridine-sensitive targets in T4 development is four (197). Three acridine-resistant mutants of T2H have been isolated and designated pr, q and prq. The mutant pr allowed growth of infected cells in AF and PF while q allowed growth in mepacrine. Mutant prq was a double mutation controlling resistance to all three dyes (104).

G/. Adaptation Theory

Dean (65) has proposed that acridine resistance may arise as a result of physiological adaptation rather than by selection of preformed mutants. He has demonstrated that by growing K. aerogenes in a variety of acridine concentrations, a whole series of strains can be isolated showing a complete spectrum of drug resistance. Cells were resistant to dye concentrations up to that in which they were trained and lost their resistance very easily in low concentrations of dye. He has also shown that

whole populations of cells adapted to PF when the dye was added gradually to growing cultures. He attributed this phenomenon, known as bulk response, to the production of increased amounts of sensitive enzymes or to the development of pathways not susceptible to the drug (65,187). Resistance to PF, chloramphenicol, oxytetracycline and crystal violet has been developed in this manner while streptomycin resistance, on the other hand, appeared to be due to selection of mutants (65). Grant (95) has trained K. aerogenes to resist EB and PF in a similar manner and has likewise suggested that resistance was due to adaptation rather than mutation.

There is no doubt that acridine resistance can arise from mutations on single genes (159,171,202,269); however, this theory does not explain how whole populations of cells become resistant to acridines (65). It is possible that acridine resistance may arise by adaptation; however, this does not appear to be a common phenomenon and is probably of relatively little importance.

Aims of Thesis

Previous work from our laboratory has been concerned with elucidating the mechanism of PF inhibition of growth of E. coli B. Initial studies showed that when whole

cells were treated with inhibitory concentrations of PF, the dye was bound to particulate matter other than the cell wall, which could be either nucleic acids or membrane fragments (134). In order to determine the nature of the intracellular binding sites available for PF, DNA, RNA and protein were individually removed from all cell fragments by enzymatic digestion prior to PF binding. These studies showed that a correlation did not exist between PF binding and the presence of any one class of macromolecule; however, they suggested that other PF binding sites existed in the cell in addition to nucleic acids (90).

More recent studies have shown a correlation between PF inhibition of growth and of glucose metabolism in PF-sensitive cells (153). Aerobic and anaerobic glucose metabolism was inhibited in PF-sensitive but not -resistant cells (153) suggesting that glucose metabolism may be the primary site of PF inhibition of E. coli B.

The first aim of this thesis was to determine the site of PF inhibition of glucose metabolism and to assess the importance of this inhibition for the inhibition of growth. This problem was studied by examining the PF sensitivity of various processes involved in glucose metabolism including transport and the glycolytic enzymes, especially pyruvate kinase. As part of this work, the effect of

growth medium composition on PF sensitivity was examined also. In order to determine if glucose metabolism was the main site of PF action, the PF sensitivity of the former was then compared to that of DNA replication in logarithmically growing cells.

Other experiments were performed to determine the ability of PF to act as an uncoupler of membrane function. These experiments involved the effect of PF on whole cell respiration and intracellular ATP levels, active transport and membrane-bound and solubilized ATPase. Sensitive and resistant cells were compared in these studies.

Earlier work from our laboratory has also been concerned with PF resistance in _E. coli_. It was suggested that resistance was due, at least in part, to the energy-dependent release of bound dye by PF-resistant cells (134). This was not thought to be the only line of defense since resistant cells grew while binding levels of PF capable of inhibiting sensitive cells (134). More recently, it was proposed that permeability barriers were not involved in resistance (96) and that resistance at the metabolic level may determine whether or not growth is inhibited (96).

The second aim of this thesis was therefore to assess the role of permeability barriers in the control of PF resistance in _E. coli_ B. Some of the studies

described above were related to this question. In addition, this problem was studied by means of experiments in which PF-resistant cells were tested for cross-resistance to a variety of antibiotics.

Chapter 2

MATERIALS AND METHODS

MATERIALS AND METHODS

MATERIALS

9-amino-2-hydroxyacridine, 2,6-diaminoacridine and 3-amino-6-iodoacridine were the generous gifts of Dr. A. Albert. ^{14}C-αMG (3 Ci/mole), D-(U-^{14}C) glucose (283 Ci/mole), L-(U-^{14}C) aspartic acid (244 Ci/mole), L-(U-^{14}C) tyrosine (552 Ci/mole) and (6-^{3}H) thymine (24 000 Ci/mole) were obtained from Amersham/Searle. ^{14}C-TMG (14.4 Ci/mole) was obtained from New England Nuclear. Trypticase Soy Broth (TSB) was from Baltimore Biological Laboratories (BBL). Acridine dyes were obtained from Allied Chemical, Aldrich Chemical, Fisher Scientific, K and K Laboratories and Sigma Chemical. Scintiverse was obtained from Fisher and filters were provided by Milipore. Antibiotic discs were obtained from either BBL or Difco. The sodium salt of Penicillin G (1650 U/mg) was from Sigma Chemical. All other biochemicals were from Sigma Chemical and all inorganic chemicals were of reagent grade.

METHODS

Strains

PF-sensitive and -resistant strains (*E. coli* B and B/Pr respectively) were those described previously (134). B/Pr and other strains resistant to 1.0 mM PF were isolated

from B after growth in TSB containing successively 0.02, 0.05, 0.10, 0.50 and 1.0 mM PF. Strains resistant to 0.1 mM PF were isolated from plates of Trypticase Soy Agar (TSA) (TSB containing 1.5 % agar) containing 0.1 mM PF that had been heavily innoculated with E. coli B. Mutants resistant to 25 U/ml of penicillin were isolated in a similar fashion. Mg^{2+}, Ca^{2+}- ATPase-deficient strains were isolated from mutants of E. coli B capable of growth on 0.5 % glucose mineral salts (134) containing 1.5 % agar and 50 µg/ml neomycin sulfate. Neomycin selects for respiratory-deficient mutants, some of which may be unable to couple respiration to ATP synthesis due to the absence of ATPase (121a). Neomycin-resistant mutants were screened for the ability to grow aerobically on 0.5 % glycerol but not on 0.5 % succinate mineral salts agar, for the inability to grow anaerobically on 0.5 % glucose mineral salts agar and for low aerobic yield on limiting (5 mM) glucose mineral salts (59). ATPase-deficient mutants cannot grow anaerobically on glucose since this enzyme is essential for anaerobic growth (282). One mutant fulfilling these requirements (GN402) posessed only 20 % of the ATPase activity found in E. coli B. E. coli AN180 (arg E3, thi-1, str^R) employed in some experiments was obtained from Dr. F. Gibson, Australian National University, Canberra, Australia.

Growth Conditions

For most experiments, cells were grown at 37C on a reciprocal shaker at 100 strokes/min in flasks containing one fifth vol of TSB. Cells were harvested in log phase by centrifugation, washed twice in cold 20 mM tris(hydroxymethyl)-aminomethane (Tris) buffer pH 7.5, resuspended in the same buffer to 2 mg dry wt/ml and stored at 4C for no longer than 2h unless otherwise specified. These preparations will be refered to as "washed cells".

Cells were grown in a similar fashion in 0.5 % glucose mineral salts for the preparation of membrane vesicles and for all whole cell transport experiments unless otherwise specified. Vesicles for TMG transport were prepared from cells grown in 0.5 % glycerol mineral salts containing 0.5 mM isopropyl-β-D-thioglactopyranoside (IPTG).

Preparation of Crude Extracts

Washed cells were resuspended to 25 % w/v in 20 mM Tris buffer pH 7.5 and were disrupted by passage through an Aminco French Pressure Cell at 20 000 psi. Unbroken cells were removed by centrifugation at 3 000 x g for 10 min and the supernatant was clarified by centrifugation at 280 000 x g for 90 min. The extract was used immediately or stored at -80C until required for enzyme asseys or purification of pyruvate kinase I (PKI).

PKI Purification

PKI was purified to homogeneity from crude extracts of *E. coli* B and B/Pr essentially by the method of Malcovati *et al* (148) with the exception that the impure enzyme was treated for 10 instead of 45 min at 55C. The purified enzyme was stored at -80C and was used freshly thawed.

Preparation of Membrane Fractions

Purified membrane vesicles for transport experiments were prepared as described by Kaback (119). Membrane fragments for the determination of ATPase activity were prepared by the method of Evans (77). Both membrane preparations were stored frozen at -80C and thawed immediately before use.

Solubilization of ATPase

ATPase was solubilized from membrane fragments by a modification of the low ionic strength wash method of Roisin and Kepes (201) and Nelson, Kanner and Gutnick (173). Freshly thawed membranes were washed in five times the original volume of 20 mM Tris buffer pH 7.5, then resuspended in 20 vol of 1.0 mM Tris buffer pH 7.5 containing 0.5 mM EDTA. After centrifugation at 27 000 x g for 20 min, the supernatant was used as the source of the solubilized enzyme. ATPase-depleted membranes were resuspended in 20 mM Tris buffer pH 7.5 containing 1.0 mM $MgSO_4$.

Enzyme Assays

The effect of PF on the activity of glycolytic enzymes was determined at 25C and was monitored in a Beckman DB Spectrophotometer at 340 nm in glass cuvets in a final vol of 2.0 ml. Only initial velocities were employed in the calculations.

The enzymes tested and references to the methods employed are as follows; hexokinase (180); phosphohexose isomerase (166); aldolase (18); phosphofructokinase (18); triosphosphate isomerase (22); glyceraldehyde-3-phosphate dehydrogenase (257); phosphoglycerate kinase (42); phosphoglycerate mutase (by coupling 2-phosphoglycerate formed to nicotinamide adenine dinucleotide (NADH) oxidation by lactic dehydrogenase (LDH) in the presence of excess enolase and pyruvate kinase); enolase (by coupling phosphoenol puruvate (PEP) formed to NADH oxidation by LDH in the presence of excess pyruvate kinase) and pyruvate kinase (43).

For studies with PKI, enzyme activity was determined by a modification of the method of Bücher and Pfleiderer (43) unless stated otherwise. In a final vol of 1.0 ml, the reaction mixture was; 20 mM morpholinopropane sulfonic acid (MOPS) pH 7.5; 10.0 mM $MgSO_4$; 75.0 mM KCl; 10.0 mM PEP; 1.0 mM ADP; 0.125 mM NADH; 25 µg LDH and 1 µg PKI. Other compounds including PF and fructose-1,6-diphosphate (FDP) were added as required before the addition of either PEP or ADP. The reaction was started by the addition of ADP and

the initial velocity at 25C was determined within 30 sec and converted to μmoles PEP utilized/min/mg PKI.

Membrane-bound and solubilized ATPase activity was determined by a modification of the method of Roisen and Kepes (201). The reaction mixture contained, in a final volume of 1.0 ml; 0.1 M Tris buffer pH 7.5; 1.0 mM $MgSO_4$; 2.0 mM ATP; membrane or solubilized protein and various concentrations of PF. The complete reaction mixture minus ATP was preincubated in a circulating water bath for 10 min at 37C. ATP was added to start the reaction and after 2 min, the reaction was terminated with 1.0 ml of 10 % trichloroacetic acid (TCA). After chilling on ice for 5 min, precipitated protein was removed by centrifugation at 37 000 x g for 15 min. The resulting supernatants were neutralized with 6 N NaOH and volumes of 0.3-0.8 ml were assayed for orthophosphate by the method of Eibl and Lands (74).

Three PF-resistant strains (B/Pr, PrA and PrC) were tested for β-lactamase activity. Each strain was grown overnight at 37C in 500 ml Erlenmeyer flasks containing one fifth vol of 0.5 % glucose mineral salts then reinnoculated into fresh media and incubated until the cells reached early log phase. In some experiments, penicillin was added (25 U/ml) and growth was allowed to continue for 2½ h. It was demonstrated that a control culture of <u>E. coli</u> B lysed after penicillin addition. One half ml

of whole cells or equal volumes of culture that had been treated with the Branson Sonifier at full power for four 30 sec periods were assayed for β-lactamase by the method of Novick (179).

Measurement of PF Binding

Samples of whole cells containing bound PF were centrifuged at 27 000 x g for 10 min at 4C. The supernatants were poured off and the cell pellets were wiped dry. PF was extracted quantitatively from the pellets by treatment with 2 % SDS at 60C for 10 min. The absorbance at 445 nm of the resulting solutions was determined and converted to nmoles PF/ml using a standard curve. Correction was made for the presence of light absorbing materials present in SDS extracts of control cells not treated with PF. The absorbance at 600 nm of the original cell suspensions was converted to mg dry wt cells/ml using a standard curve. Bound PF was then expressed as nmoles PF bound/mg dry wt cells.

Polyacrylamide Disc Gel Electrophoresis

Polyacrylamide disc gel electrophoresis was carried out essentially by the method of Davis (62). Gels were fixed in 12.5 % TCA, stained in 0.2 % Coomassie Blue, destained and stored in 10 % acetic acid.

Protein Determinations

Protein determinations were performed by the method of Lowry et al (144) using bovine serum albumin as the standard.

Preparation of Samples for ATP and Glucose Determinations

Samples of washed cells were treated with 500 µg/ml of lysozyme and 1.0 mM EDTA for 15 sec on a vortex mixer then boiled for 10 min. After centrifugation at 37 000 x g for 10 min to remove cell debris, the supernatants were used for ATP and glucose determinations. In experiments in which ATP levels were not measured, cells were removed prior to the determination of glucose by precipitation with 0.5 vol of both 5 % $ZnSO_4 \cdot 7H_2O$ and 0.3 N $Ba(OH)_2$ followed by centrifugation at 37 000 x g for 10 min.

ATP Determinations

Cell-free supernatants were assayed for ATP by a modification of the firefly procedure (81a). Dessicated firefly tails were homogenized, at a concentration of 20 mg/ml, in 25 mM arsenate-sulfate buffer pH 7.5 containing 10 mM $MgSO_4$, followed by centrifugation at 37 000 x g for 30 min. Two ml samples in 10 mM phosphate buffer pH 7.5 and 6 mM $MgSO_4$ were mixed with 0.1 ml of the firefly tail extract and total light emmitted was determined after 15 sec in a Turner Fluorometer. Readings were corrected for the presence of PF

and for the fluorescence of the firefly extract and were converted to µg ATP using standard ATP solutions.

Glucose Determinations

Cell-free supernatants were assayed for glucose by a modification of the method outlined in the Sigma Bulletin No. 510. Samples of 0.25 ml were added to 2.5 ml of a mixture of buffered glucose oxidase and peroxidase containing 0.158 mM o-dianisidine dihydrochloride. After incubation at 37C for 30 min, the absorbance of each sample was determined at 495 nm in a Beckman DB Spectrophotometer and converted to µmoles glucose using standard glucose solutions. PF did not absorb significantly at 495 nm.

Role of Growth Media in PF Sensitivity

The effect of growth media on PF sensitivity of *E. coli* B was determined in two ways. *E. coli* B was grown overnight at 37C with shaking in 500 ml Erlenmeyer flasks containing one fifth vol of mineral salts and either 30 mM glucose or 60 mM acetate, succinate, pyruvate or glycerol. Flasks were fitted with 18 mm (O.D.) side arms. Cells were innoculated (0.05 vol) into fresh media containing different concentrations of PF, and after incubation for 17 h, absorbance at 600 nm was determined for each flask and expressed as a percent of the control in the absence of PF.

E. coli B was also grown as described above in mineral salts containing either 7 mM glucose or 15 mM acetate, succinate, pyruvate or glycerol. Cells were innoculated (0.05 or 0.10 vol) into fresh media and when the cultures had reached mid log phase ($Absorbance_{660\ nm}$ = 0.10-0.15), PF was added to 0.05 mM. Growth was measured as absorbance at 660 nm at various times. Generation times (t) for cells grown on each substrate in the presence and absence of PF were calculated from the formula;

$$t = \frac{T_2 - T_1}{\log_2 A_2 - \log_2 A_1}$$

where T and A refer to time in hours and absorbance at 660 nm respectively.

In other experiments, PF was added to a final concentration of 0.10 and 0.02 mM to cells growing logarithmically in 7 mM glucose and 15 mM acetate respectively. Absorbance at 660 nm was measured at various intervals and growth was expressed as a percent of the controls not treated with PF. Samples of 3.0 to 9.0 ml were taken at each time and centrifuged at 27 000 x g for 10 min at 25C. PF was determined in the pellet as described earlier and expressed as nmoles bound/ mg dry wt cells.

Relative PF Sensitivity of Growth, Glucose Utilization and DNA Synthesis

E. coli B was grown in 7 mM glucose mineral salts to mid log phase as described above, at which time different concentrations of PF were added. At various intervals, the absorbance at 600 nm was measured and samples of the cultures were removed and assayed for total glucose remaining/ml. In some experiments, 1.5 mM 2'-deoxyadenosine and 8 μM ^{3}H-thymine (187.5 Ci/mole) were added at the same time as PF. One ml samples were taken in duplicate at various intervals and added to 10 ml of cold 10 % TCA containing 100 μg/ml thymine. After 10 min on ice, the samples were filtered onto 0.45 μ Milipore filters, washed with 10 ml each of 5 % TCA containing 100 μg/ml thymine, 80 % ethanol, 1 N NaOH, and 5 % TCA containing 100 μg/ml thymine. Filters were dried and counted in 10 ml of Scintiverse (a prepared scintillation fluor) in a Beckman Liquid Scintillation Counter.

Effect of PF on Respiration

In these experiments, washed cells were employed at concentrations of 0.35 to 0.50 mg dry wt. cells/ml. Cells were treated at 37C for 30 min with 1.0 μl toluene and 20 μM PF, dinitrophenol (DNP) or carbonylcyanide m-chlorophenylhydrazone (CCCP) or 100 μM PF. Three ml volumes of

treated cells were equilibrated at 37C and 0.4 mM NADH was added. Values for decrease in percent oxygen saturation for a 3 min period were determined using a Yellow Springs oxygen electrode.

Effect of PF on Intracellular ATP Levels and Glucose Utilization

Washed cells at a concentration of 2 mg dry wt cells/ml were employed for all experiments in this section.

In order to show the effect of PF on ATP levels over a period of time, cells were preincubated with shaking at 37C for 15 min, then exposed to PF at a concentration of 0.07 mM Samples were removed at different time intervals and the intracellular ATP levels were determined as µg ATP/mg dry wt cells and expressed as a percent of the control with no PF. Similar experiments were also performed with washed cells that had been treated overnight at 4C with different concentrations of dicyclohexylcarbodiimide (DCCD).

Time course experiments were also performed with washed cells metabolizing glucose. Cells were preincubated with shaking at 37C for 15 min at which time glucose was added to 1.0 mM. After 7 min, PF was added to 0.07 mM. Samples were removed at intervals after glucose and PF addition and determinations of ATP in µg/mg dry wt cells and total glucose/ml were made. Experiments were also performed under nitrogen and in some of these, iodoacetate at a concentration of 1.43 mM replaced PF. In other experiments, washed cells

were exposed to different concentrations of PF with shaking at 37C for 30 min, at which time determinations of ATP in µg/mg dry wt cells and bound PF in nmoles/mg dry wt cells were made. Glucose was added to duplicate samples of PF-treated cells to a final concentration of 1.0 mM. Rates of glucose utilization were determined over a 15 min period and expressed as a percent of the control with no PF. In experiments designed to compare the effect of PF on glucose utilization and α-methyl glucoside (αMG) accumulation in whole cells, rates of glucose utilization were determined in cells that were not shaken.

Effect of PF on Amino Acid and Sugar Transport

Accumulation of glucose by membrane vesicles was determined, as described by Kaback (119), in a 100 µl reaction mixture consisting of; 70 mM phosphate buffer pH 6.6; 20 mM PEP; 10 mM $MgSO_4$; 0.3 M LiCl; 2.5 mg vesicle protein and different concentrations of PF. After equilibration at 30C for 15 min, the reaction was started by the addition of U(^{14}C)-glucose (35.4 µM at 283 Ci/mole). The reaction was terminated by dilution with 5 ml of 0.5 M LiCl then the vesicles were collected on 0.45 µ Milipore filters. The filters were washed with 5 ml of 0.5 M LiCl, dried and counted as described earlier.

Tyrosine, aspartate, and TMG accumulation were measured (119) in 100 µl reaction mixtures containing; 50 mM phosphate

buffer pH 6.6; 10 mM $MgSO_4$; 20 mM D-lactate; 100 µg vesicle protein and different concentrations of PF or CCCP. The samples were equilibrated for 15 min at 30C, then either ^{14}C-tyrosine (10 µM at 522 Ci/mole), ^{14}C-aspartate (11.1 µM at 224 Ci/mole) or ^{14}C-TMG (200 µM at 14.44 Ci/mole) were added. The reactions were terminated at 10, 2 and 30 min for tyrosine, aspartate and TMG respectively by dilution with 5 ml of 0.5 M LiCl. The samples were filtered, washed and counted as before.

Whole cells for amino acid accumulation experiments were grown as described and washed twice in mineral salts containing 100 µg/ml of chloramphenicol. The reaction mixtures contained, in a final vol of 100 µl; 50 mM phosphate buffer pH 6.6; 10 or 100 µg dry wt cells; 100 µg/ml chloramphenicol and different concentrations of PF. In some experiments, DNP or CCCP replaced PF and in others, 20 mM D-lactate was added as an energy source. The samples were preincubated at 30C for 15 min prior to the addition of either ^{14}C-tyrosine (10 µM at 522 Ci/mole) or ^{14}C-aspartate (11.1 µM at 224 Ci/mole). The reactions were terminated after 1 and 2 min for tyrosine and aspartate respectively by dilution with 5 ml of 50 mM phosphate buffer pH 6.6. The samples were filtered, washed with an additional 5 ml of buffer and counted as described earlier. Transport rates were linear over the experimental period.

Time course accumulation of aspartate by whole cells was measured in a reaction mixture of 0.5 ml containing; 50 mM phosphate buffer pH 6.6; 1.0 mg dry wt cells/ml and 100 µg/ml chloramphenicol. ^{14}C-aspartate (224 Ci/mole) was added to 22.2 µM and at various times, 100 µl samples were removed, diluted into 5 ml of 0.5 M LiCl, filtered, washed and counted as described above.

αMG accumulation was determined in washed cells prepared as described earlier. One half ml samples of cells at 2 mg dry wt/ml were exposed to various concentrations of PF at 37C for 30 min. ^{14}C-αMG (83 µM at 3.0 Ci/mole) was added and after 0.5 and 5 min, 0.125 ml vol were diluted with 10 ml of mineral salts, filtered, washed with a further 10 ml and counted. α -methyl glucoside phosphate (αMGP) was determined by diluting duplicate 0.125 ml vol with 10 ml of 37 mM $BaCl_2$ in 80 % ethanol. After chilling on ice for 10 min, the samples were filtered, washed with 10 ml of cold 80 % ethanol and counted.

For all accumulation studies, counts were corrected for background by filtering duplicate samples immediately after addition of the isotope.

Antibiotic Resistance Studies

Strains to be tested for antibiotic resistance were grown overnight in TSB at 37C and innoculated onto plates

of TSA. Antibiotic discs were placed, four to a plate, on the agar surface and after incubation at 37C for 24 h, the diameters of the clear zones were measured.

The effect of penicillin on growth of PF-sensitive and -resistant strains was determined by innoculating tubes containing 10 ml of TSB and different penicillin concentrations with the strains to be tested. After incubation at 37C for 24 h, growth was measured as absorbance at 660 nm and expressed as a percent of the control containing no penicillin.

The frequency of cross-resistance between PF and penicillin was determined by replica plating mutants resistant to either 0.1 mM PF or 25 U/ml penicillin from selection plates to plates containing the other drug. After incubation at 37C for 24 h, colonies resistant to the other drug were expressed as a percent of the number of colonies transfered.

Spontaneous resistance to PF or penicillin was determined by replica plating colonies of *E. coli* B from TSA to plates containing either PF or penicillin. After incubation at 37C for 24 h, mutants resistant to PF or penicillin were expressed as a percent of the colonies transfered.

Each data point in this thesis was the mean of 4 to 6 determinations, obtained from two or more experiments. Individual determinations did not vary by more than 10 percent.

Chapter 3

MECHANISM OF INHIBITION OF GLUCOSE UTILIZATION BY PF

MECHANISM OF INHIBITION OF GLUCOSE UTILIZATION BY PF

Introduction

Previous work had shown that PF inhibited glucose metabolism in sensitive but not resistant *E. coli* B (153). In order to determine the site of PF action, we examined its effect on glucose transport and on the enzymes involved in glycolysis. The major part of this chapter will be concerned with the PF inhibition of FDP-regulated pyruvate kinase (PKI), the only sensitive enzyme found.

Results

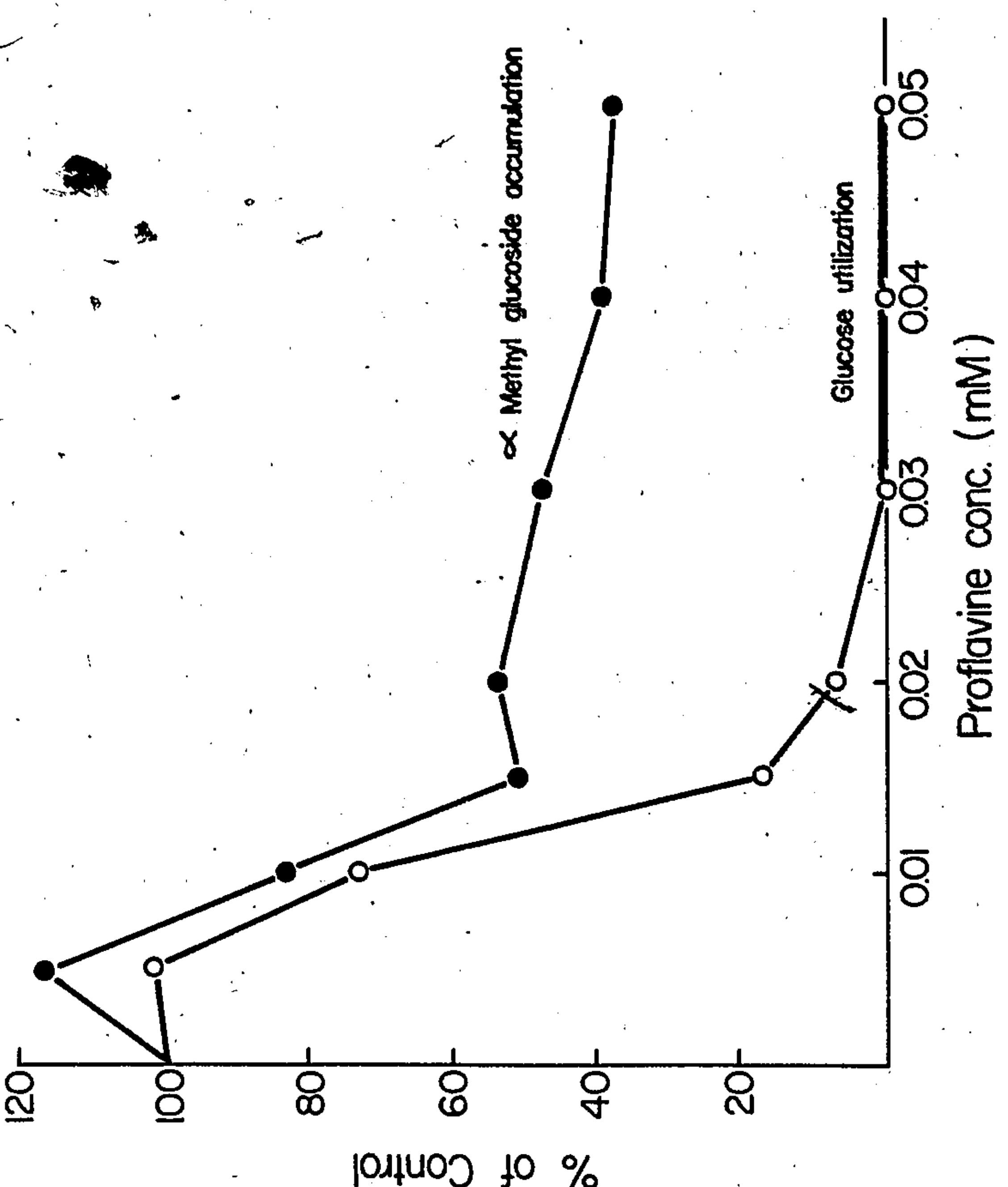
A/. Effect of PF on Glucose Transport

Glucose transport could not be studied directly in whole cells since glucose is utilized so rapidly. Instead, αMG, a non-metabolizable analog of glucose, whose transport is thought to involve the same system as glucose (86) was used.

Fig. 2 shows the effect of different concentrations of PF on the rate of glucose utilization and on accumulation of ^{14}C-αMG in washed cells of *E. coli* B. Both processes were similarly affected by low concentrations of PF; however, at 0.03 mM PF, αMG accumulation was inhibited by only 55 % while glucose utilization was

Fig. 2. Effect of PF concentration on αMG Accumulation and Glucose Utilization by Whole Cells of *E. coli* B.

Control rate of αMG accumulation was 2.38 nmoles/mg dry wt./5 min.
Control rate of glucose utilization was 0.457 µmoles utilized/mg dry wt./15 min.



% of Control
120
100
80
60
40
20
0.01
0.02
0.03
0.04
0.05
Proflavine conc. (mM)
∝ Methyl glucoside accumulation
Glucose utilization

completely blocked. Further addition of PF had no effect on αMG accumulation. The data in Fig. 2 show αMG accumulation after 5 min. Similar results were obtained after 0.5 min.

Gachelin (86) has proposed that phosphorylation of αMG at the expense of PEP was essential for accumulation by whole cells. In spite of this, it was thought possible that PF may permit limited accumulation while inhibiting phosphorylation. In such a case, αMG accumulated by PF-treated cells may be chiefly in the unphosphorylated form.

αMGP levels were determined, as described in Materials and Methods, in cells that had accumulated ^{14}C-αMG in the presence and absence of 0.05 mM PF. Table 1 shows that in both cases, 50 % of the total label incorporated was in the phosphorylated form indicating that PF probably did not directly block phosphorylation of αMG.

These results show that cells can accumulated and phosphorylate αMG (and therefore glucose) in the presence of inhibitory levels of PF and suggest that glucose transport _per se_ is not the major site of PF inhibition. αMG accumulation is an energy-requiring process, and therefore the observed inhibition by PF may be due to the blocking of energy production by this dye.

Table 1

Effect of PF on Accumulation of αMG and αMGP

by E. coli B

	PF(0.05mM)	nmoles accumulated/ mg dry wt.	% of Total
αMG + αMGP	-	2.16	100.0
	+	1.00	100.00
αMGP	-	1.17	54.2
	+	0.52	52.0

α MGP was determined as the Ba^{2+}-insoluble portion of the total label accumulated.

B/. Effect of PF on Glycolytic Enzymes

All glycolytic enzymes up to and including pyruvate kinase were tested for sensitivity to PF in cell-free extracts of *E. coli* B. As shown in Table 2, pyruvate kinase was the only enzyme significantly inhibited under my conditions of assay, losing 86 % of its activity in the presence of 0.4 mM PF. Inhibition of other enzymes such as phosphohexose isomerase, aldolase, triosphosphate isomerase and enolase was minimal and took place only at much higher concentrations of PF. Extensive precipitation of protein occured at high concentrations of PF and may account for the inhibition observed at this level of the dye.

Two pyruvate kinases exist in *E. coli* (147,262); PKI which is regulated by FDP and is involved in glycolysis (148,261) and PKII which is regulated by AMP and ribose phosphate and serves a role in glucogenesis (148,265).

Further studies were initiated to determine the relative sensitivity of these two enzymes to PF. PKI and PKII were separated on DEAE cellulose as described by Malcovati *et al* (148) and it was found that while PKI was completely inhibited by 0.5 mM PF, PKI was inhibited by only 52 % (Table 3). Since PKI is involved in glycolysis, studies were carried out to determine the nature of the inhibition of this enzyme by PF and its possible role in the inhibition of glucose utilization.

Table 2

Effect of PF on Glycolytic Enzymes

Enzyme \ PF conc. (mM)	Velocity (% of Control)					
	0	0.2	0.3	0.4	0.6	0.8
Hexokinase	100(.024)	116.0	ND	89.6	ND	ND
Phosphohexose isomerase	100(.403)	ND	ND	103.7	87.3	71.7
Phosphofructokinase	100(.520)	ND	ND	90.9	87.3	87.3
Aldolase	100(.719)	ND	ND	110.3	89.3	68.4
Triosephosphate isomerase	100(20.3)	ND	ND	83.7	69.9	65.0
3-glyceraldehyde phosphate dehydrogenase	100(2.14)	ND	ND	125.2	125.2	125.2
Phosphoglycerate kinase	100(2.58)	ND	ND	81.4	75.2	84.5
Phosphoglycerate mutase	100(2.08)	ND	ND	111.1	100.0	93.3
Enolase	100(1.98)	96.0	96.0	96.0	76.8	76.8
Pyruvate kinase	100(.268)	28.4	28.4	14.2	ND	ND

Bracketed values are control velocities in µmoles substrate utilized/min/mg protein.

ND = not done

All assays were performed with saturating levels of substrate.

Table 3

Effect of PF on PKI and PKII from E. coli B

Enzyme	0.5mM PF	Velocity (ΔA340/min/25µl)	% of Control
PKI	-	0.230	100.0
	+	0	0
PKII	-	0.125	100.0
	+	0.060	48.0

C/. Effect of PF on PKI

i. Purification of PKI

PKI was purified by the method of Malcovati *et al* (148) as described in Materials and Methods and was found to be homogeneous by polyacrylamide disc gel electrophoresis.

ii. Kinetic Characterization of PKI

Other workers have demonstrated (148,261) that PKI from *E. coli* K12 is an allosteric enzyme and it seemed likely that the enzyme from *E. coli* B would have similar properties. We would therefore expect the saturation curve for the allosteric substrate to be sigmoidal, indicating that homotrophic interactions between substrate molecules are taking place. A value for the Hill number (n) may then be obtained from the linear form of the Hill equation;

$$\log \frac{V}{V_{max}-V} = n \log S - \log K \ ,$$

where V refers to velocity and S to substrate concentration. This value, when greater than 1.0 is a direct measure of the strength of the homotrophic interactions (161). In general, in the presence of an allosteric activator, the substrate saturation curve becomes

hyperbolic and the Hill number reduces to 1.0 indicating that no homotrophic interactions are taking place (161). In the presence of the allosteric inhibitor, on the other hand, both the sigmoidality of the substrate saturation curve and the Hill number increase (161).

Saturation curves for allosteric activators and inhibitors are also sigmoidal. The homotrophic interactions of one are increased in the presence of the other resulting in antagonism of action (161).

Michalis constants (K_m) may be calculated for hyperbolic saturation curves. A similar value, $K_{\frac{1}{2}}$, defined as the concentration of substrate producing half maximal activity may be calculated from sigmoidal curves (261).

For PKI from E. coli B, a plot of enzyme velocity against PEP concentration (Fig. 3) gave a $K_{\frac{1}{2}}$ of 5.0 mM and a V_{max} of 31.5 μmoles PEP utilized/min/mg PKI. Fig. 4 shows a Hill plot of the data from Fig. 3. An n value of 3.36 was determined indicating that PEP is an allosteric substrate (161). Fig. 5 shows the effect of the allosteric activator FDP (148,261) on the PEP saturation curve. It was observed that FDP changed the shape of the curve from sigmoidal (Fig. 3) to hyperbolic (Fig 5). Using the weighted analysis method of Wilkinson (268a), K_m and V_{max} values of 0.081 mM and

Fig.3. Effect of PEP Concentration on PKI Activity.

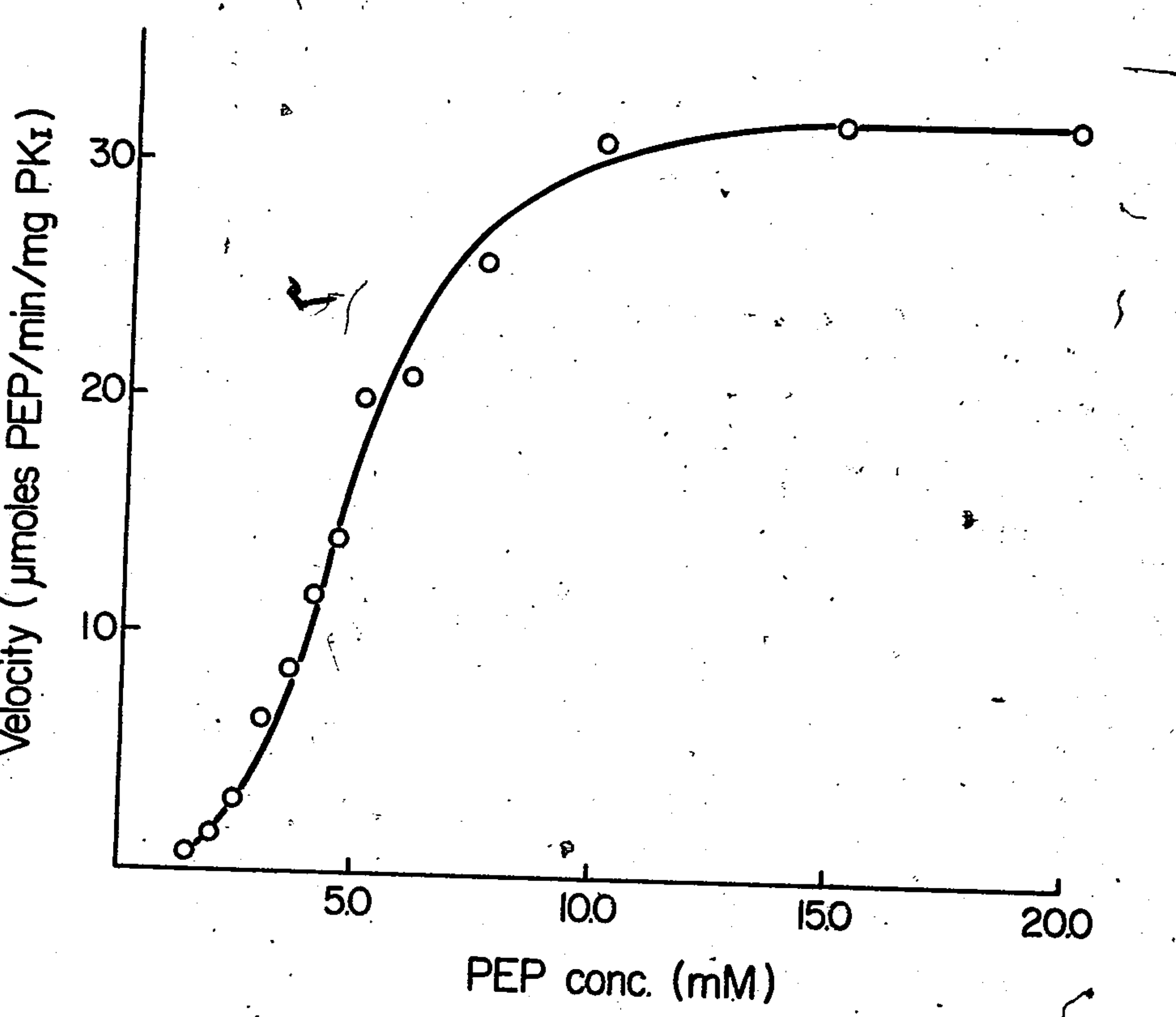
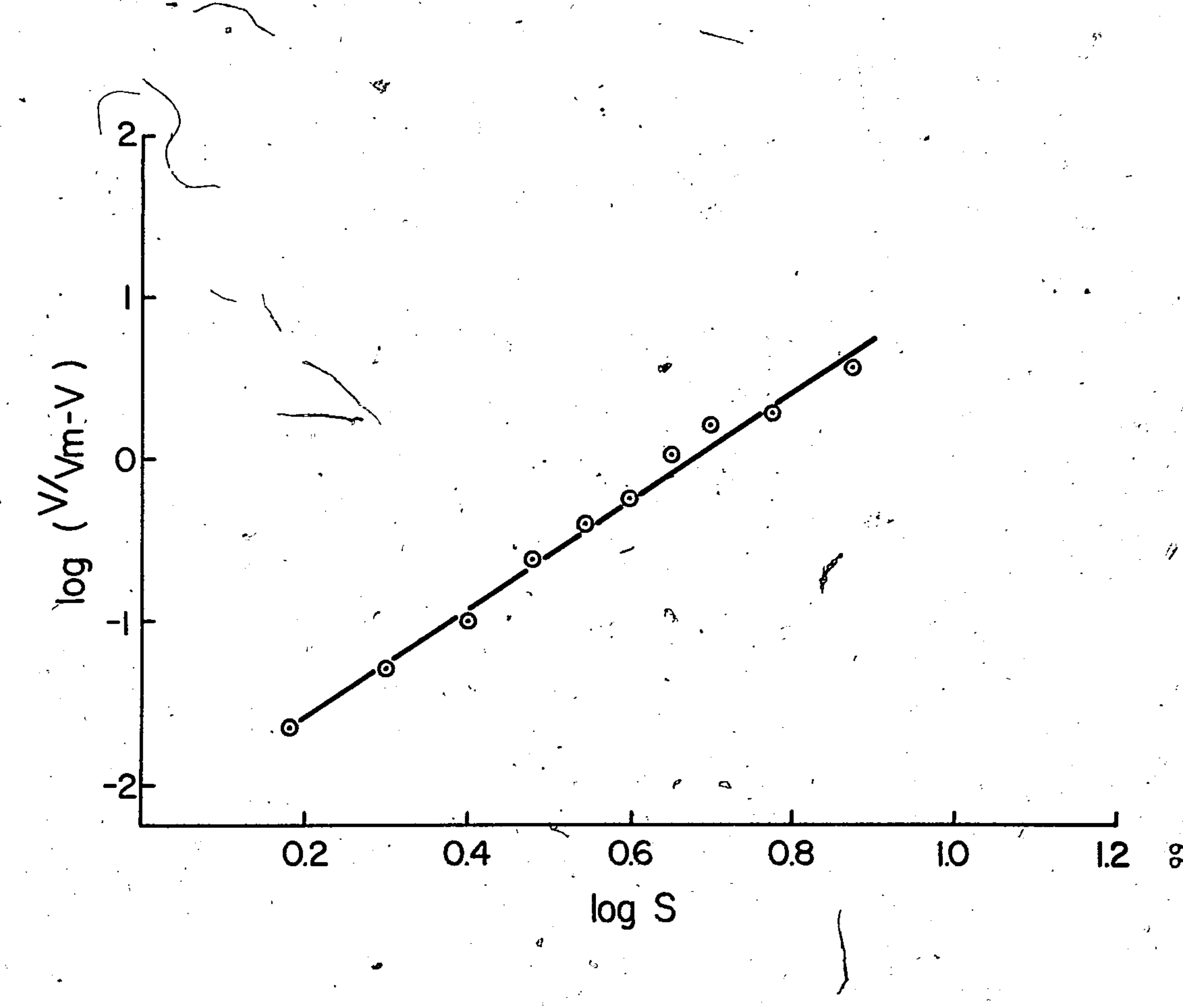

Velocity (μmoles PEP/min/mg PKI)
30
20
10
5.0
10.0
15.0
20.0
PEP conc. (mM)

Fig. 4. Determination of the Hill Number for PEP.

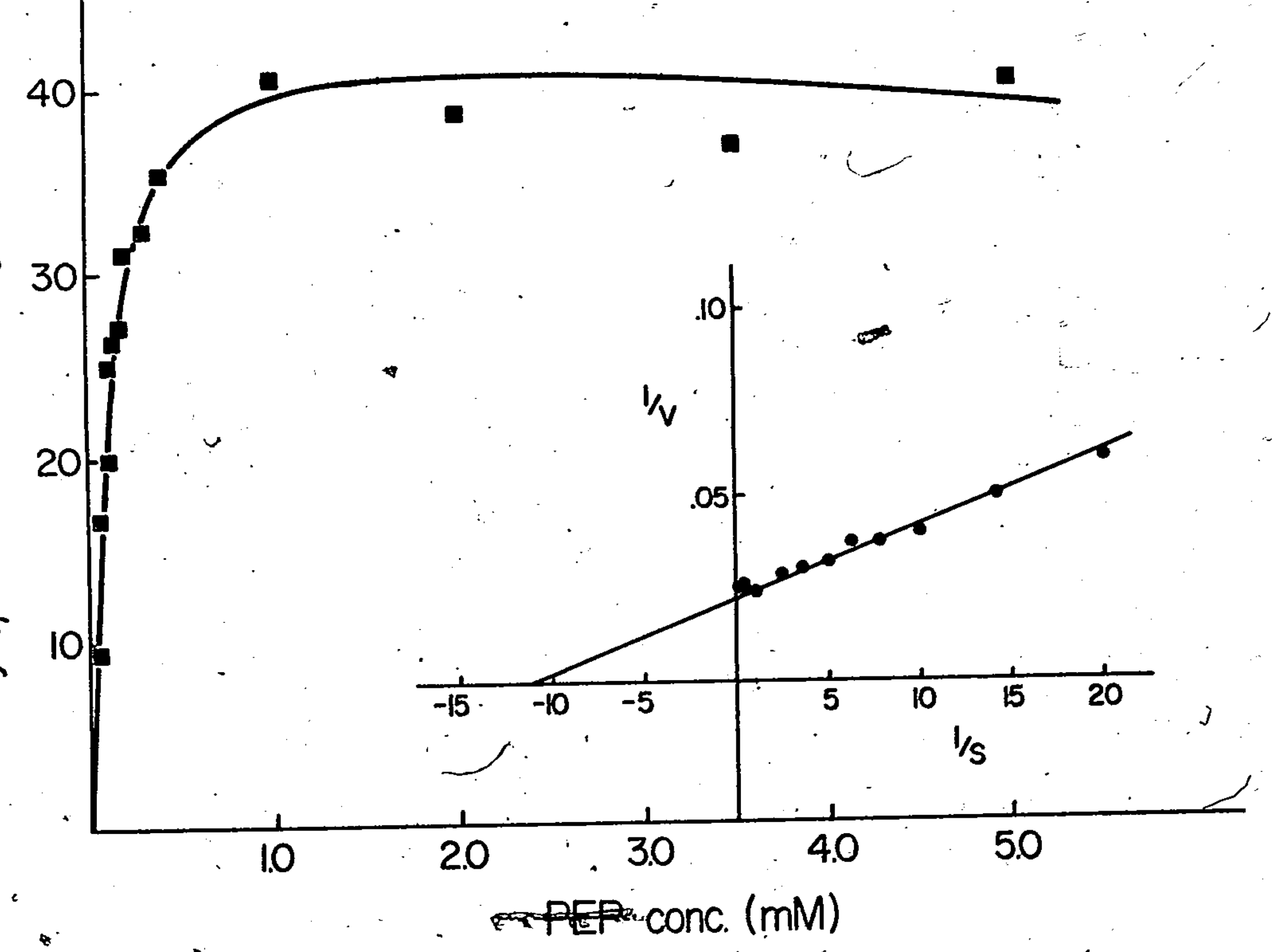
Data obtained from Fig. 3.



log (V/Vm-V)
2
1
0
-1
-2
0.2
0.4
0.6
0.8
1.0
1.2
log S

Fig.5. Determination of the K_M for PEP.

Assays were performed in the presence of 1.0 mM FDP. Inset is a double reciprocal plot of the data. V refers to the velocity and S refers to the PEP concentration.


Velocity (μmoles PEP/min/mg PKi)
PEP conc. (mM)
1/V
1/S
10
20
30
40
1.0
2.0
3.0
4.0
5.0
.10
.05
-15
-10
-5
5
10
15
20

and 42.7 μmoles PEP utilized/min/mg PKI respectively were calculated from double reciprocal plots of the data (Fig. 5; inset).

A similar plot of enzyme velocity against ADP concentration (Fig. 6) gave a K_m of 0.229 mM and a V_{max} of 39.6 μmoles PEP utilized/min/mg PKI. ADP appeared to be inhibitory at concentrations in excess of 1.0 mM.

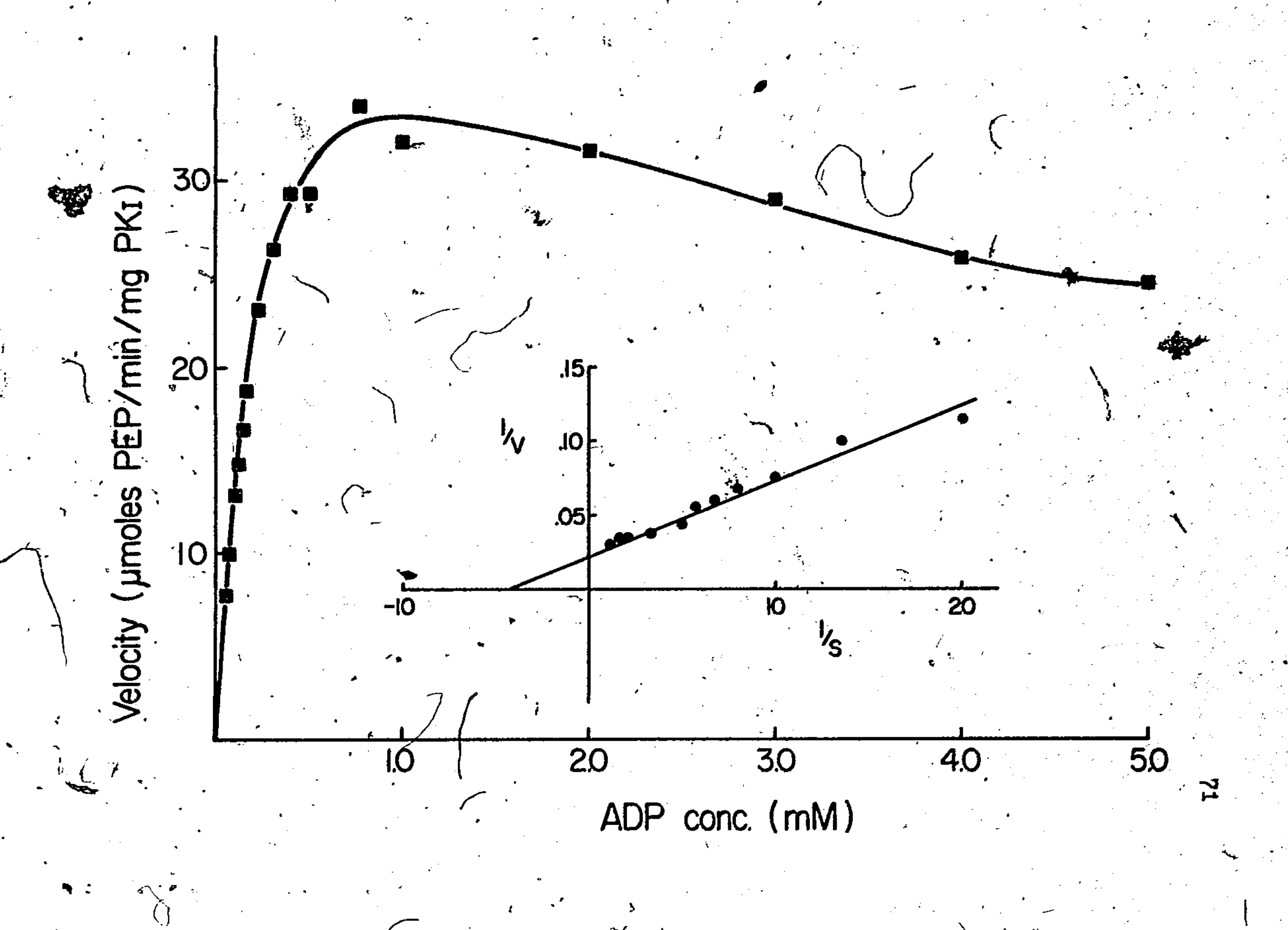
Homotrophic interactions for the allosteric activator FDP (161) were observed in the saturation curve illustrated in Fig. 7. An n value of 2.56 was calculated from the Hill plot (Fig. 8).

Using PKI isolated from a strain of E. coli K12, Waygood and Sanwal (261) have reported the following kinetic values; $K_{\frac{1}{2}PEP}$ = 4.0 mM; K_{mPEP} = 0.03 mM; n_{PEP} = 3.0; K_{mADP} = 0.24 mM and V_{max} = 55 μmoles PEP utilized/min/mg PKI. With the exception of the K_m for PEP, these values are in fairly good agreement with the ones we have presented here.

When PEP and PKI were mixed to start the reaction, the initial velocity observed was non-linear. This may have been due to a conformational change in the enzyme induced by PEP binding (51). This phenonenon was not observed when ADP replaced PEP; therefore ADP was used to initiate the reaction in all further experiments.

Fig. 6. Determination of the K_M for ADP.

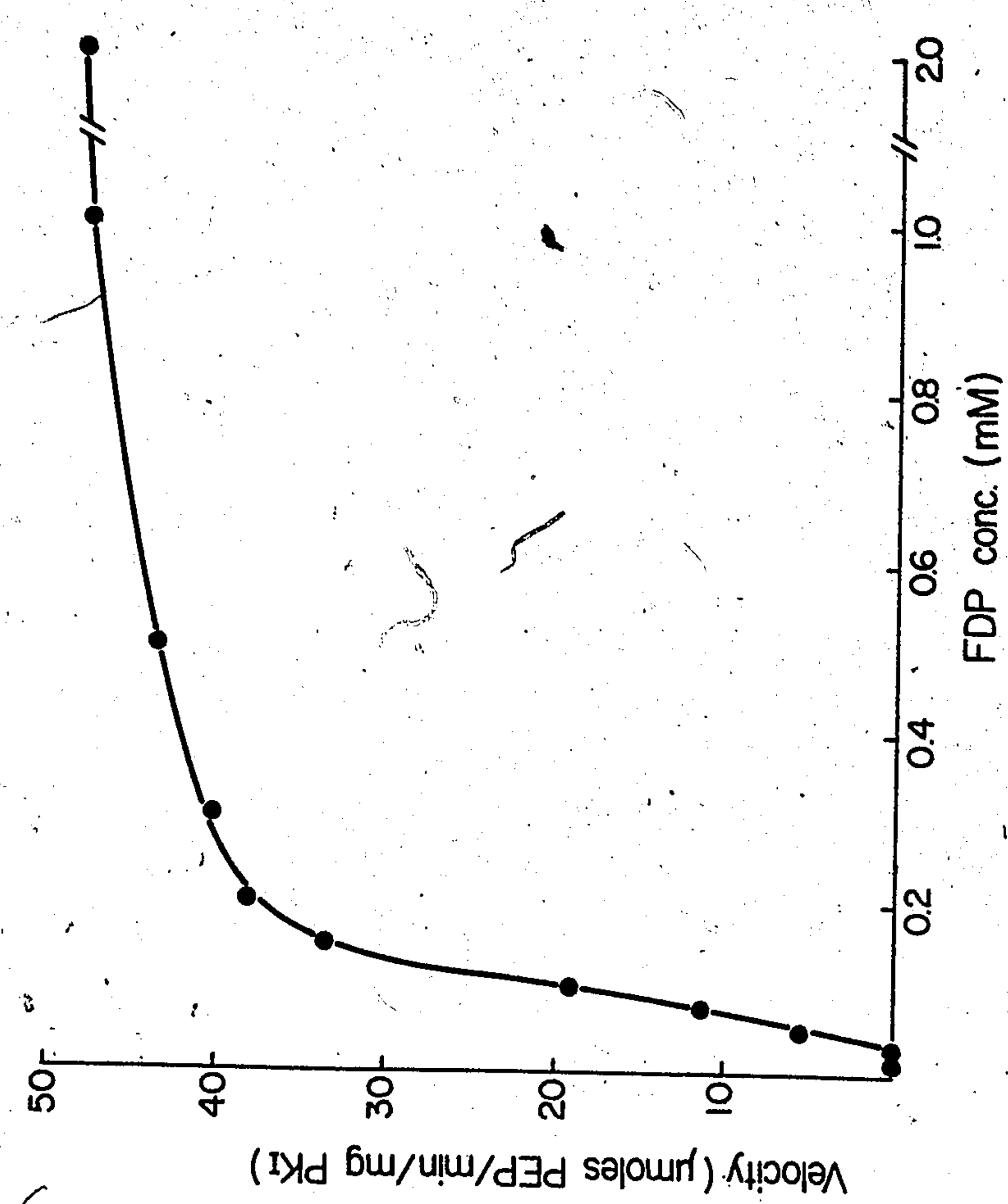
The inset is a double reciprocal plot of the data. where V refers to the enzyme velocity and S refers to the ADP concentration.



Velocity (μmoles PEP/min/mg PKI)
30
20
10
1.0
2.0
3.0
4.0
5.0
ADP conc. (mM)
1/V
.15
.10
.05
-10
10
20
1/S

Fig. 7. Effect of FDP concentration on PKI Activity.

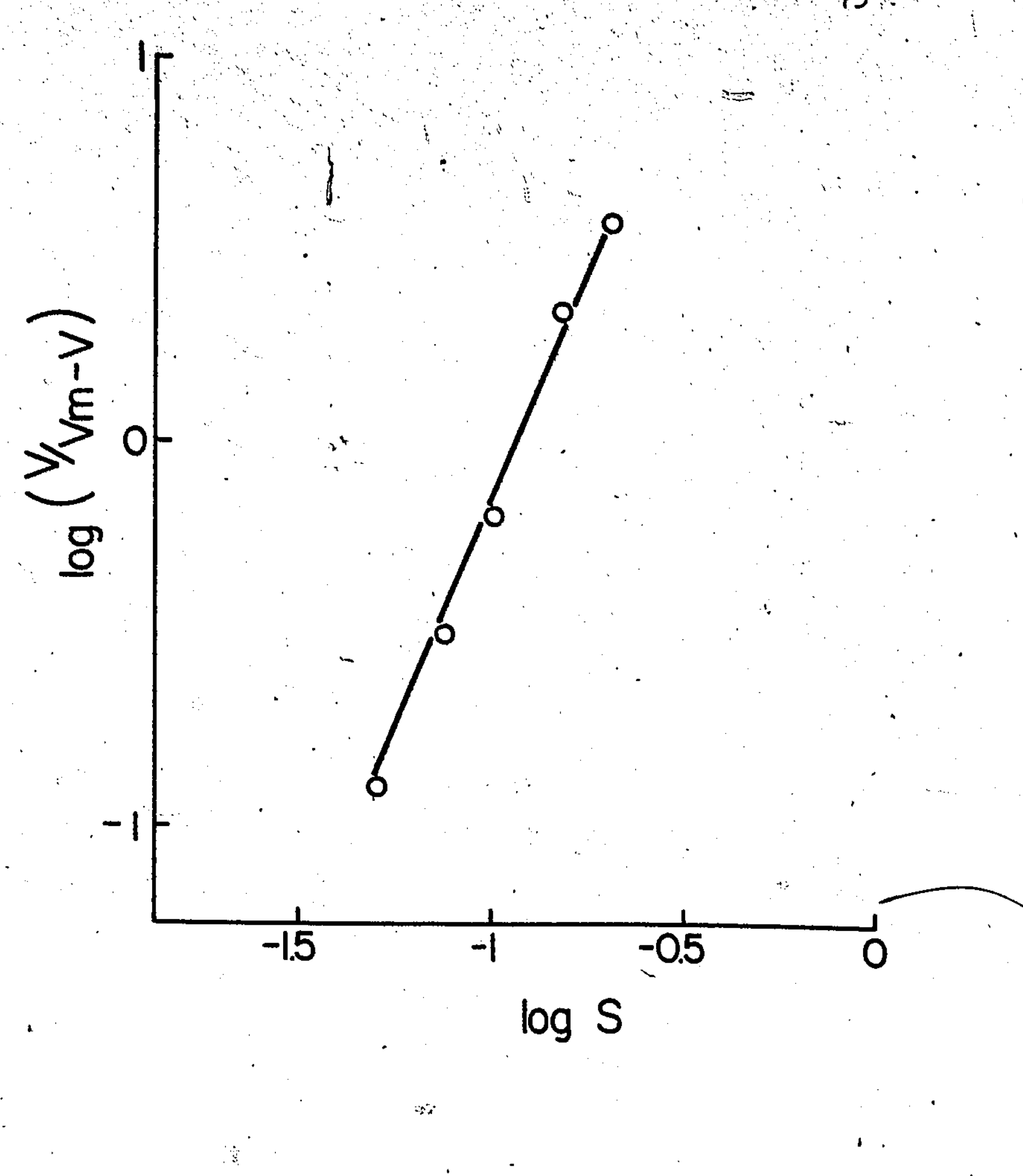
PEP was employed at a concentration of 0.5 mM. All other reagents were as described in Materials and Methods.



Velocity (µmoles PEP/min/mg PKI)
50
40
30
20
10
0.2
0.4
0.6
0.8
1.0
20
FDP conc. (mM)

Fig. 8. Determination of the Hill Number for FDP.

Data was obtained from Fig.7.


1
0
-1
log (V/Vm-V)
-1.5
-1
-0.5
0
log S

When the assay conditions for PKI were employed as described in Materials and Methods, a plot of velocity against enzyme concentration (Fig. 9) produced a straight line.

Preliminary experiments indicated that PKI from E. coli B had an absolute requirement for K^+. This phenomenon was not observed with the enzyme from E. coli K12 (148,261). Maximum activity was attained at 75 mM K^+ (Fig. 10). The saturation curve in Fig 10 was sigmoidal suggesting that K^+ was an allosteric cofactor for PKI and an n value of 2.51 was obtained from the Hill plot (Fig.11).

iii. Inhibition of PKI by PF

PKI was completely inhibited by 0.8 mM PF (Fig. 12). The inhibition curve was sigmoidal and a Hill plot of these data (Fig. 13) gave an n value of 1.96 indicating that PF is an allosteric inhibitor of PKI (161).

iv. Effect of PF on PEP Homotrophic Interactions

According to the model for allosteric enzymes proposed by Monod et al (161), an allosteric inhibitor should increase the homotrophic interactions of the allosteric substrate (i.e. increase the n value).

Fig. 14 shows the effect of PF on the PEP saturation

Fig. 9. Effect of Protein Concentration on PKI Activity.

Velocity (µmoles PEP/min)

80
70
60
50
40
30
20
10

.5
1.0
1.5
2.0
2.5

PKI conc. (µg/ml)

Fig.10. Effect of K^+ concentration on PKI Activity.

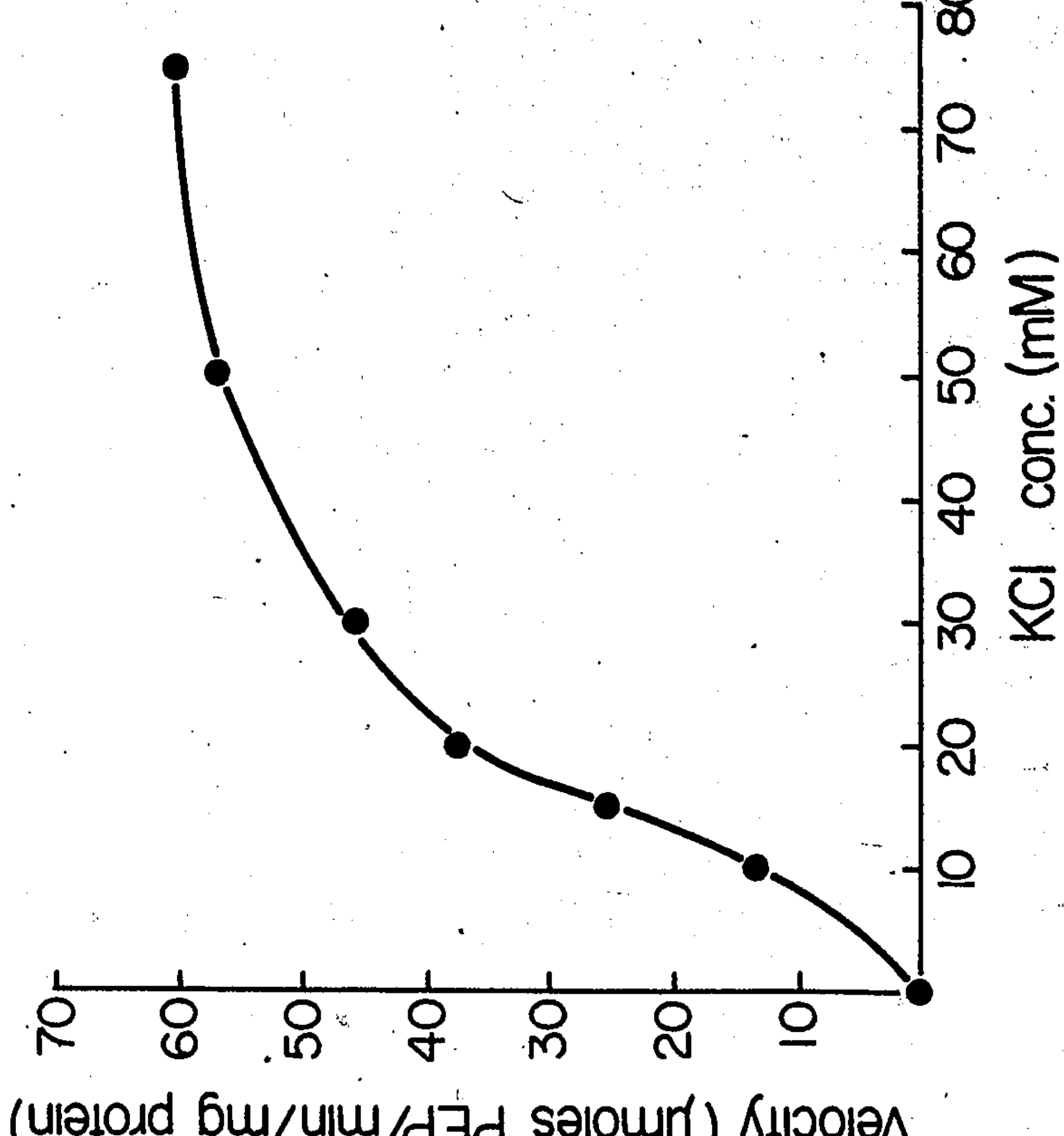

Velocity (μmoles PEP/min/mg protein)
10
20
30
40
50
60
70
KCl conc. (mM)
10
20
30
40
50
60
70
80

Fig. 11. Determination of the Hill Number for K^+.

Data was obtained from Fig. 10.

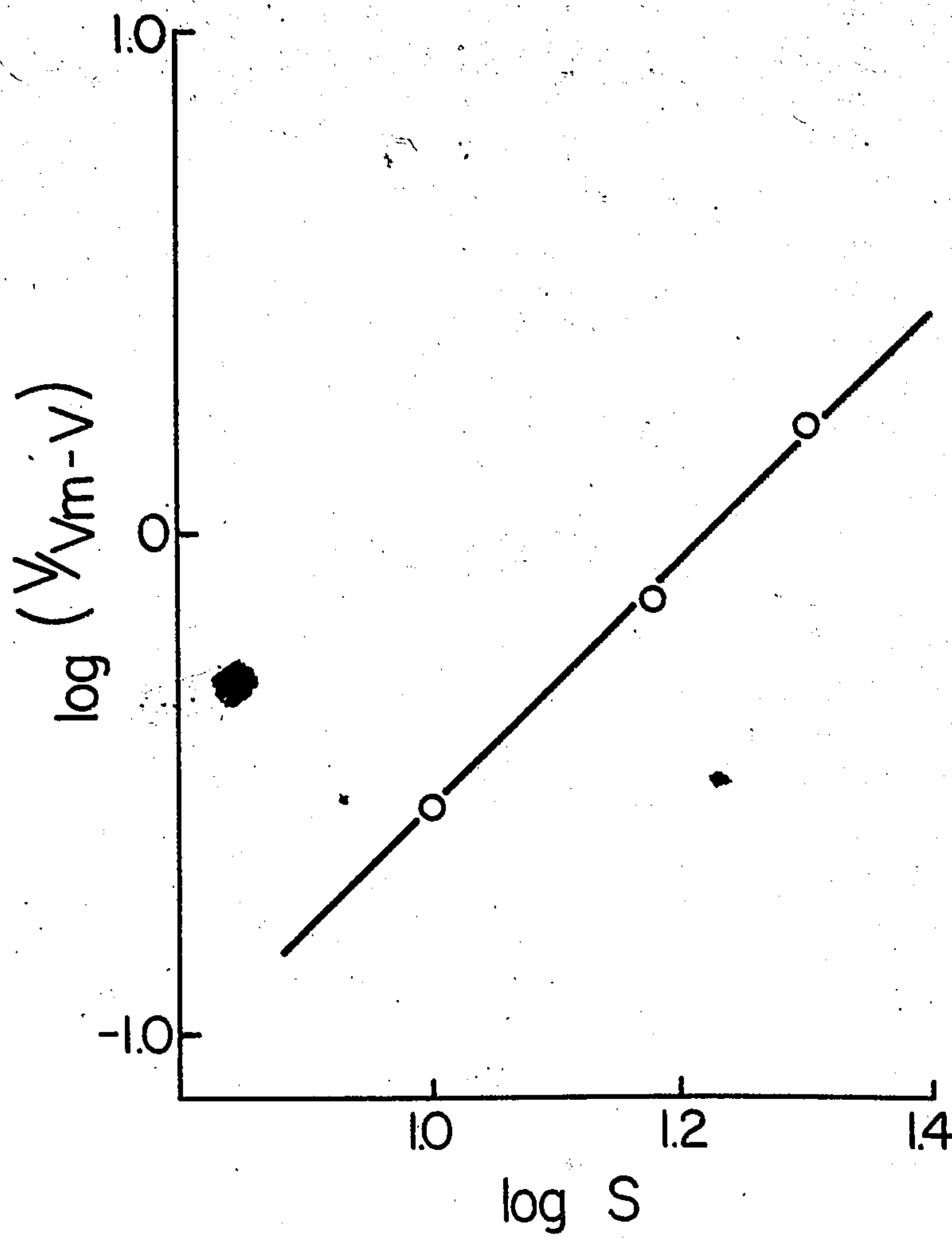

1.0
0
-1.0
log (V/Vm-V)
1.0
1.2
1.4
log S

Fig. 12. Effect of PF Concentration on PKI Activity.

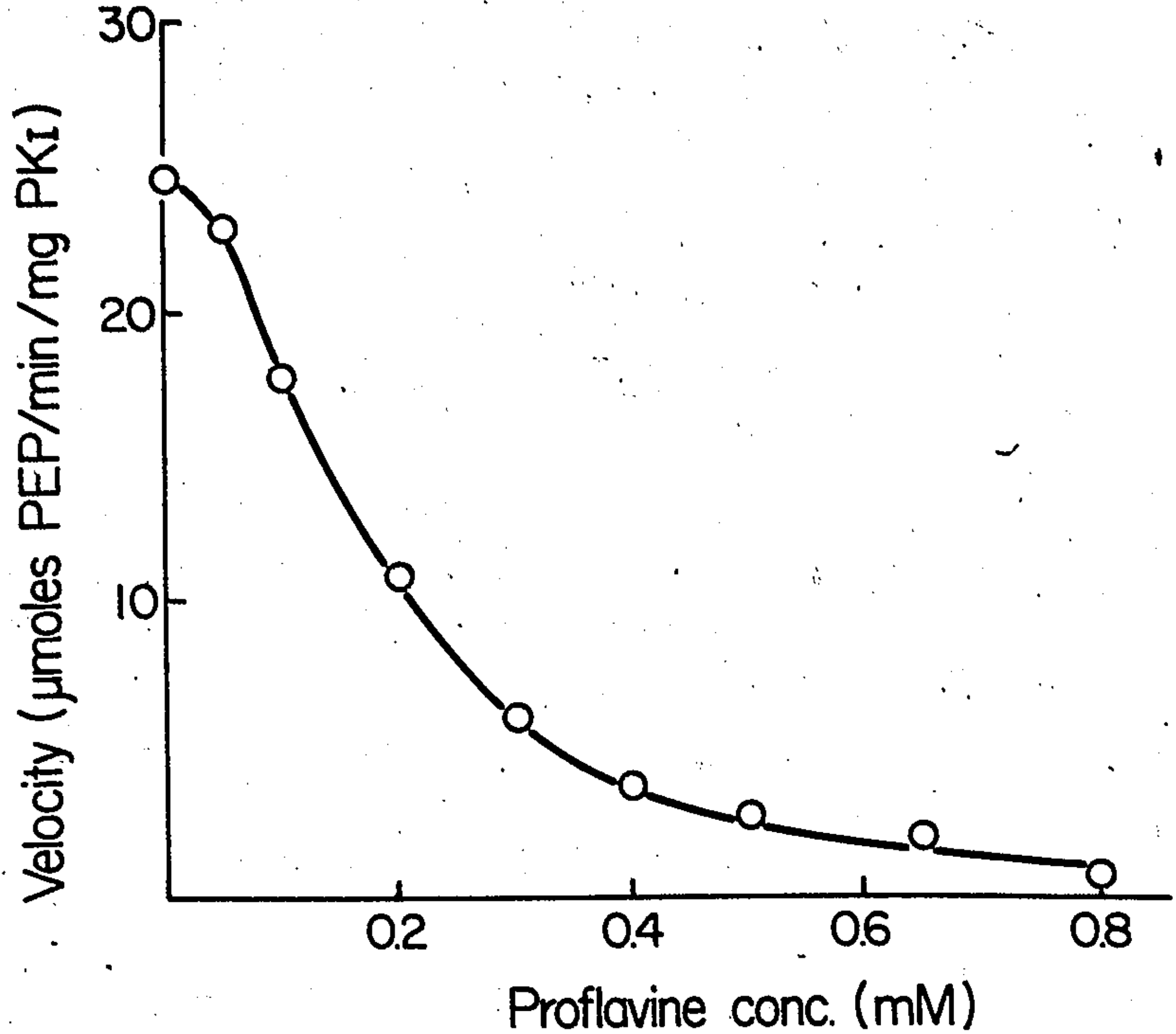
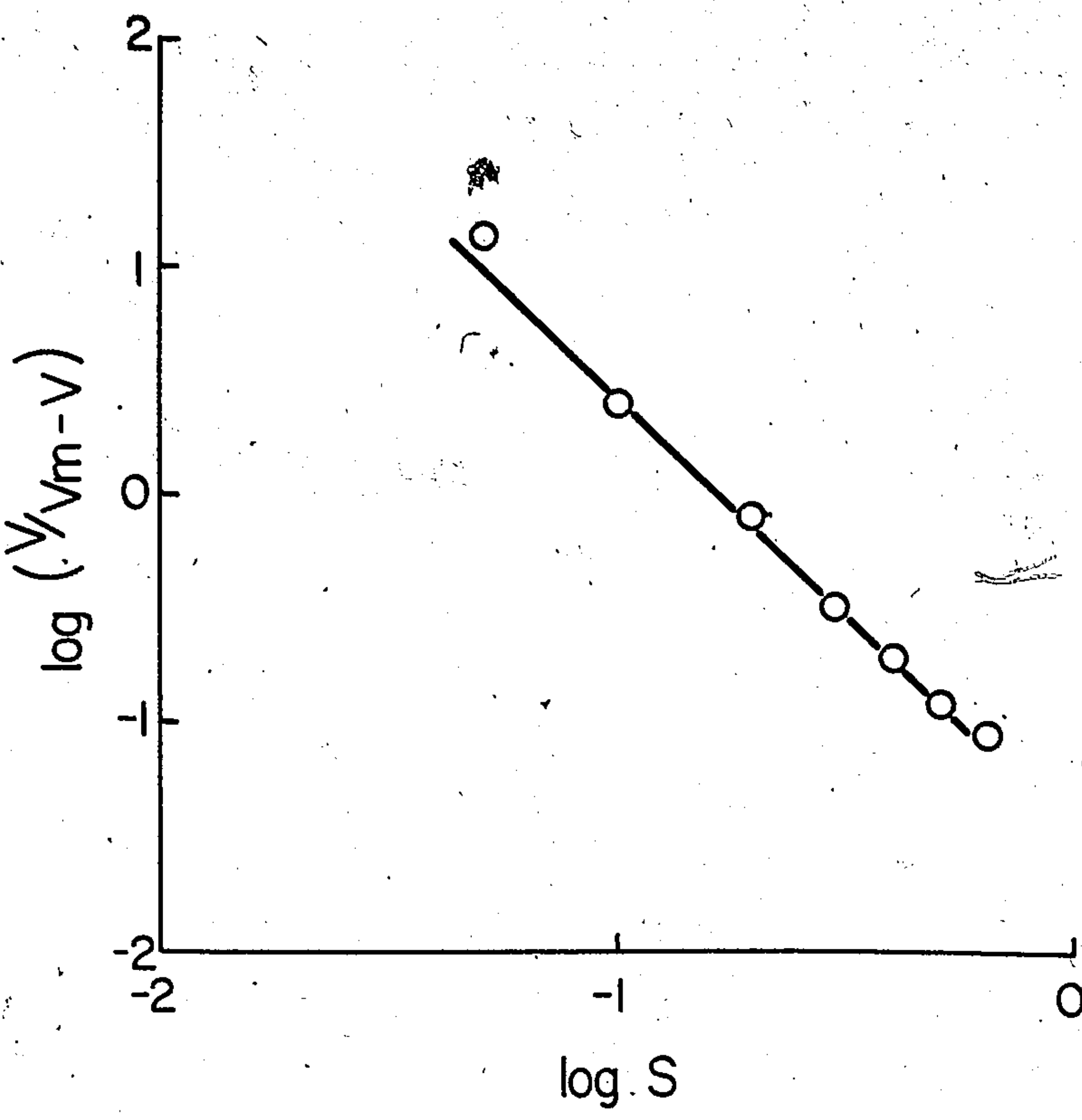

30
20
10
Velocity (μmoles PEP/min/mg PKI)
0.2
0.4
0.6
0.8
Proflavine conc. (mM)

Fig. 13. Determination of the Hill Number For PF.

Data was obtained from Fig.12.



2
1
0
-1
-2
-2
-1
0
log (V/Vm-V)
log S

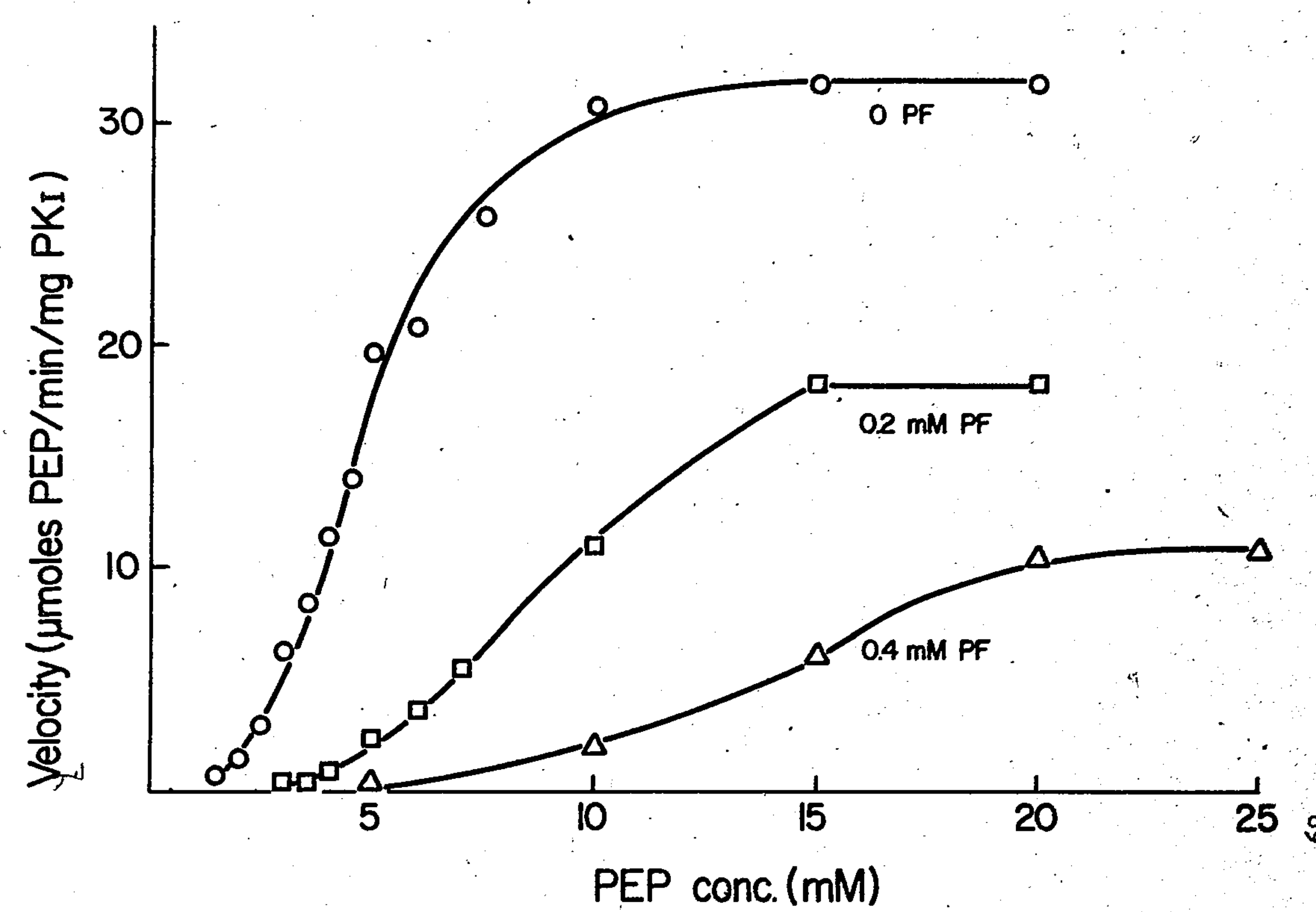
curve. It was observed that 0.2 mM PF increased the $K_{\frac{1}{2}}$ for PEP as expected for an allosteric inhibitor (161); however, it had no effect on the n value (Fig. 15). Homotrophic interactions for PEP are already quite strong (i.e. the n value of 3.36 is close to 4.0, the number of subunits (261)) and Rubin and Changeaux (205) have shown that when these interactions are strong, the allosteric inhibitor would not be expected to increase the substrate n value.

Fig. 14 also shows that PF significantly lowered the V_{max} for PKI; however, allosteric inhibitors would not be expected to affect the V_{max} (161). A similar phenomenon has been previously observed for other allosteric enzymes (145,205) and may be due to either "dead-end" inhibition at the substrate active site (145) or to "non-exclusive" ligand binding (205). The latter term refers to the ability of the inhibitor to bind to both conformational forms of the enzyme, albeit with different affinities (205).

v. Effect of FDP on PF Inhibition of PKI

Monod et al (161) have suggested that an allosteric activator will antagonize or reverse the action of an allosteric inhibitor. In accordance with this, it was found that low concentrations of FDP could significantly

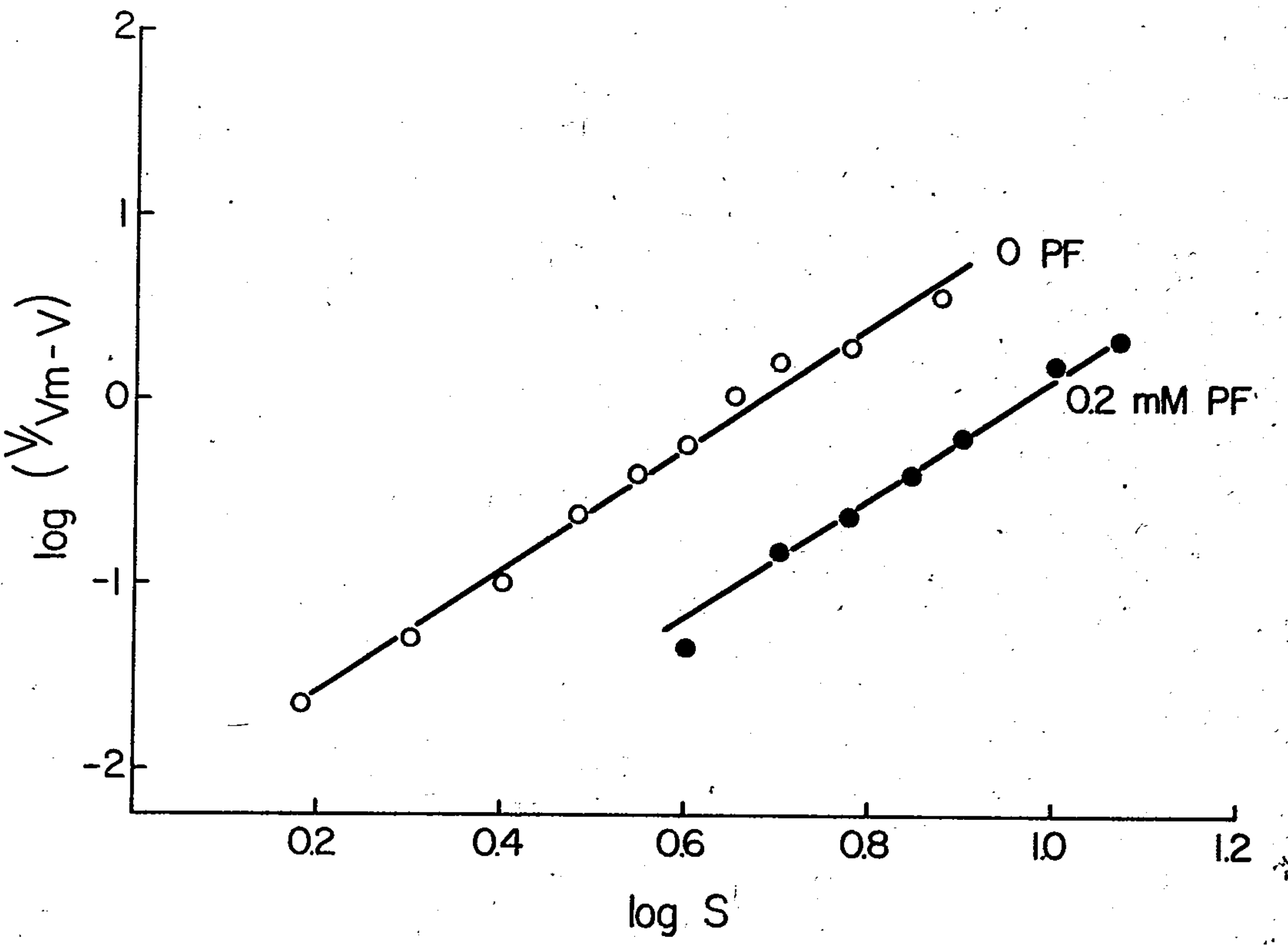
Fig. 14. Effect of PF Concentration on the PEP Saturation Curve.



Velocity (µmoles PEP/min/mg PKI)
30
20
10
0 PF
0.2 mM PF
0.4 mM PF
5
10
15
20
25
PEP conc. (mM)

Fig. 15. Effect of PF on the Hill Number for PEP.

PF concentration was 0.2 mM.
Data was obtained from Fig. 14.



log (V/Vm-V)
2
1
0
-1
-2
0.2
0.4
0.6
0.8
1.0
1.2
log S
0 PF
0.2 mM PF

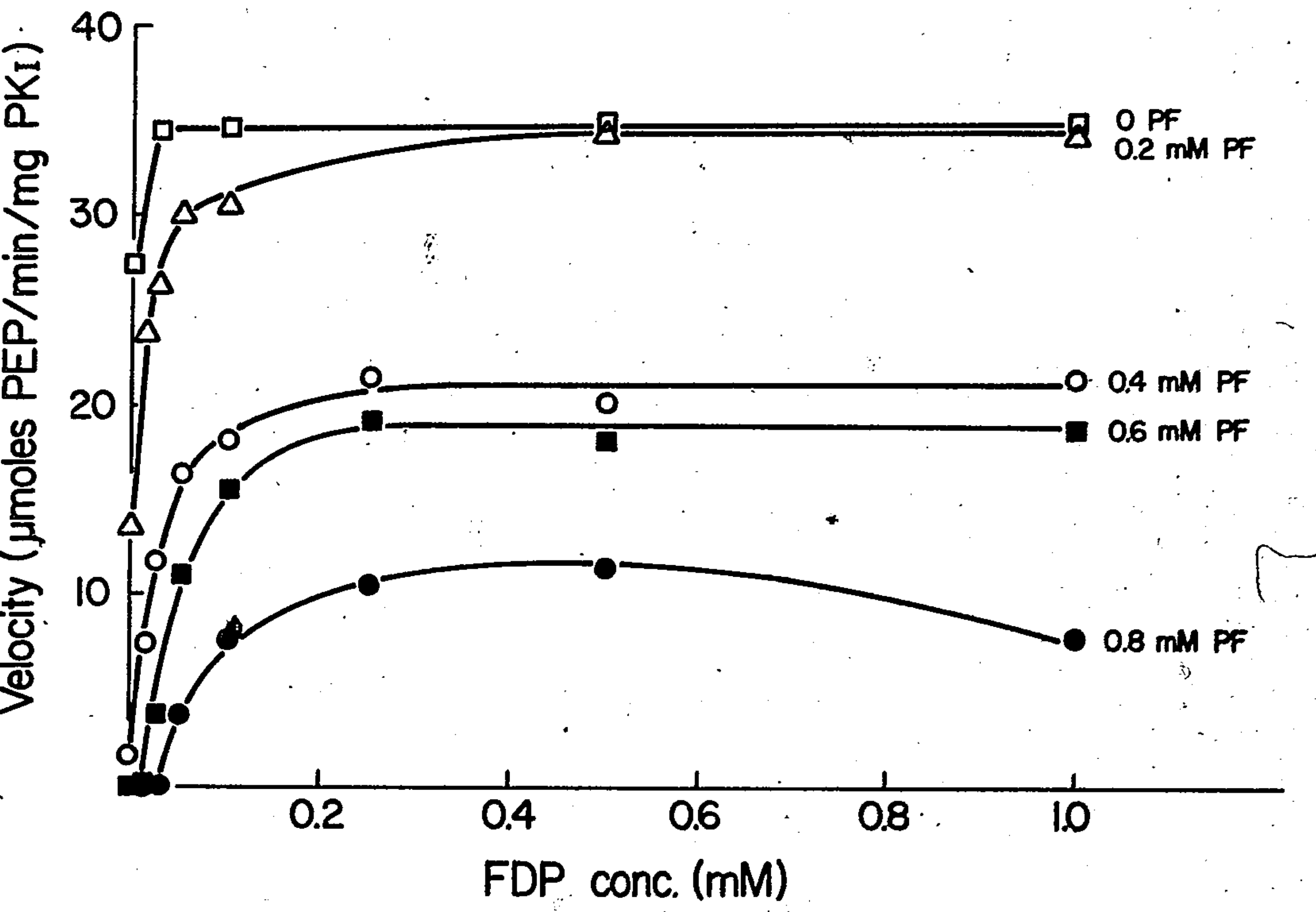
reverse the inhibition produced by 0.2 - 0.8 mM PF (Fig. 16). Maximum antagonism was observed at 0.25 mM FDP for all concentrations of PF, suggesting that PF does not bind to the same site as FDP. At high levels of PF, FDP was unable to completely reverse the inhibition. This phenomenon may be due to "dead-end" inhibition or the "non-exclusive" binding mentioned earlier.

vi. Effect of Other Metabolites on PF Inhibition of PKI

Several other metabolites namely cyclic AMP, AMP, fructose-6-phosphate (F6P) and dithiothreitol (DTT) were tested for their ability to substitute for FDP as PF antagonists. Cyclic AMP was employed since it was shown to relieve the inhibition of RNA synthesis by PF (57a). ATP is an allosteric inhibitor of PKI (261) while AMP is an allosteric activator of PKII (263). F6P was tested since it is similar in structure to FDP and DTT was used to determine if sulfhydryl groups are involved in PF inhibition of PKI. These compounds were tested at 1.0 mM and were found to be ineffective in reversing PF action, suggesting that the reversal phenomenon is a property of the allosteric activator only.

Fig. 16. Antagonism of PF Inhibition of PKI by FDP.

PEP concentration was 8.0 mM.



Velocity (μmoles PEP/min/mg PKI)
40
30
20
10
0.2
0.4
0.6
0.8
1.0
FDP conc. (mM)
0 PF
0.2 mM PF
0.4 mM PF
0.6 mM PF
0.8 mM PF

vii. Effect of Dilution on PF Inhibition of PKI

The effect of dilution on PF inhibition of PKI was also determined. Enzyme activity was determined in the presence of 0.15 and 0.30 mM PF (Table 4; top line). Duplicate samples were diluted with the reaction mixture prior to assaying in order to give a 1:1 dilution of PF and PKI (Table 4; bottom line). Control samples containing no PF were diluted in a similar fashion. The results show that activity increased on dilution and that the level of inhibition corresponded to the final rather than the initial PF concentration suggesting that at low levels of PF, inhibition was essentially reversible.

viii. Effect of Order of Reagent Addition

In several experiments, the order of addition of PEP, PF, PKI and FDP was varied prior to initiation of the reaction with ADP. It was found that the order of addition did not affect the extent of PF inhibition.

ix. Effect of PF on PKI from E. coli B/Pr

We wanted to find out if a modified PKI was responsible for PF-resistant glucose utilization in *E. coli* B/Pr. The enzyme was purified from *E. coli* B/Pr as described previously for the *E. coli* B enzyme. The two enzymes were found to be chromatographically and electrophoretically identical.

The enzyme from *E. coli* B/Pr was characterized

Table 4

Effect of Dilution on PF Inhibition of PKI

Procedure \ Initial PF conc (mM)	Velocity (% of Control) 0.15	0.30
PF added then assayed	45.40	17.86
PF added, diluted 1:1 then assayed	69.00	41.10

Control velocity was 75.03 μmoles PEP utilized/min/mg PKI.

All values have been corrected for dilution.

kinetically as described previously and the data in Table 5 shows that the enzymes from the two strains have similar kinetic properties. Fig.17 shows the effect of PF on the two enzymes in the presence and absence of FDP. PKI from B/Pr did not show any significant resistance to PF under the conditions studied, suggesting that PF resistance does not occur at the level of this particular enzyme.

x. Effect of Various Acridines on PKI from E. coli B

In order to determine the structural requirements for inhibition of PKI by PF, a variety of acridines related to PF were tested for their ability to inhibit PKI from *E. coli* B (Table 6).

3-amino-6-iodoacridine and unsubstituted acridine were effective inhibitors of PKI. Mepacrine, acriflavine, acridine yellow, 9-aminoacridine and 9-amino-2-hydroxy-acridine produced significantly less inhibition than PF while acridine orange and 2,6-diaminoacridine were essentially ineffective inhibitors of PKI. Ethidium bromide, a planar molecule having a similar effect as acridines on nucleic acids (91a) was unable to inhibit PKI.

These results suggest that a complete acridine ring is required for effective inhibition. Acridines ionizing as cations are most effective if they posess a substi-

Table 5

Comparison of the Kinetic values for PKI from

E coli B and B/Pr

	B	B/Pr
n_{PEP}	3.36	3.61
n_{FDP}	2.56	2.72
n_{PF}	1.96	2.09
$K_{\frac{1}{2}}PEP$	5.0	4.0
V_{max}	31.5 - 96.5	45.0 - 49.2

Values for $K_{\frac{1}{2}}$ are given in mM.

Values for V_{max} are given in µmoles PEP utilized/min/mg PKI.

The wide range in the Vmax for the E.coli B enzyme is due to variations in the activity of the different enzyme preparations.

Fig. 17. Effect of PF Concentration on PKI Activity from *E. coli* B and B/Pr in the Presence and Absence of FDP.

FDP concentration was 1.0 mM.

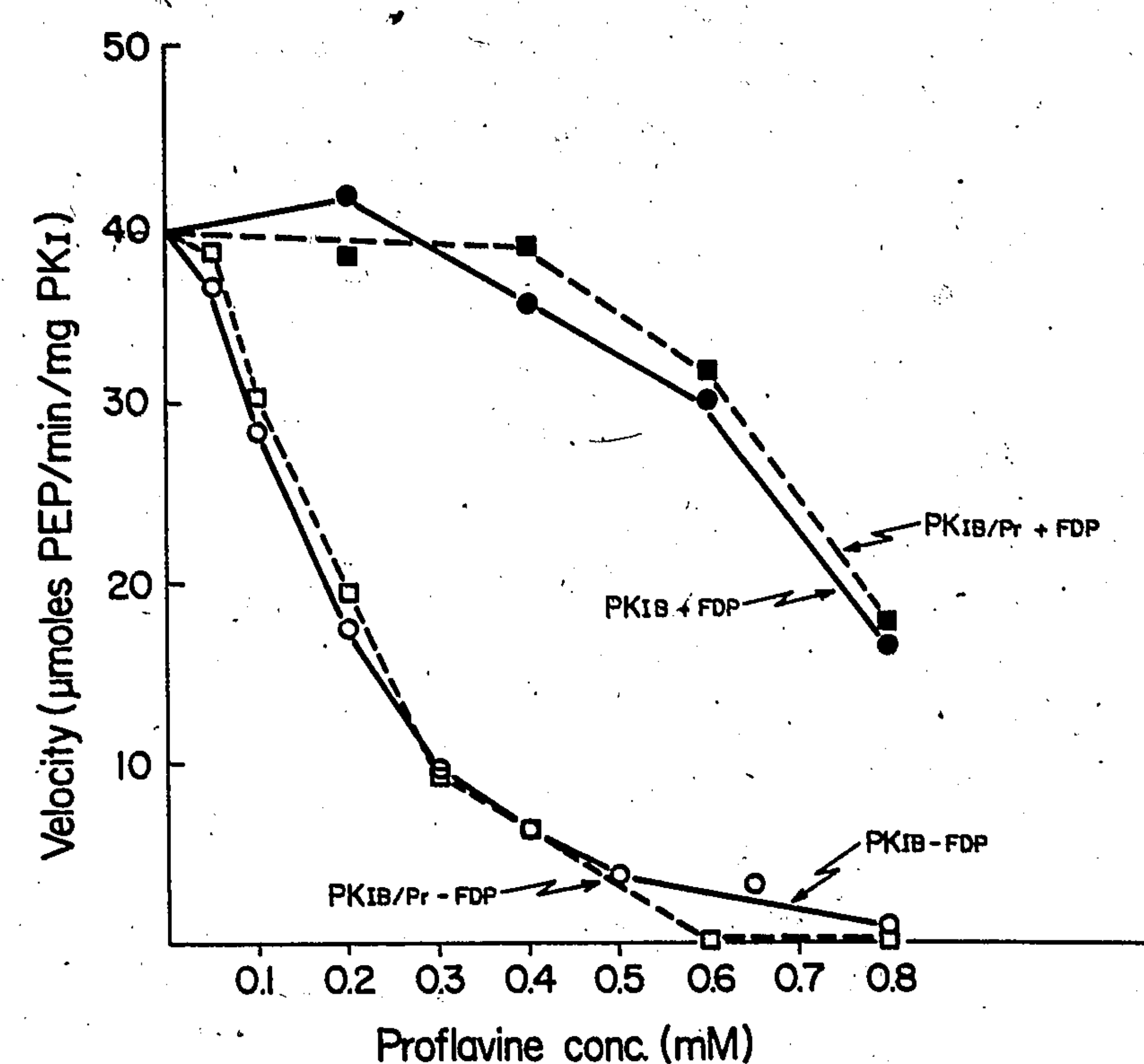

Velocity (µmoles PEP/min/mg PKI)
50
40
30
20
10
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Proflavine conc. (mM)
PKIB/Pr + FDP
PKIB + FDP
PKIB - FDP
PKIB/Pr - FDP

Table 6

Inhibition of PKI by Various Substituted Acridines

Acridine (0.2mM)	% Inhibition
3-amino-6-iodoacridine	63.26
Proflavine	57.67
Acridine	50.48
Mepacrine	24.92
Acriflavine	24.91
Acridine Yellow	12.14
9-aminoacridine	5.75
9-amino-2-hydroxyacridine	5.75
Acridine Orange	0
2, 6-diaminoacridine	0
Ethidium bromide	0

Control velocity was 89.75 µmoles PEP utilized/min/mg PKI.

tution at the 3 position. Unionized, unsubstituted acridine was an effective inhibitor of PKI reflecting the possible role of hydrophobic enzyme sites in inhibition. The presence of bulky side groups on some of the acridines (mepacrine and acridine orange) may account for their reduced effectivness as inhibitors.

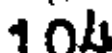
D/. Role of Growth Media on PF Sensitivity

When *E. coli* was grown on glucogenic carbon sources such as pyruvate, succinate, acetate and glycerol, PKI was not present in large amounts and was relatively unimportant for growth (148,261). Since PKI appears to be an important PF-sensitive site in *E. coli* B, it was thought that cells grown in the absence of glucose might be less sensitive to the inhibitory effects of PF than cells grown in glucose. Studies were then initiated to determine the effect of PF on stationary and logarithmic cells in a variety of growth media.

For initial experiments, stationary cells from a particular growth media were added to flasks containing fresh media and different concentrations of PF. Growth was then determined after 17 h at 37C. Fig.18 shows that acetate- and succinate-grown cells were very sensitive to PF and were completely inhibited at a concentration of 0.02 mM. Dye concentrations of 0.05 mM

Fig. 18. Effect of PF Concentration on Growth of *E. coli* B in Various Media.

Growth was determined at 17 h. Control growth values, in A_{660} units were: glucose, .46; pyruvate, .64; glycerol, .63; acetate, .68; and succinate, .61.



Pyruvate
Glucose
Glycerol
Succinate
Acetate
Growth (% of control)
100
80
60
40
20
.025
.050
.10
Proflavine conc. (mM)

or greater were required to inhibit cells grown in glucose, glycerol and pyruvate.

PF was also added to cells growing in the logarithmic phase in various growth media. Generation times for cells on each media in the presence and absence of 0.05 mM PF were calculated and are presented in Table 7. E. coli grew well in glucose and glycerol with generation times of approximately 1 h and was essentially unaffected by 0.05 mM PF. Generation times in succinate and acetate were 2.0 h and 2.5 h respectively. In these media, cells grew poorly and were completely inhibited by PF.

Our results show that PF sensitivity cannot be related directly to PKI content. Cells grown in glucose and glycerol are equally sensitive to PF (Fig. 18) but contain different levels of PKI. A close correlation exists between PF sensitivity and generation time suggesting that cells with a low metabolic rate are more sensitive to the dye.

It is possible that cells grown in different media are equally sensitive to bound PF. The apparent high sensitivity of acetate- and succinate-grown cells would be due to the inability of these cells to generate sufficient energy to actively release the dye. If this is the case, equimolar levels of bound PF would be required to produce the same level of inhibition in all growth media.

Table 7

Effect of PF(0.05mM) on Generation Time of E coli B in Various Media

Carbon Source	Generation Time (h)	Generation Time +PF(h)
Glucose (7mM)	0.91	1.24
Glycerol (15mM)	1.16	1.30
Pyruvate (15mM)	1.66	3.03
Succinate (15mM)	2.00	∞
Acetate (15mM)	2.50	∞

Table 8 shows that five times more PF had to be added to glucose-grown cells than to cells growing in acetate to achieve 50 - 60 % inhibition. Cells growing in glucose bound proportionally more of the dye. These results suggest that if PF inhibits the same site regardless of the media, this site may be more sensitive in acetate-grown cells due to their low rate of metabolism. It is also possible that specific pathways required for growth on acetate are highly PF-sensitive.

Discussion

Pyruvate kinase has been isolated from several eucariotic sources including *Neurospora crassa* (125), human erythrocytes (47), dog erythrocytes (21), rat liver and muscle (245), yeast (106,110,260) and *Rana pipens* (81) as well as from a variety of bacteria (23, 57,58,69,72,139,147,175,183,255,280). *E. coli* has been shown to possess two pyruvate kinases; PKI and PKII (147,262). Growth on glucose promotes the induction of high levels of PKI (148) and the formation of high concentrations of FDP which is the allosteric activator for the enzyme (143). This suggests that PKI plays an important role in glycolysis. PKII, on the other hand, is constitutive and is involved in glucogenesis (148,263). This enzyme is regulated by AMP and may be responsible for producing ATP at the expense of PEP under conditions of low energy charge (148).

Table 8

Relationship Between PF Binding and Inhibition of Growth of E coli B in Two Different Media

Carbon Source	PF (mM)	Time after PF addition (h)	nmoles PF bound/ mg dry wt. cells	Growth (% of Control)
Glucose	0.10	1.0	12.0	28.8 (.090)
"	"	1.5	11.6	38.5 (.130)
"	"	2.0	13.7	37.5 (.160)
Acetate	0.02	2.0	2.60	47.6 (.105)
"	"	3.0	1.55	44.8 (.145)
"	"	4.0	2.36	36.9 (.230)

Bracketed figures represent growth, in A660 units, of control cells not treated with PF.

In this chapter, we have shown that accumulation of αMG by whole cells of E. coli was only partially inhibited by PF while all glycolytic enzymes with the exception of PKI were little affected by the dye. We have also found that LDH, mediating the breakdown of pyruvate to lactate was not inhibited by up to 0.4 mM PF. PKI is the only PF-sensitive site between glucose and lactate and we therefore propose that inhibition of PKI by PF is responsible for the inhibition of glucose utilization.

It would be useful to be able to demonstrate that inhibition of glucose utilization in vivo is a direct result of inhibition of PKI. Unfortunately, determinations of intracellular concentrations of PF cannot easily be made due to the number of cellular binding sites available to the dye (96). Using data from Fig. 23 and assuming an intracellular volume of 2.7 ml/g dry wt cells (270a), we have calculated that, under conditions of complete inhibition of glucose utilization, the intracellular PF concentration was 4.0 mM, assuming all the dye wasunbound. This is unlikely to be the case; however, PKI would be inhibited by 85 % if only 10 % of the total PF in the cell were free.

The intracellular concentration of FDP in logarithmically growing E. coli is 2.4 mM (143), much higher

than the level required to produce maximum reversal of PF inhibition suggesting that PF cannot completely inhibit PKI in vivo. Since this enzyme is involved in the regulation of glycolysis, however, partial inhibition by PF could result in a significant and possibly complete inhibition of glucose utilization.

We have presented evidence that PF is an allosteric inhibitor for PKI and as such would be expected to occupy specific inhibitor binding sites (161). PF does not appear to bind directly to the FDP site but it may be involved to some extent with the active site for PEP. As a result, PF may inhibit PKI by acting at several unrelated sites. 1,8-anilino-naphthalene sulfonate (ANS), an inhibitor of N. crassa pyruvate kinase (123,124), has been associated with the sites for FDP and PEP as well as other unrelated sites (123,124).

The requirements for acridine binding to PKI have yet to be fully determined. Preliminary results have indicated that acridine binding to, and inhibition of, PKI requires substitution at the 3 position on the acridine ring. The reduced inhibitory effeciency of some acridines such as mepacrine and acridine orange may be attributed to the presence of bulky side groups preventing entry of these dyes to the binding site.

Unsubstituted acridine, an effective inhibitor of PKI, is highly lipophilic and may bind to hydrophobic regions of the enzyme. ANS has been shown to inhibit pyruvate kinase from N. crassa by interacting with hydrophobic sites (124) and it is possible that the inhibition of PKI by acridine dyes involves hydrophobic regions of the enzyme to some extent.

Fluorescent probes have been used extensively to study the reaction mechanism of allosteric enzymes. ANS has been employed in studies on pyruvate kinase from N. crassa (123,124) and rabbit muscle (115), phosphofructokinase (141,32) and glyceraldehyde-3-phosphate dehydrogenase (117) from E. coli as well as transaldolase (37) and cholinesterase (49). N-methyl-acridinium iodide has also been used as a fluorescent probe for horse serum cholinesterase (46). As mentioned in the General Introduction, PF was found to inhibit αCT and stimulate papain and ficin and has been used to elucidate the reaction mechanism of these enzymes. We have presented the first evidence to suggest that this dye can inhibit an important regulatory enzyme and we propose that this phenomenon could be useful in studying the structure-function relationships of PKI.

We have shown that PKI from E. coli B/Pr is not

significantly resistant to PF. This is somewhat disappointing in light of the fact that an in vitro acridine-resistant site has yet to be demonstrated in any bacterium. Acridine resistance in bacteria, as mentioned in the General Introduction, has often been ascribed to changes in cell permeability or dye binding capacity. It is therefore not unreasonable to believe that PF resistance in *E. coli* B/Pr lies at the membrane rather than the enzyme level.

We have found that PKI from *E. coli* B had an absolute requirement for K^+. Recently, Waygood *et al* (260a) have shown that K^+ is required for PEP binding to the enzyme from *E. coli* K12. Pyruvate kinase from *Bacillus sp.* has also been shown to have a K^+ requirement (69,255).

Monovalent cations have been shown to regulate glycolysis in mammalian systems (21) and it may be suggested that K^+ serves a similar function in *E. coli*. Regulation of glycolysis is being studied in *E. coli* (261); however, the role of K^+ has not yet been taken into account.

Mixing PKI and PEP to start the enzyme reaction

resulted in a non-linear initial velocity and we have suggested that PEP binding produced a conformational change similar to that described by Citri (51). This phenomenon has not been previously reported in studies of pyruvate kinase from other bacteria, however, conformational changes due to the presence of Tris have been observed in the pyruvate kinase of _N. crassa_ (126).

According to the model of Monod _et al_ (161), conformational changes in allosteric enzymes are not rate-limiting. Our enzyme may represent a deviation from this particular aspect of the model or possibly PEP is involved in more than one kind of conformational change. In all our studies with PKI, conformational changes in the enzyme produced by PEP, FDP and PF (if any) were allowed to take place prior to initiation of the reaction.

It is difficult to assess the importance of PKI inhibition in the inhibition of growth by PF. We found that cells growing poorly (i.e. long generation time) in a particular carbon source appear more sensitive to PF than cells growing in a media that supports rapid growth. Slow growing cells may have low metabolic activity and as such might have fewer PF-sensitive sites. Inhibition would therefore take place at lower levels of bound PF than in rapidly growing cells. It

is also possible that these cells may require specific enzymes for growth that are highly sensitive to PF. Cells growing in glucose and glycerol are equally sensitive to PF; however, glycerol-grown cells have low levels of PKI (262) and FDP (143). PF may therefore inhibit a common site in glucose- and glycerol-grown cells in which case the inhibition of glucose utilization by the effect of PF on PKI may not make a significant contribution to the inhibition of growth.

Chapter 4

RELATIVE PF SENSITIVITY OF GROWTH, GLUCOSE UTILIZATION AND DNA SYNTHESIS

RELATIVE PF SENSITIVITY OF GROWTH, GLUCOSE UTILIZATION AND DNA REPLICATION

Introduction

Considerable evidence implicates DNA replication as the primary site of acridine inhibition. Since we have shown that PF inhibited glucose utilization in washed cells of sensitive but not resistant E. coli, we wanted to determine if glucose utilization in growing sensitive cells was more or less sensitive than DNA replication to PF. Experiments were performed to determine the rate of growth, glucose utilization and DNA replication in cells of E. coli B growing logarithmically in the presence and absence of PF.

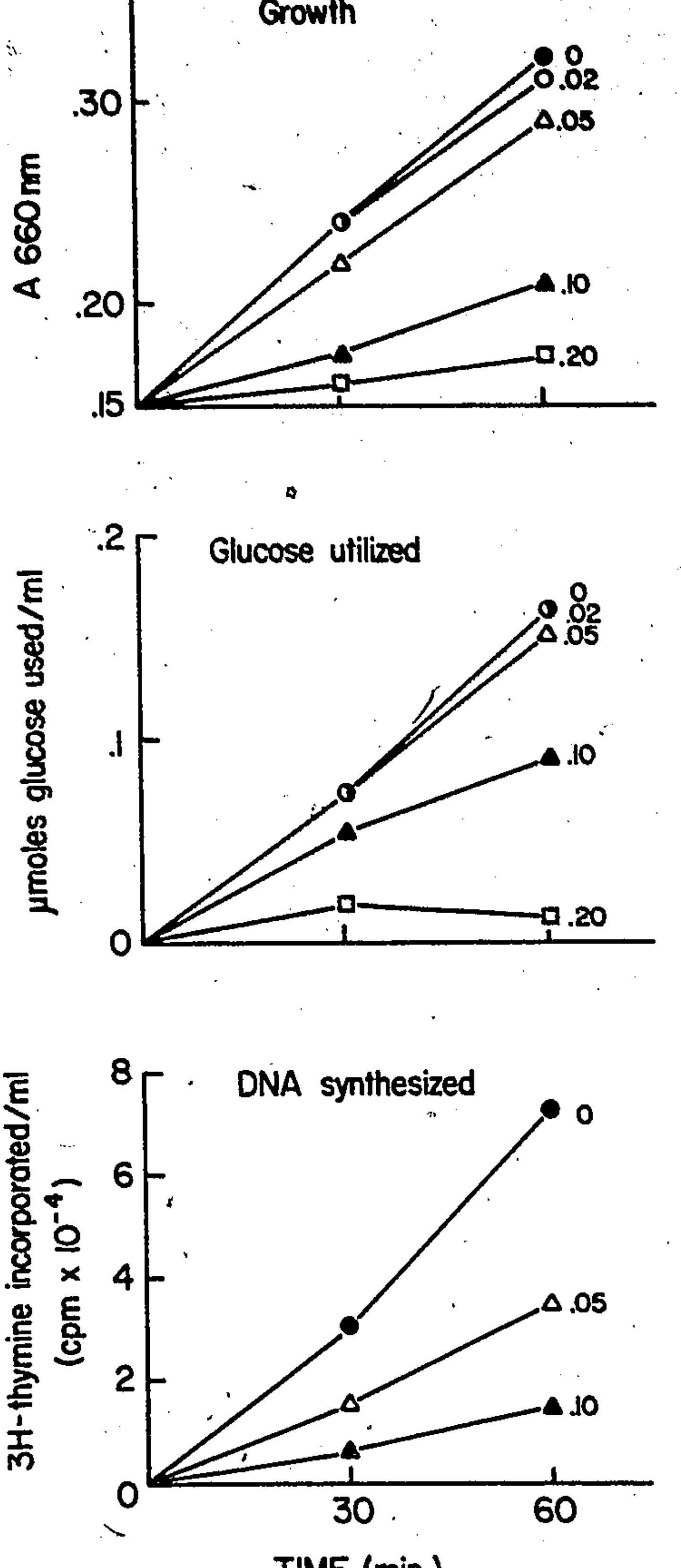
Results

Fig. 19 shows the effect of different concentrations of PF on growth, glucose utilization and DNA replication of E. coli B growing logarithmically on glucose. At 0.05 mM PF, growth was slightly affected and DNA replication was inhibited by 50 % while glucose utilization was unaffected. At 0.10 mM PF, glucose utilization was only slightly inhibited while both growth and DNA replication were strongly affected.

Microscopic examination of cells grown in the

Fig. 19. Effect of PF Concentration on Growth, Glucose Utilization and DNA Synthesis in *E. coli* B.

PF concentrations are given in mM.


Growth
A 660 nm
.30
.20
.15
0
.02
.05
.10
.20
Glucose utilized
μmoles glucose used/ml
.2
.1
0
0
.02
.05
.10
.20
DNA synthesized
3H-thymine incorporated/ml
(cpm x 10^-4)
8
6
4
2
0
0
.05
.10
30
60
TIME (min.)

presence of PF showed that 0.05 mM PF produced cells that were 1.5-2 times as long as control cells. In 0.10 mM PF, cells were 5 times as long as control cells. Elongation of cells is indicative of normal RNA and protein synthesis in the absence of DNA replication (44,50,275) suggesting that DNA replication in *E. coli* B is more sensitive to PF than RNA and protein synthesis.

Discussion

It was previously thought that inhibition of growth in *E. coli* B by PF was due to inhibition of glucose utilization (153). The data presented in this chapter indicates that inhibition of growth by PF may be more accurately correlated with inhibition of DNA replication. This is in agreement with the hypothesis proposed by a number of other workers (19,50,149,229,275). It would appear, therefore, that inhibition of glucose utilization may be of secondary importance.

It should be pointed out, however, that the data presented here are not extensive enough to allow us to conclusively state that DNA replication is the major site of PF action.

Chapter 5

POSSIBLE ROLE FOR PF AS AN UNCOUPLER OF MEMBRANE FUNCTION

POSSIBLE ROLE FOR PF AS AN UNCOUPLER OF MEMBRANE FUNCTION

Introduction

Several workers have proposed that acridine dyes can act as uncoupling agents (12,109,112,129). While studying the effect of PF on enzymes, we discovered that PF stimulated the rate of NADH oxidation by whole cells of E. coli B. Since uncouplers of oxidative phosphorylation can stimulate oxidation in mitochondria (100a,226a), our observations suggest that PF may be acting as a membrane uncoupler. Further studies were then initiated to determine the effects of PF on membrane function and to assess their importance in inhibition of growth.

In this chapter, we have looked at the effect of PF on oxygen uptake by whole cells and the effect of PF on amino acid and sugar transport in whole cells and membrane vesicles. In view of the reported effect of uncouplers on ATP levels (231,128) and ATPase activity (100a,226a), we have also studied the effect of PF on ATP levels in resting cells and cells metabolizing glucose, and on membrane-bound and solubilized ATPase.

Results

A/. Effect of PF on Respiration

During the preceeding studies of glycolytic enzymes, attempts were made to render cell membranes more permeable by treating them with toluene so that the intracellular action of glucose-6-phosphate dehydrogenase and pyruvate kinase could be studied. We were unable to detect these enzymes after toluene treatment; however, it was found that NADH oxidation increased markedly after treatment with toluene. This procedure was therefore used to prepare cells for experiments designed to illustrate the effect of PF on NADH oxidation in whole cells.

Table 9 shows that PF stimulated NADH-dependent oxygen uptake by toluene-treated whole cells of *E. coli* B by 45 - 75 %. The classical uncouplers DNP and CCCP produced a similar stimulation.

B/. Effect of PF on ATP Levels

Membrane uncouplers may be expected to affect intracellular energy levels (128,231). Therefore the effect of PF on intracellular ATP levels was studied under different conditions.

Table 9

Stimulation of Oxygen Uptake by PF in "Permeabilized"

E coli B

Experiment	Control	PF(0.02mM)	PF(0.10mM)	DNP(0.02mM)	CCCP(0.02mM)
1	12.33 ± 1.43	21.33 ± 2.45	ND	19.60 ± 4.69	23.17 ± 1.54
2	21.63 ± 1.15	31.25 ± 1.09	31.33 ± 2.54	ND	ND

Values are in Δ% O_2 saturation/3min and are the means of 6 determinations with 95% confidence intervals.

Substrate was 0.4mM NADH and toluene (1μl/ml) was the permeabilizing agent.

ND = not done

i. Effect of PF on ATP Levels in Resting Cells

Fig. 20 shows the effect of PF on intracellular ATP levels in *E. coli* B, B/Pr and AN180, a strain of *E. coli* K12 resistant to 1.0 mM PF. In *E. coli* B, PF stimulated the rapid and complete loss of ATP from the cells. In both B/Pr and AN180, ATP levels dropped to 20 and 50 % of the initial levels respectively then rose slightly before leveling off. These data suggest that loss of ATP could be related to inhibition of growth and that resistance may be due to the ability of the cells to maintain ATP levels in the presence of the dye.

ii. Effect of PF on ATP Levels in Cells Metabolizing Glucose

It was thought that the presence of a readily metabolizible energy source could exert some influence on the effect of PF on intracellular ATP levels. Fig. 21 shows that cells of *E. coli* B stop metabolizing glucose within 2 min of PF addition. Complete loss of ATP was observed after 5 min. On the other hand, PF had no effect on the ability of B/Pr cells to metabolize glucose (Fig. 22). In these cells, in the presence of glucose, ATP did not fall after PF addition.

Fig.20. Effect of PF on Intracellular ATP Levels in E. coli B, B/Pr and AN 180.

Control ATP levels were, in µg/mg dry wt cells; B, 0.379; B/Pr, 0.379 and AN 180, 0.727. PF was added to 0.07 mM at t = 0. Concentration of PF bound by B and B/Pr after 30 min was 30 nmoles/mg dry wt cells.

ATP levels (% of control)

100
75
50
25

AN 180

B/Pr

B

5 10 15 20 25 30

Time (min)

Fig. 21. Effect of PF on Intracellular ATP Levels in *E. coli* B in the Presence of Glucose.

Control ATP levels were 0.379 µg/mg dry wt cells. PF was added, as indicated by the arrows, to a final concentration of 0.07 mM. Bound PF was 30 nmoles/mg dry wt cells at 15 min.

Key to symbols;

●—● - glucose (µmoles/ml)

■—■ - ATP levels (% of control)

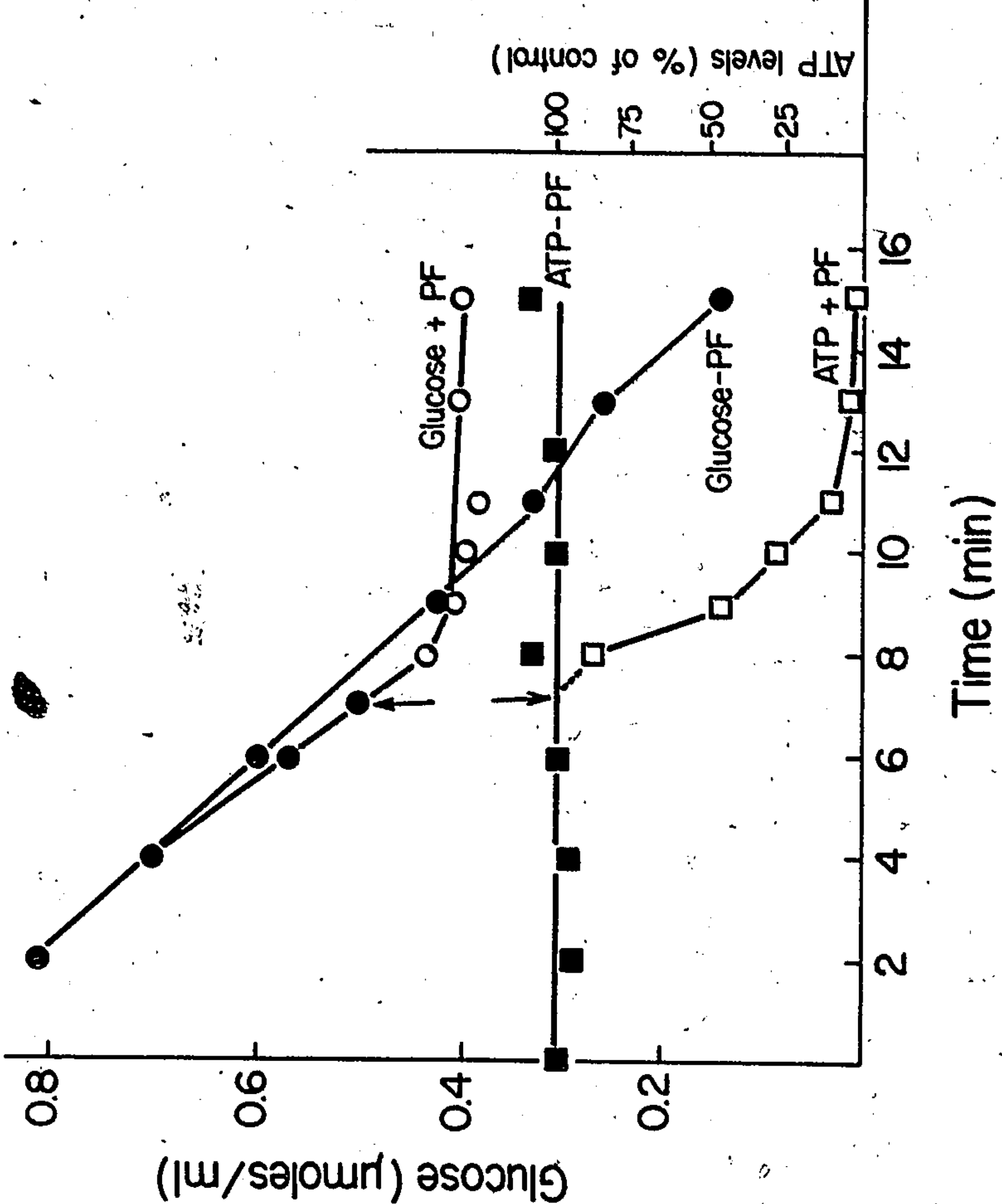

Glucose (μmoles/ml)
0.8
0.6
0.4
0.2
ATP levels (% of control)
100
75
50
25
Glucose + PF
ATP-PF
Glucose-PF
ATP + PF
2
4
6
8
10
12
14
16
Time (min)

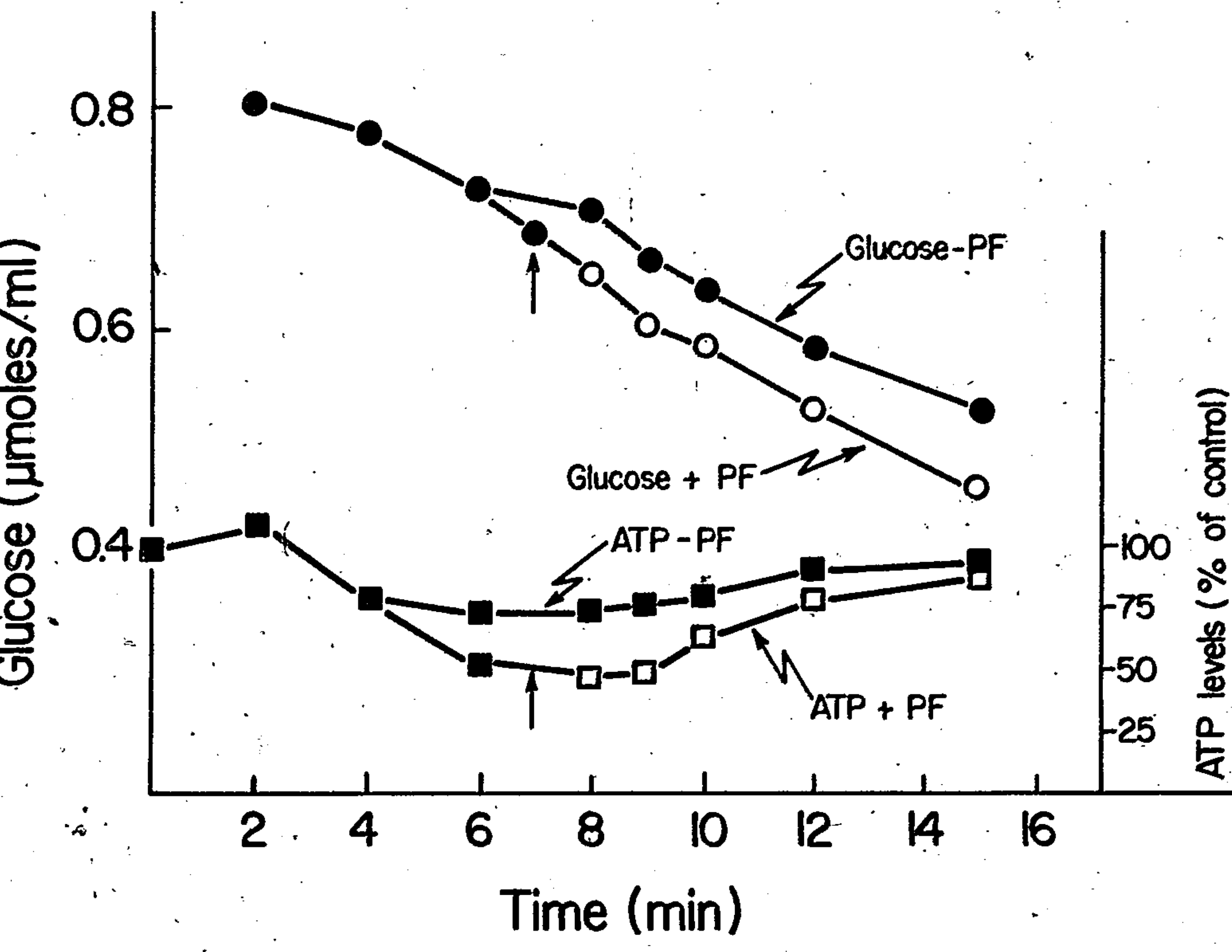
Fig. 22. Effect of PF on Intracellular ATP Levels in E. coli B/Pr in the Presence of Glucose.

Control ATP levels were 0.341 µg/mg dry wt cells. PF was added, as indicated by the arrows, to a final concentration of 0.07 mM. Bound PF was 30 nmoles/mg dry wt cells at 15 min.

Key to symbols;

●—● - glucose (umoles/ml)

■—■ - ATP levels (% of control)



Glucose-PF
Glucose + PF
ATP - PF
ATP + PF
0.8
0.6
0.4
Glucose (µmoles/ml)
100
75
50
25
ATP levels (% of control)
2
4
6
8
10
12
14
16
Time (min)

iii. Effect of Different PF Concentrations on ATP Levels and Glucose Utilization

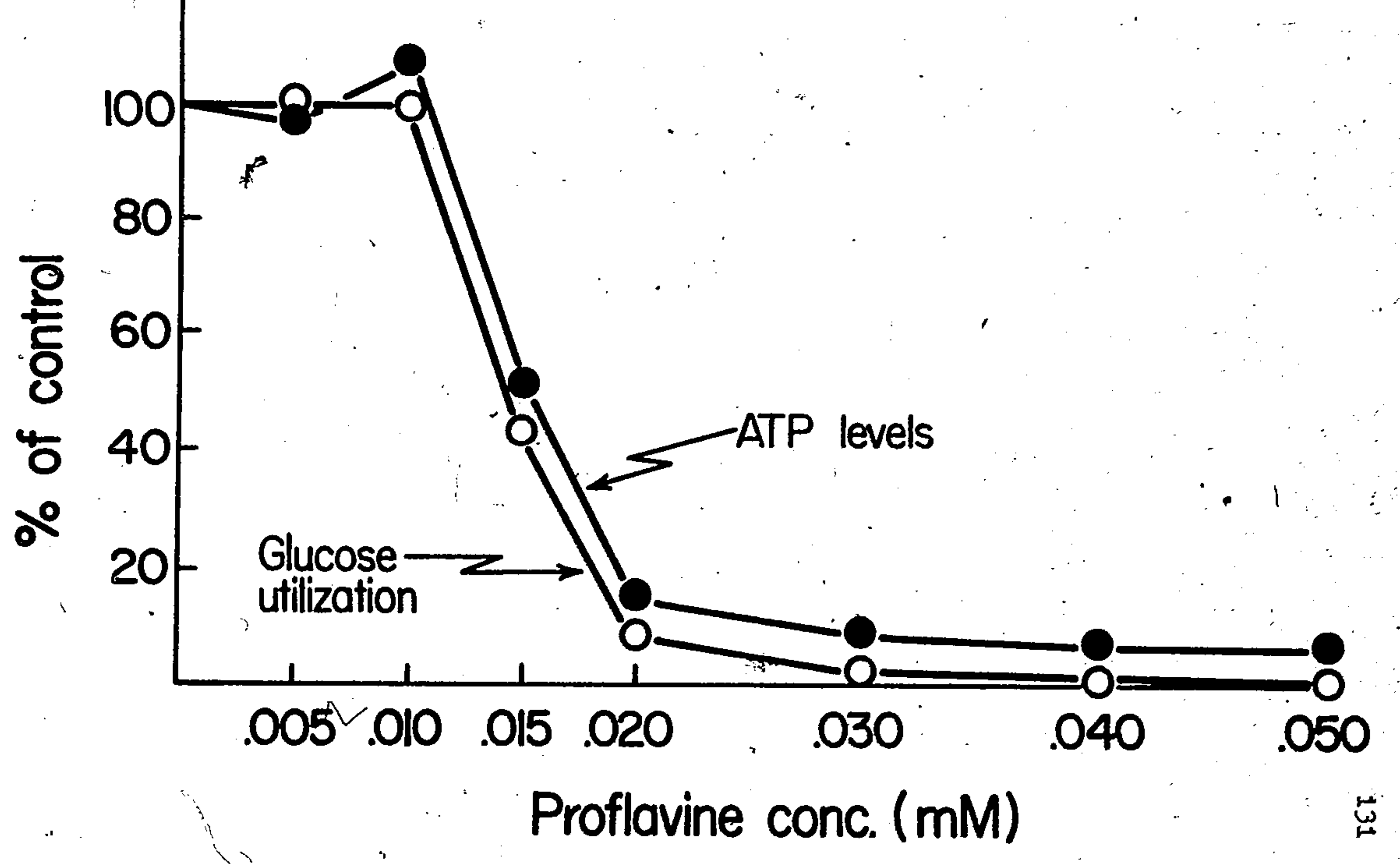
In further experiments, the effects of different PF concentrations on glucose utilization and ATP levels in *E. coli* B were examined. A close correlation between inhibition of glucose utilization and loss of ATP was observed within the range of 0.01 and 0.03 mM PF (Fig. 23). This range corresponded to 4.5-12.5 nmoles of PF bound/mg dry wt cells. A concentration of 0.03 mM PF completely inhibited glucose utilization and abolished intracellular ATP levels.

These data suggest that the inability of PF-sensitive cells to maintain ATP levels in the presence of PF is a consequence of their inability to utilize glucose.

On the other hand, it is possible that glucose transport and metabolism are regulated by the ATP levels. A partial loss of ATP in the presence of PF might then result in a partial loss of glucose utilizing ability. Complete loss of ATP would certainly prevent glucose utilization since ATP is required for the phosphorylation of F6P; however, Atkinson (11) has shown that a reduction in ATP levels within certain limits results in increased rather than decreased rates of glucose utilization.

Fig.23. Effect of PF Concentration on Intracellular ATP Levels and Glucose Utilization in *E. coli* B.

Control ATP levels were 0.262 µg/mg dry wt cells. Control rate of glucose utilization was 0.639 µmoles/ml/15 min. For each added concentration of PF (mM), levels of bound dye were, in nmoles/mg dry wt cells; 0.005, 2.38; 0.010, 4.50; 0.015, 6.50; 0.020, 7.96; 0.030, 12.50; 0.040, 16.70; 0.050, 20.95.


% of control
100
80
60
40
20
ATP levels
Glucose utilization
.005
.010
.015
.020
.030
.040
.050
Proflavine conc. (mM)

iv. Effect of Iodoacetate on ATP Levels in Cells Utilizing Glucose Anaerobically

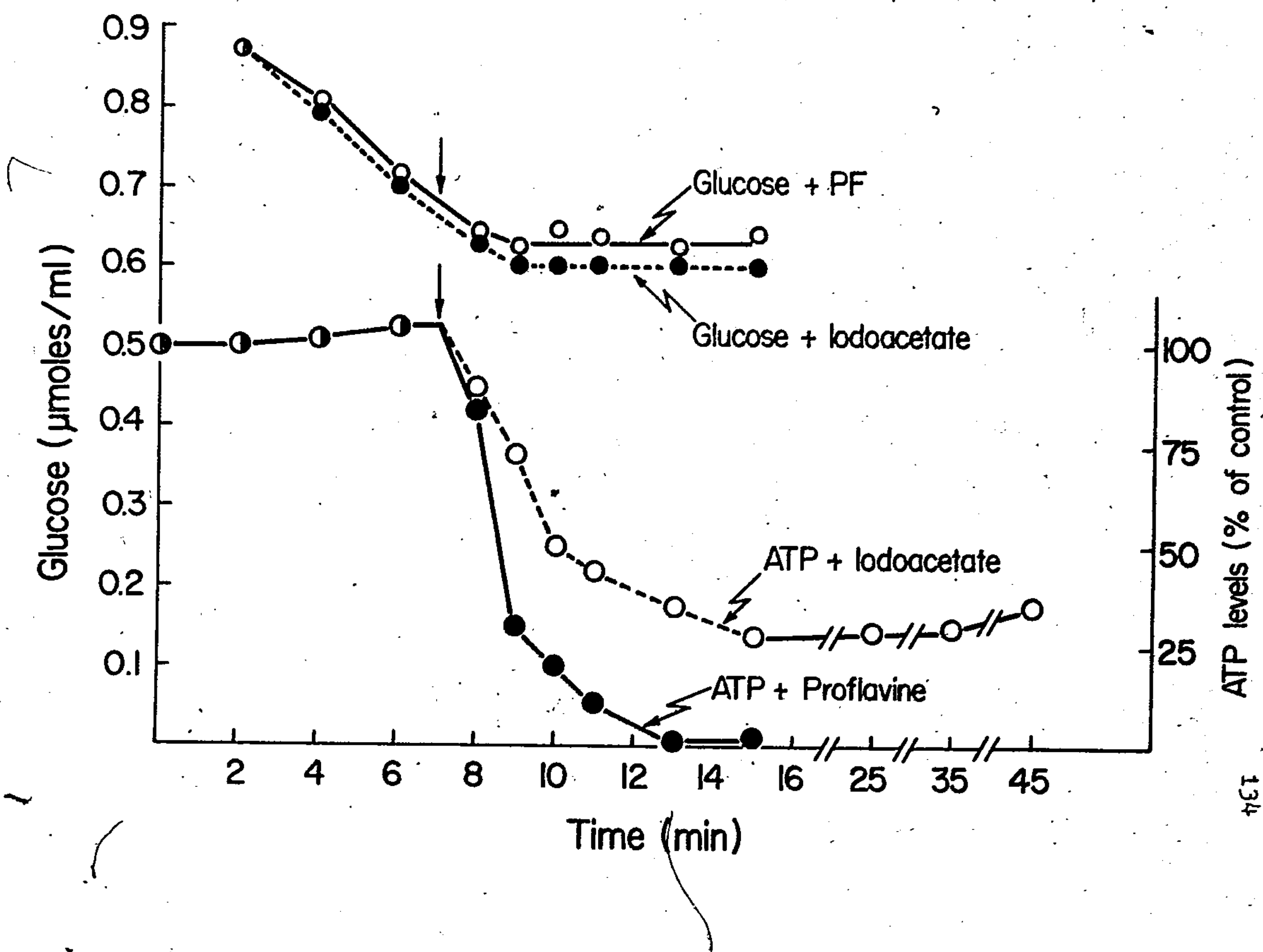
It was thought possible that the effect of PF on ATP levels was a secondary one, due to inhibition of metabolism and that ATP fell through turnover. In order to test this possibility, the effects of PF were compared with those of iodoacetate, believed to be a specific inhibitor of glyceraldehyde-3-phosphate dehydrogenase (144a). Under anaerobic conditions, this inhibitor prevents generation of energy from glucose and as a result any loss of ATP would be attributed to turnover. The relationship may not be that clear, however, since iodoacetate may have some direct effect on intracellular PEP and glucose transport as well (128).

Fig. 24 shows that under anaerobic conditions, both inhibitors blocked glucose utilization in E. coli B within 2 min of addition. In the presence of iodoacetate, however, ATP levels dropped much more slowly than with PF and remained at approximately 30 % of the initial levels 38 min after addition of the inhibitor.

This experiment suggests that although some of the loss of ATP following PF addition may be due to turnover, PF has a further direct effect on ATP levels.

Fig.24. Effect of PF and Iodoacetate on Intracellular ATP Levels and Glucose Utilization Under Anaerobic Conditions.

Control ATP levels were 0.364 µg/mg dry wt cells. PF and iodoacetate were added, as indicated by the arrows, to 0.07 mM and 1.43 mM final concentrations respectively. Bound PF was 30 nmoles/mg dry wt cells at 15 min.



Glucose (μmoles/ml)
0.9
0.8
0.7
0.6
0.50
0.4
0.3
0.2
0.1
ATP levels (% of control)
100
75
50
25
Time (min)
2
4
6
8
10
12
14
16
25
35
45
Glucose + PF
Glucose + Iodoacetate
ATP + Iodoacetate
ATP + Proflavine

C/. The Role of ATPase in the PF-stimulated Loss of ATP

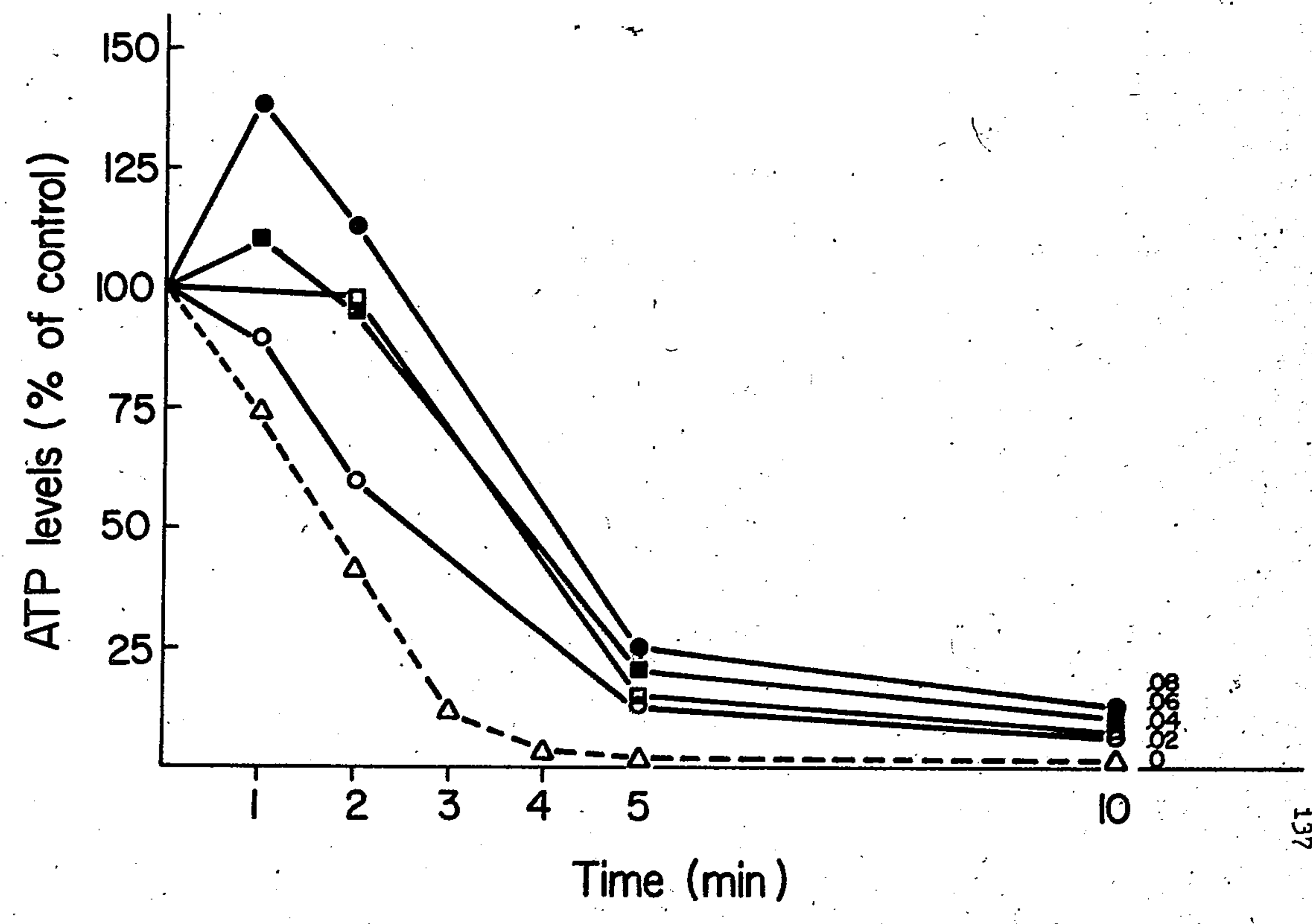
The loss of intracellular ATP in *E. coli* in the presence of colicin K seemed due to the ability of this inhibitor to stimulate the hydrolysis of ATP via the membrane-bound Mg^{2+}, Ca^{2+}- ATPase (193). A number of experiments were carried out to test the possibility that this enzyme was involved in the PF-stimulated loss of ATP in *E. coli* B. These included the effect of PF on ATP in whole cells treated with DCCD and whole cells of GN402, an ATPase-deficient mutant and on membrane-bound and solubilized ATPase in cell-free systems.

i. Effect of PF on DCCD-treated Cells of E. coli B

When PF was added to washed cells pre-treated with low concentrations of DCCD, a specific inhibitor of ATPase, the initial rate of ATP loss was reduced (Fig.25). AT higher DCCD concentrations, addition of PF produced a slight rise in ATP levels; however, PF essentially eliminated ATP from the cell after 10 min, despite the presence of DCCD. These results are complicated by the fact that DCCD alone reduced ATP levels by up to 60 % at a concentration of 0.08 mM; however, since DCCD does reduce PF-stimulated ATP loss, it is possible that ATPase may be involved to some extent in the PF-stimulated loss of ATP.

Fig. 25. Effect of PF on Intracellular ATP Levels in *E. coli* B Treated with DCCD.

For each DCCD concentration given in mmoles/liter, control ATP levels were, in µg/mg dry wt cells; 0, 0.379; 0.02, 0.285; 0.04, 0.250; 0.06, 0.189; 0.08, 0.151. PF was added to a final concentration of 0.07 mM at t = 0.


ATP levels (% of control)
150
125
100
75
50
25
1
2
3
4
5
10
Time (min)
.08
.06
.04
.02
0

ii. Effect of PF on ATP Levels in *E. coli* GN 402

GN 402 was an ATPase-deficient mutant of *E. coli* B isolated as described in Materials and Methods. When PF was added to washed cells of GN 402, ATP levels rose initially then fell rapidly to zero within 10 min of PF addition (Fig.26). The initial delay in ATP loss in this mutant suggests that stimulation of ATPase by PF may account for the ATP depletion in *E. coli* B.

iii. Effect of PF on Membrane-bound and Solubilized ATPase

In order to elucidate the effects of PF on ATPase activity, washed membrane particles were prepared from whole cells of *E. coli* B and B/Pr. For some experiments, the ATPase enzyme was solubilized by the low ionic strength wash described in Materials and Methods. By comparing the activity found in the solubilized and the depleted membrane fractions, it was determined that 75-85 % of the ATPase had been removed from the membrane.

Plots of velocity against enzyme concentration for membrane-bound (Fig.27) and solubilized (Fig.28) ATPase from *E. coli* B gave straight lines.

Fig.29 shows the effect of different concentrations of PF on the specific activity of membrane-bound ATPase from *E. coli* B and B/Pr. The enzyme from both strains was stimulated by up to approximately 50 % by PF, this

Fig. 26. Effect of PF on Intracellular ATP Levels in *E. coli* GN402.

Control ATP level was 0.082 μg/mg dry wt cells. PF was added to a final concentration of 0.07 mM at t = 0.

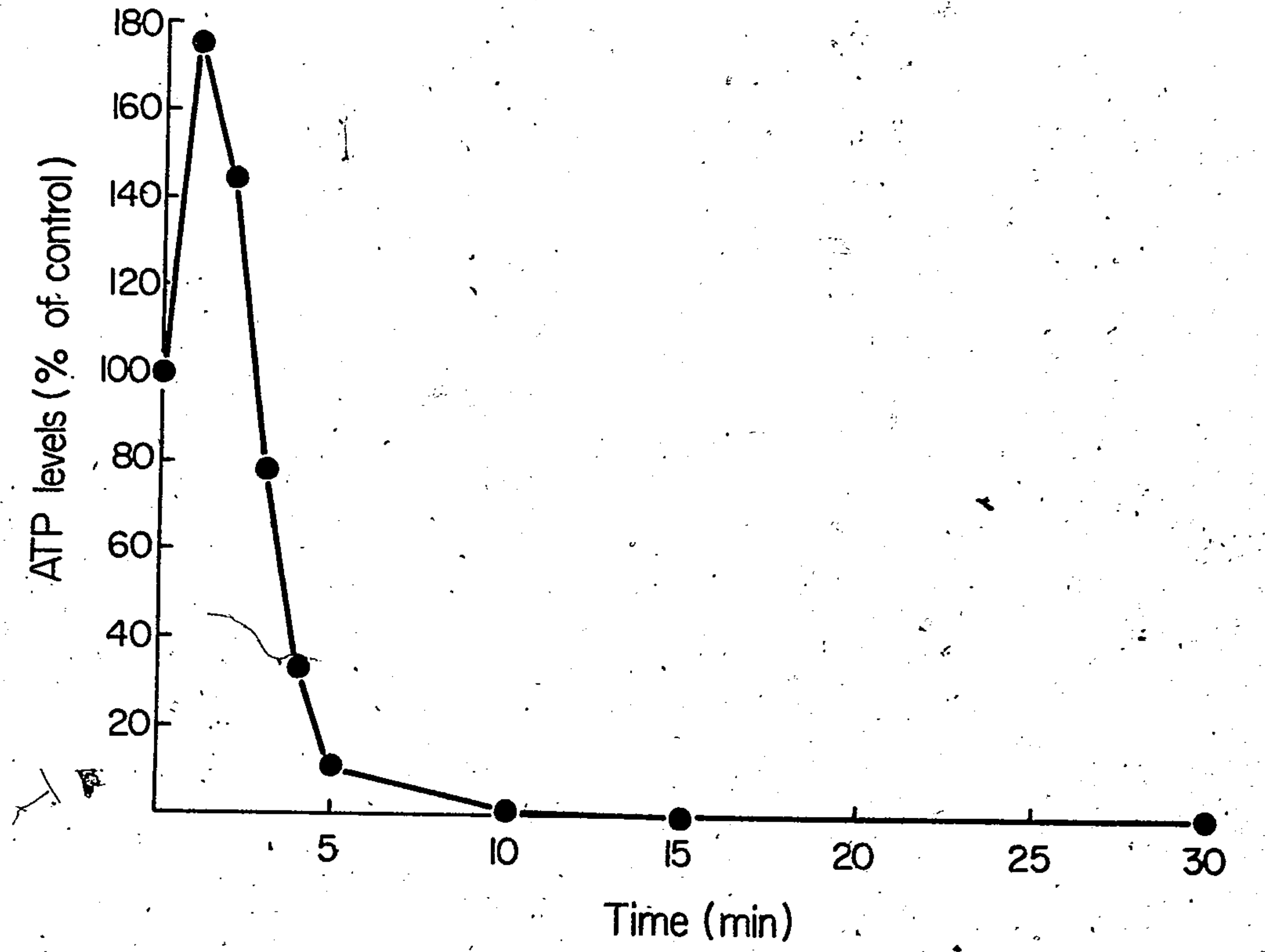
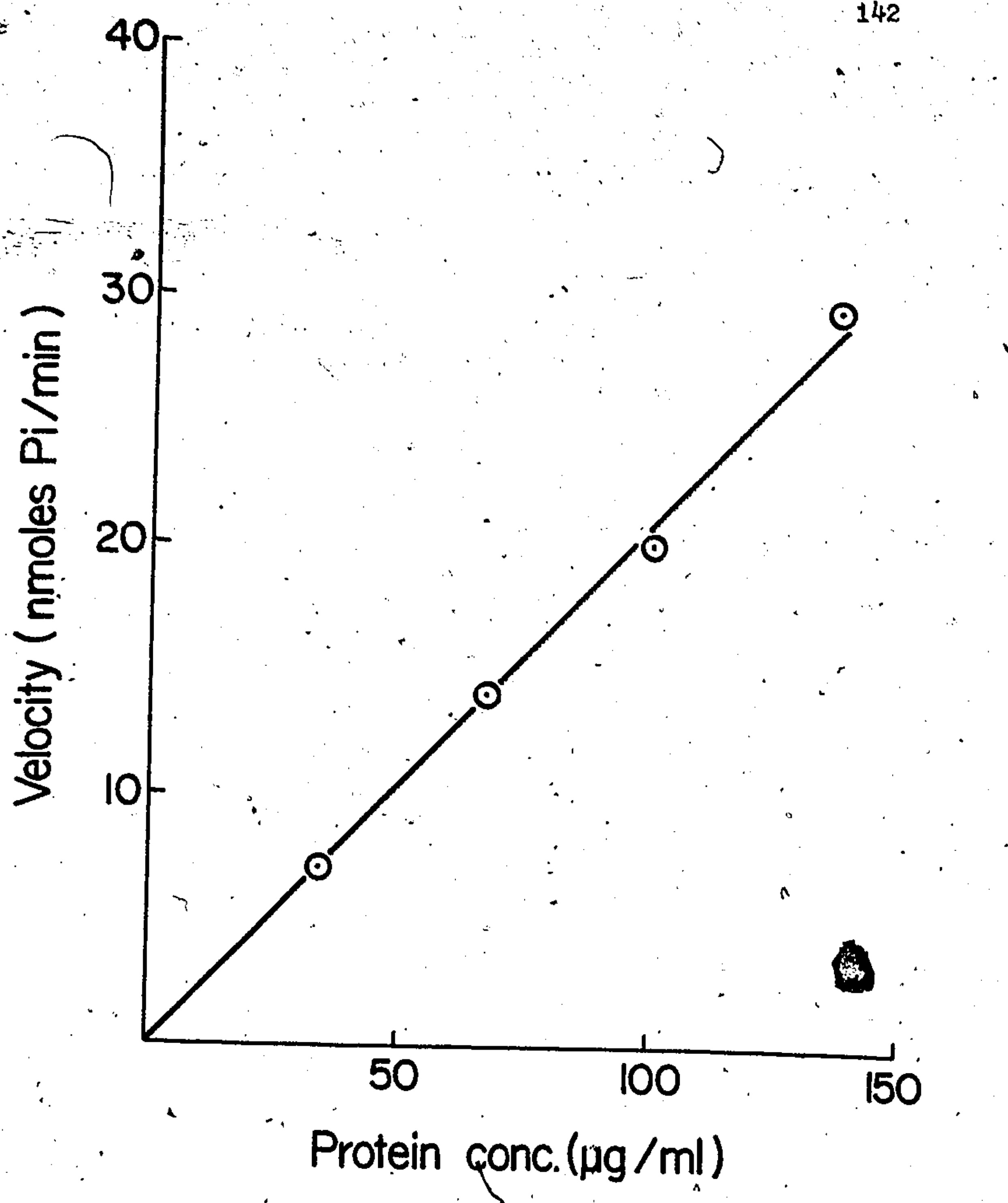
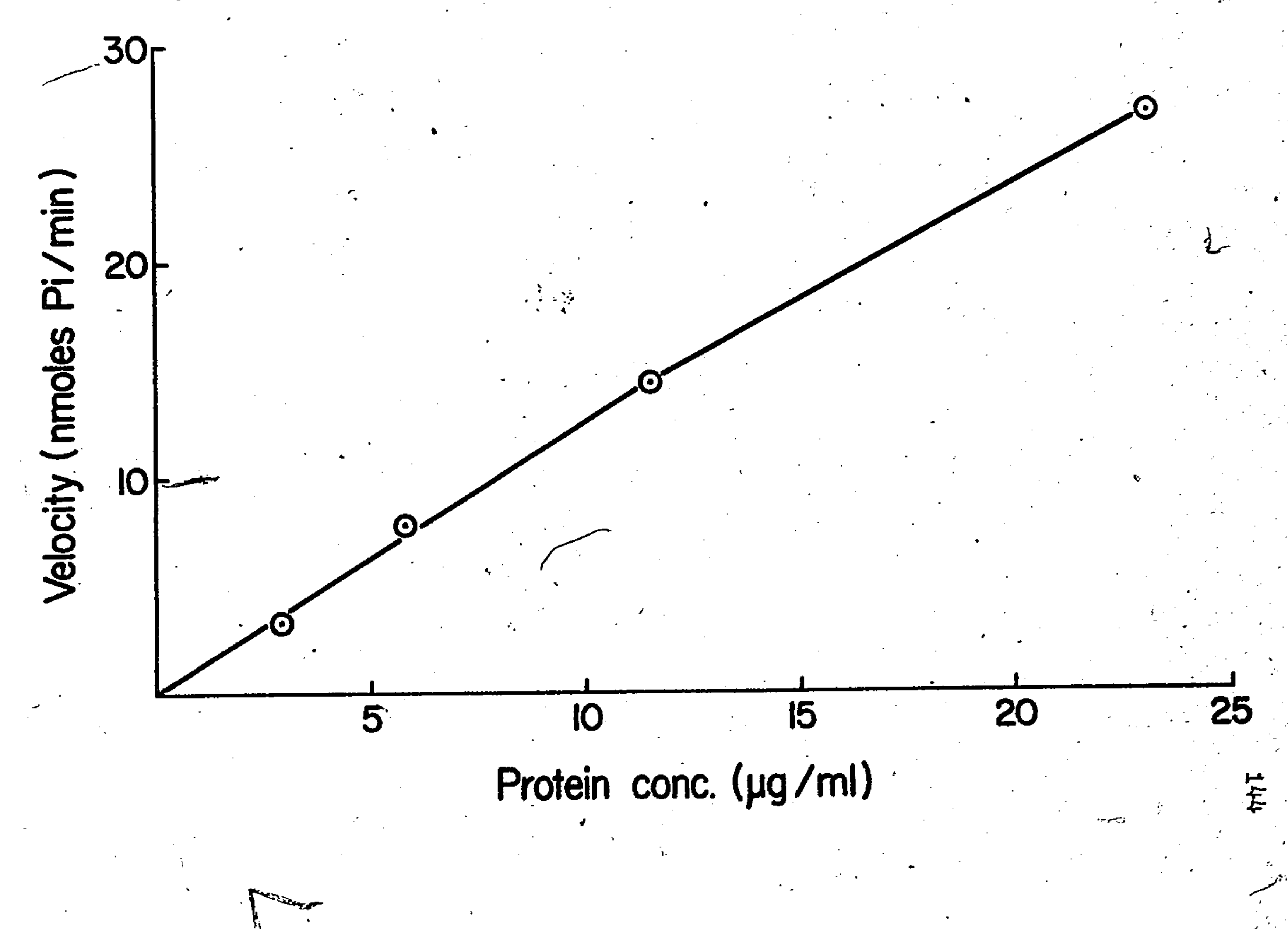

ATP levels (% of control)
180
160
140
120
100
80
60
40
20
Time (min)
5
10
15
20
25
30

Fig. 27. Effect of Protein Concentration on the Activity of Membrane-bound ATPase from *E. coli* B.



40
30
20
10
Velocity (nmoles Pi/min)
50
100
150
Protein conc. (μg/ml)

Fig. 28. Effect of Protein Concentration on the Activity of Solubilized ATPase from *E. coli* B.



30
20
10
Velocity (nmoles Pi/min)
5
10
15
20
25
Protein conc. (µg/ml)

maximum occuring at 0.3 and 0.4 mM PF for the enzyme from E. coli B/Pr and B respectively. At 0.5 mM PF, ATPase from E. coli B/Pr was much less affected by PF, being stimulated 5.9 % as compared to 42.7 % for the enzyme from E. coli B. Fig. 29 also shows that the solubilized enzyme from E. coli B was essentially unaffected by PF.

PF increased both the K_m and V_{max} for the membrane-bound enzyme from E. coli B (Fig. 30). The data was analyzed by the method of Wilkinson (268a). The presence of substrate inhibition prevented an accurate assessment of the type of stimulation produced by PF; however, the stimulation appeared to be uncompetitive.

The results obtained with ATPase suggested that PF may contribute to the loss of ATP from the cell by stimulating membrane-bound ATPase. PF may act on ATPase indirectly since it has no effect on the solubilized enzyme.

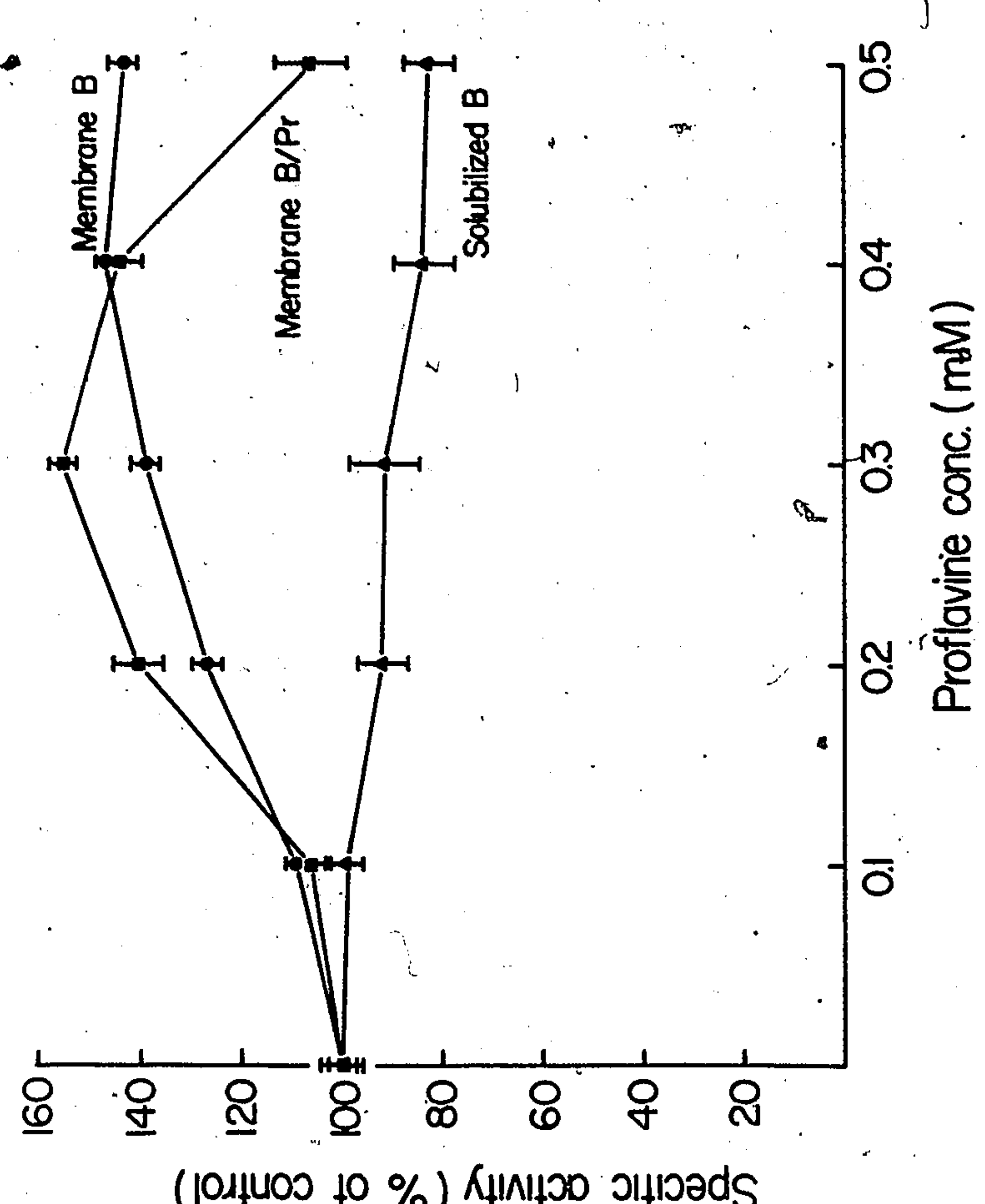
D/. Effect of PF on Amino Acid and Sugar Transport

One of the most important functions of an uncoupler is to prevent the energization of active transport (100a). PF was therefore tested for the ability to inhibit amino acid and sugar transport into membrane vesicles and whole cells of E. coli B and B/Pr.

Transport of glucose into vesicles of E. coli B was essentially unaffected by PF while the accumulation

Fig. 29. Effect of PF Concentration on the Specific Activity of Membrane-bound ATPase from *E. coli* B and B/Pr and Solubilized ATPase from *E. coli* B.

Control specific activities, in μmoles Pi/min/mg protein were; membrane-bound from B, 0.244; membrane-bound from B/Pr, 0.288; solubilized from B, 2.29.
95 % confidence intervals were calculated from a total of 12 determinations for each point.



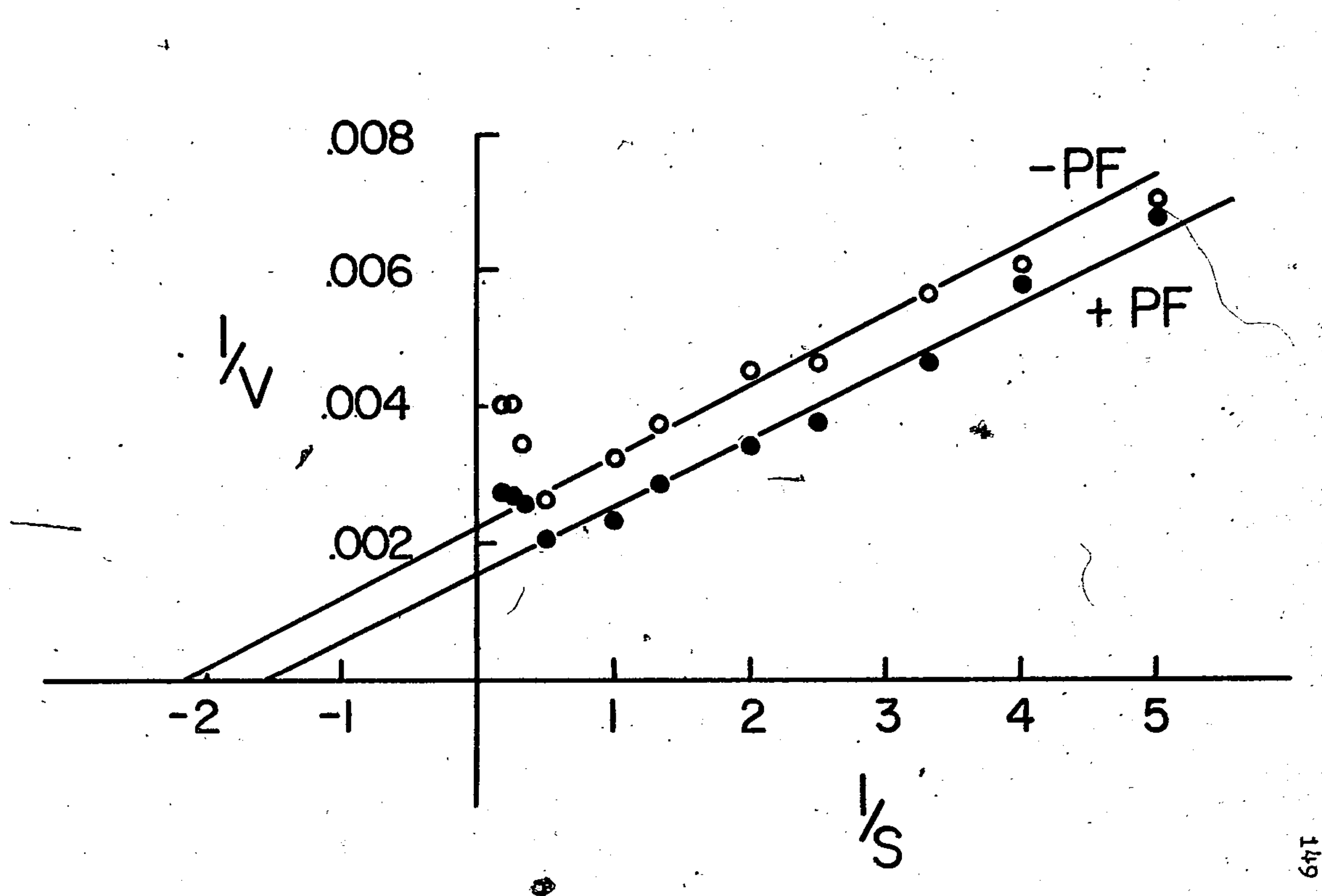
Specific activity (% of control)
160
140
120
100
80
60
40
20
0.1
0.2
0.3
0.4
0.5
Proflavine conc. (mM)
Membrane B
Membrane B/Pr
Solubilized B

Fig. 30. Determination of the K_m for Membrane-bound ATPase from E. coli B in the Presence and Absence of PF.

PF was employed at 0.3 mM.

1/v is the reciprocal of the enzyme velocity in µmoles/min/mg protein^{-1}.

1/s is the reciprocal of the substrate concentration in mmoles/liter^{-1}.



-PF
+PF
1/V
.008
.006
.004
.002
1/S
-2
-1
1
2
3
4
5

of tyrosine, aspartate and TMG was inhibited by up to 60 % by 0.5 mM PF (Fig. 31). At lower concentrations of PF, the dye had less effect on transport of TMG than amino acids. It was also found that TMG transport was less sensitive than aspartate transport to the uncoupling action of CCCP (Fig. 32). The effect of PF on amino acid transport by *E. coli* B/Pr vesicles was also examined. Aspartate and tyrosine accumulation by these vesicles were only slightly less sensitive to PF than in the vesicles from *E. coli* B (Fig. 33).

Accumulation of aspartate by cells of *E. coli* B and B/Pr were equally sensitive to PF (Fig. 34). Since PF concentrations which completely blocked growth only partially blocked transport, these experiments suggest that while PF affects transport to some extent, interference with this process is not likely the most important mechanism of inhibition.

Tyrosine accumulation by cells of *E. coli* B was less sensitive to PF in the presence of D-lactate. A concentration of 0.075 mM PF produced an 83 % inhibition of accumulation in the absence and a 54 % inhibition in the presence of 20 mM D-lactate (Table 10). These results suggest that PF may inhibit tyrosine accumulation in part by affecting endogenous energy levels.

Fig. 31. Effect of PF Concentration on Amino Acid and Sugar Transport by Membrane Vesicles of E. coli B

Control transport rates were, in nmoles accumulated/min/mg protein; glucose, 0.096; TMG, 0.064; aspartate, 0.215, tyrosine, 0.012.

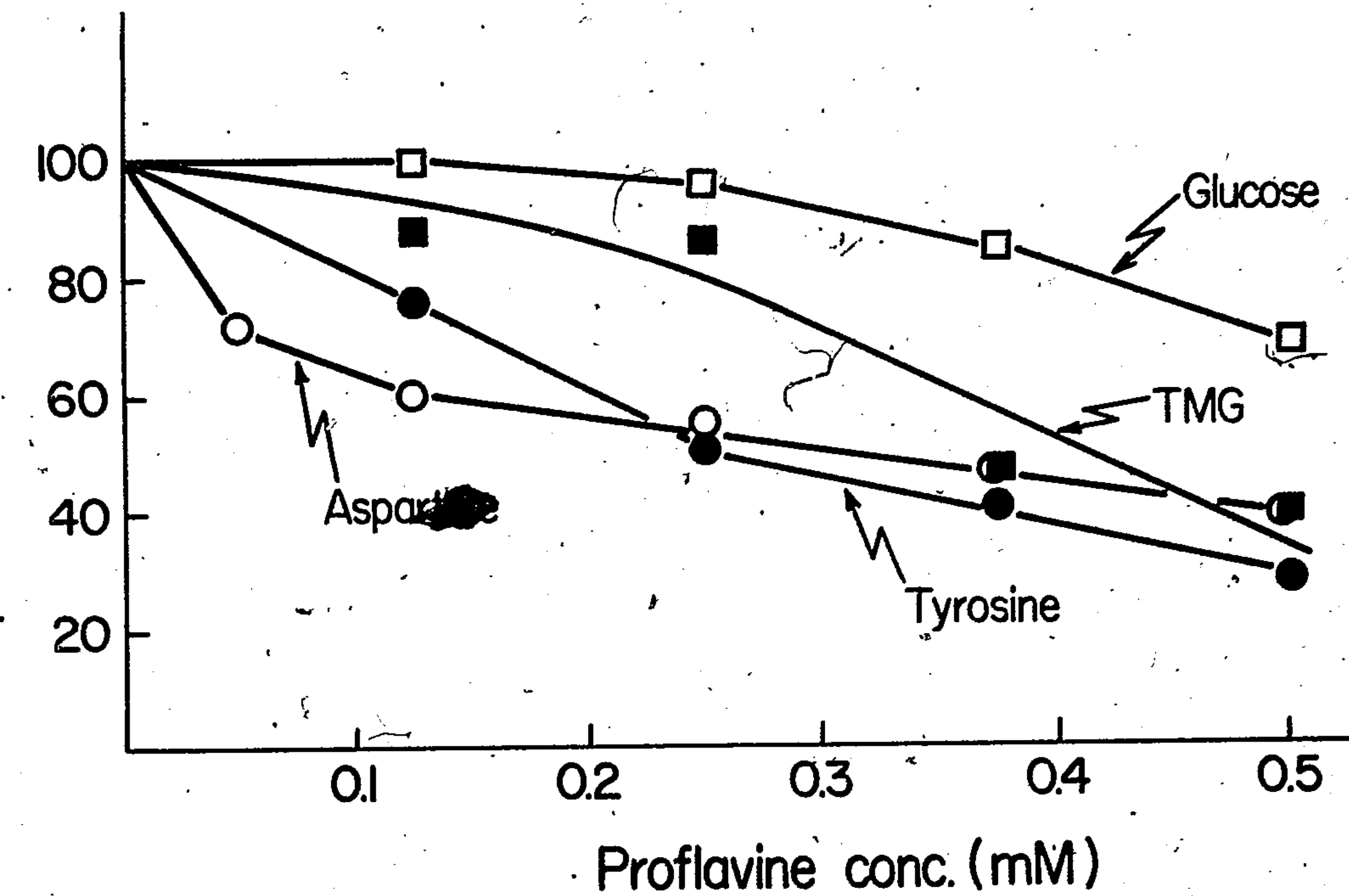
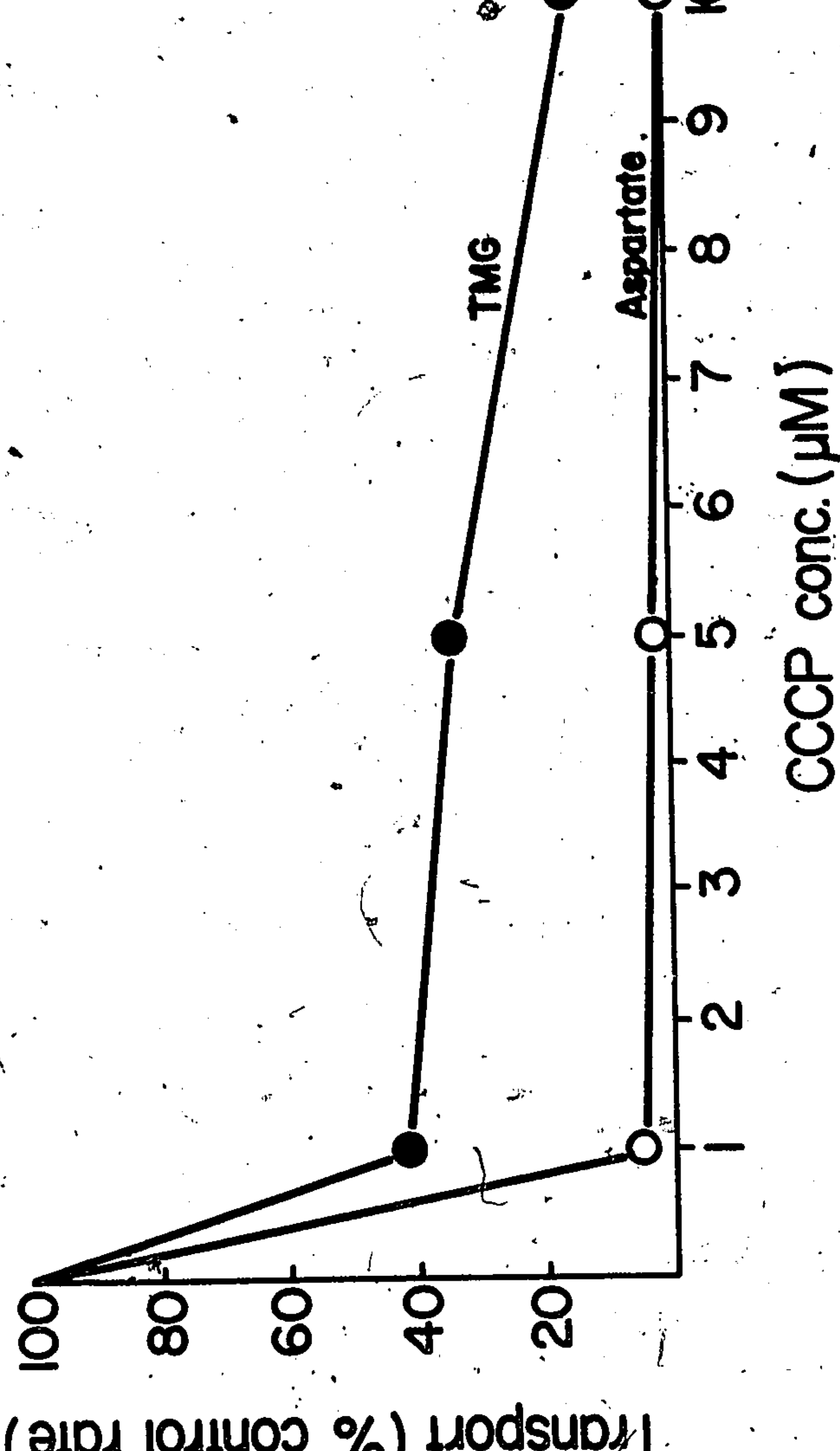

Glucose
TMG
Tyrosine
Aspar
100
80
60
40
20
Transport (% of control rate)
0.1
0.2
0.3
0.4
0.5
Proflavine conc. (mM)

Fig. 32. Effect of CCCP Concentration on Aspartate and TMG Transport by Membrane Vesicles of E. coli B.

Control transport rates were, in nmoles accumulated/min/mg protein; aspartate, 0.147; TMG, 0.107.



Transport (% control rate)
100
80
60
40
20
TMG
Aspartate
1
2
3
4
5
6
7
8
9
10
CCCP conc. (μM)

Fig. 33. Effect of PF Concentration on Amino Acid Transport by Membrane Vesicles of _E. coli_ B/Pr.

Control transport rates were, in nmoles accumulated/min/mg protein; tyrosine, 0.06; aspartate, 1.04.

Transport (% of control rate)

100
80
60
40
20

0.1
0.2
0.3
0.4
0.5

Proflavine conc. (mM)

Aspartate

Tyrosine

Fig. 34. Effect of PF Concentration on Aspartate Transport by Whole Cells of *E. coli* B and B/Pr.

Control transport rates were, in nmoles accumulated/min/mg dry wt cells; B, 3.87 ; B/Pr, 2.18.

Transport (% of control rate)

100
80
60
40
20

B/Pr
B

.020
.040
.060
.080
.100

Proflavine conc. (mM)

Table 10

Effect of PF on Tyrosine Accumulation by Whole Cells of E coli B

PF (0.075mM)	D-lactate (20mM)	Accumulation (nmoles/min/mg protein)	% of Control
-	-	0.354	100.00
+	-	0.060	16.95
-	+	0.349	100.00
+	+	0.136	36.10

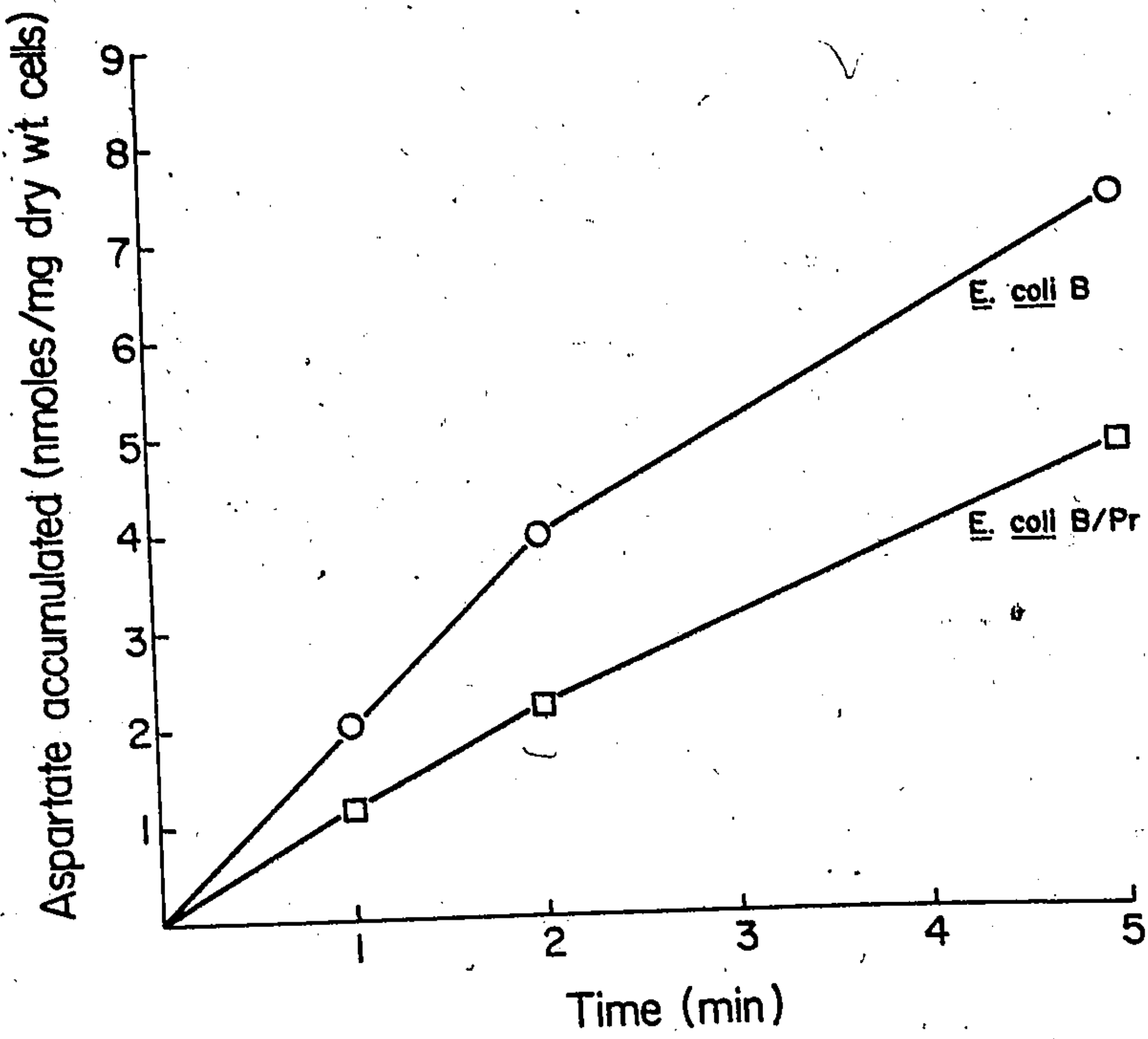
By comparing the rates of transport for tyrosine and aspartate in B and B/Pr vesicles (Fig. 31 and 33), it may be observed that B/Pr vesicles transport 5 times faster than B vesicles. On the other hand, whole cells of *E. coli* B transport aspartate faster than whole cells of *E. coli* B/Pr. (Fig. 35). This could be due to increased stability of the B/Pr vesicles during preparation or could conceivably reflect changes in these vesicles brought about as a result of the development of PF resistance.

Discussion

We have shown that PF stimulated the rapid and complete loss of intracellular ATP from *E. coli* B. This phenomenon has not been previously reported and in view of the deleterious effects that ATP loss would have on cell processes, it must be considered as a possible mechanism of inhibition. It has been demonstrated that a 50 % loss of ATP in *E. coli* was sufficient to completely inhibit DNA and RNA synthesis (193). ATP depletion by PF may possibly contribute to inhibition of growth.

Other inhibitors such as arsenate (128), cyanide (231) and colicin K (193) reduce intracellular ATP levels in *E. coli*. Arsenate, which competes with

Fig. 35. Rates of Aspartate Transport by Whole Cells of *E. coli* B and B/Pr.


Aspartate accumulated (nmoles/mg dry wt. cells)
9
8
7
6
5
4
3
2
1
E. coli B
E. coli B/Pr
1
2
3
4
5
Time (min)

orthophosphate for active sites on enzymes involved in substrate level phosphorylation, reduced ATP levels by 95 % within 5 min of addition (128). Cyanide, a cytochrome inhibitor, stimulated the loss of 70 % of the initial ATP within 20 min (231) while the addition of colicin K reduced ATP levels by 30 % within 3 min due to its effects on membrane-bound ATPase (193).

PF stimulated the loss of ATP from E. coli B/Pr; however, this strain was able to maintain low levels of ATP in the presence of the dye. PF had no effect on ATP levels in cells of E. coli B/Pr metabolizing glucose. It appears likely that these cells can maintain ATP levels by virtue of their ability to metabolize glucose and resynthesize ATP in the presence of PF. We have also shown that cells of E. coli B are able to maintain substantial ATP levels in the presence of PF provided that glucose metabolism is not completely inhibited. It appears, therefore, that inhibition of energy generation by PF may be far more serious than the direct effect of the dye on ATP levels. The primary effect of PF on metabolism may be on the ability to generate ATP rather than some direct mechanism of ATP degradation.

Some observed physiological effects of PF may, however, be attributed to degradation of ATP. Amino acid transport into whole cells was more resistant to PF

in the presence of D-lactate than in its absence. In the absence of an external energy source, intracellular ATP might be expected to provide energy for transport. As a result, transport at the expense of endogenous energy would be more sensitive to PF. αMG accumulation was also significantly inhibited by concentrations of PF capable of depleting ATP levels. This is difficult to explain in light of the fact that PEP rather than ATP acts as the phosphate donor for αMG accumulation (86). Loss of ATP in the presence of PF would likely produce a concomitant rise in AMP levels (11). According to Malcovati <u>et al</u> (148), increased AMP levels would stimulate PKII to produce ATP from PEP and thus PF may affect αMG accumulation by indirectly depleting PEP levels. Arsenate has been also shown to inhibit αMG accumulation indirectly by lowering ATP and PEP levels (128). The possiblilty that PF can directly inhibit αMG accumulation to some extent cannot be excluded, however.

Loss of ATP may be due to turnover as well as to a direct effect by PF. In the presence of iodoacetate, a specific glycolytic inhibitor, cells lost ATP at a much slower rate than with PF and did not lose all their ATP. Inhibition of oxidation by cyanide in <u>E. coli</u> resulted in the loss of ATP at a much slower rate than we have observed with PF (231). Our results

suggest that while PF directly stimulates the loss of ATP through hydrolysis or some other mechanism, turnover may be involved to some extent.

Studies with DCCD and an ATPase-deficient mutant have suggested that ATPase may play a role in PF-stimulated ATP loss. Partial reduction of ATPase activity by inhibition or mutation slowed down but did not completely prevent loss of ATP. The ATPase from E. coli has been studied by a number of authors (reviews 59 and 100a) and it has been implicated in the energy-linked transhydrogenase reaction (60), oxidative phosphorylation (195), anaerobic growth and transport (282), anaerobic DNA synthesis (155) and aerobic motility (248). A structural role in transport has also been proposed for this enzyme (203). Our results have indicated that PF stimulated membrane-bound ATPase activity by 50 % in both E. coli B and B/Pr. PF had no effect on the solubilized enzyme suggesting that the dye inhibited ATPase by binding to, and deenergizing , the membrane.

ATPase in cell-free systems was not strongly stimulated by PF. Reduction of the level of active enzyme in whole cells by inhibition or mutation did not strongly affect PF-stimulated ATP loss. It is possible that ATPase plays a very minor role in the PF-stimulated ATP loss; but it is also possible that the level of

stimulation observed in cell-free systems does not accurately reflect the level of stimulation in whole cells. The presence of ATPase activity in membrane fragments suggests that these fragments are uncoupled to some extent (100a) and as such may be less sensitive to stimulation by PF. ATPase in whole cells may be very strongly stimulated by the dye and as a result, a partial loss of enzyme activity due to inhibition or mutation would have only a slight effect on PF-stimulated ATP loss catalyzed by ATPase.

PF, as well as the classical uncouplers DNP and CCCP, stimulated oxygen uptake by whole cells of _E. coli_ B. Amino acid transport into whole cells and vesicles was also significantly inhibited by this dye. As mentioned in Chapter 1, acridine dyes have been shown to uncouple oxidative phosphorylation and deenergize membranes of mitochondria, chloroplasts and photosynthetic bacteria. We therefore propose that PF serves a similar role in _E. coli_ B. This hypothesis is supported by the observation that PF stimulated membrane-bound but not solubilized ATPase. PF may stimulate ATPase by binding to and deenergizing the membrane; however, a direct interaction between PF and the membrane portion of the ATPase complex cannot be excluded.

Uncouplers such as CCCP and DNP are believed to

act by specifically conducting protons across or into biological membranes, resulting in the dissipation of the membrane potential and the subsequent deenergization of the membrane (100a).

PF is not very lipid soluble and does not possess a pKa in the neutral range (3) and would therefore not be expected to uncouple by conducting protons. It is possible, however, that PF may deenergize the membrane by binding to nucleophilic sites at the expense of energy, a process described by Massari _et al_ (151) and Dell'Antone _et al_ (66).

PF was a much less effective uncoupler of amino acid transport than CCCP. Also, PF inhibited transport equally in sensitive and resistant cells. These results suggest that the inhibition of membrane function by PF may not contribute significantly to the bacteriostatic action of this dye.

Chapter 6

RELATIONSHIP BETWEEN PF RESISTANCE AND ANTIBIOTIC RESISTANCE

RELATIONSHIP BETWEEN PF RESISTANCE AND ANTIBIOTIC RESISTANCE

Introduction

As was mentioned earlier, cross resistance between acridines and unrelated inhibitors may be associated with a common permeability barrier. With a view to assessing the importance of permeability changes in PF resistance in *E. coli* B, cross resistance between PF and a number of antibiotics was studied.

Results

The PF-resistant strain *E. coli* B/Pr was significantly more resistant to several antibiotics than the parent *E. coli* B. These, and similar results for other PF-resistant strains, are presented in Table 11.

All PF-resistant strains were resistant to penicillin and cephalothin but only slightly resistant to ampicillin and carbenicillin. Strain B/Pr was also resistant to chloramphenicol and, to some extent, rifampicin; however, in general, all of the strains were equally resistant to most of the other antibiotics.

Fig.36 shows the effect of different concentrations of penicillin on the growth of PF-sensitive and -resistant strains. Growth of the PF-sensitive strain

Table 11

Sensitivity of PF-resistant Strains of E coli B to Antibiotics

Antibiotic	Amt./disc	Width of Clear Zone (mm)				
		B	B/Pr	PrA	PrB	PrC
Penicillin	10 units	2.33 ± .11 (6)	0 (7)	0 (4)	0 (4)	0 (4)
Cephalothin	30 mcg	6.58 ± .08 (3)	1.00 ± .14 (3)	0 (5)	1.20 ± .09 (5)	1.50 ± .35 (4)
Ampicillin	10 mcg	8.33 ± .17 (3)	3.63 ± .13 (2)	3.0 ± .20 (4)	4.90 ± .19 (5)	4.75 ± .14 (4)
Carbenicillin	100 mcg	10.83 ± .17 (3)	4.25 ± .10 (4)	7.25 ± .14(4)	6.70 ± .12 (5)	6.91 ± .15 (6)
Chloramphenicol	5 mcg	2.95 ± .10 (6)	0 (6)	2.25 ± .14(4)	1.50 ± 0 (4)	2.63 ± .24 (4)
Tetracycline	30 mcg	3.19 ± .18 (9)	2.63 ± .25 (9)	2.25 ± .14(4)	1.10 ± .16 (4)	1.00 ± 0 (4)
Streptomycin	10 mcg	1.60 ± .10 (5)	1.75 ± .30 (4)	2.00 ± .35(4)	0.67 ± .17 (3)	2.63 ± .13 (5)
Rifampicin	10 mcg	2.50 ± 0 (2)	0.88 ± .13 (2)	1.60 ± .10(5)	1.62 ± .13 (4)	1.38 ± .24 (4)
Gentamycin	10 mcg	1.58 ± .08 (3)	1.80 ± .17 (3)	NT	NT	NT
Nitrofurodantin	30 mcg	5.80 ± .08 (3)	6.00 ± .22 (3)	NT	NT	NT
Neomycin	10 mcg	1.50 ± 0 (5)	2.20 ± .17 (3)	NT	NT	NT
Septra	10 mcg	4.83 ± .17 (3)	3.17 ± .73 (3)	NT	NT	NT
Naladixic Acid	30 mcg	4.25 ± .14 (3)	4.83 ± .17 (3)	NT	NT	NT
Colistin	10 mcg	2.50 ± 0. (3)	3.30 ± .08 (3)	NT	NT	NT

Strain B is PF-sensitive, the others are resistant to 1.0 mM PF.

Values are presented with standard errors. Bracketed figures represent the number of determinations.

NT = not tested

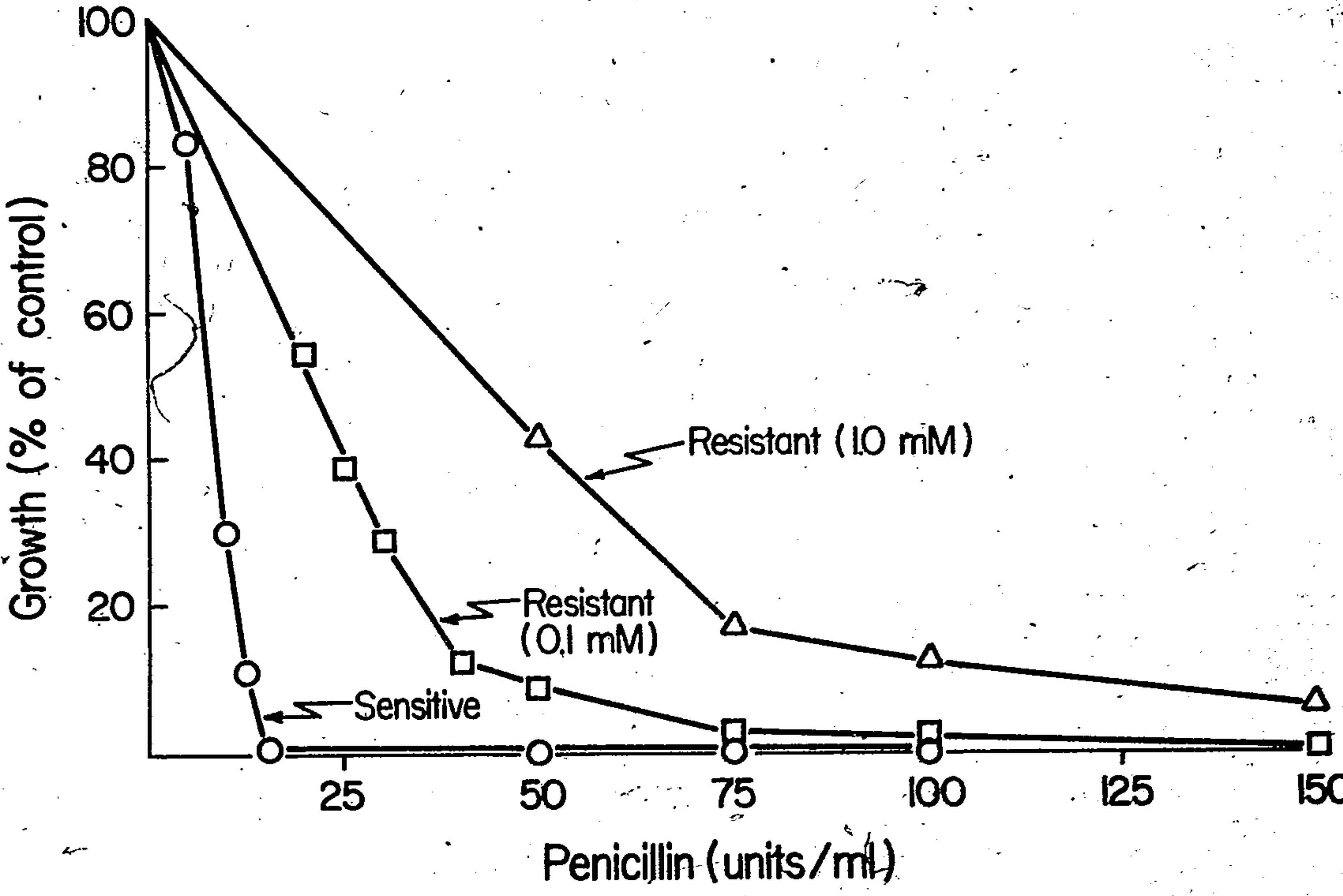
was completely inhibited by 15 U/ml penicillin while the strain resistant to 1.0 mM PF grew in penicillin concentrations of up to 150 U/ml. Resistance to 0.10 mM PF permitted growth in up to 50 U/ml of penicillin.

Penicillin resistance in E. coli is often due to β-lactamase activity (45). In order to test the importance of these enzymes, three strains resistant to 1.0 mM PF were grown in the presence and absence of penicillin and ~~both whole cell~~ and sonicated preparations were assayed for the presence of β-lactamase activity. None of the strains tested possessed detectable quantities of this enzyme. Since resistance is not due to destruction of the antibiotic, it is likely due to permeability (199).

The frequency of cross resistance between PF and penicillin was determined by replica plating. Table 12 shows that spontaneous resistance to either drug was very low. Frequency of resistance to either drug increased dramatically, however, when mutants were selected for resistance to the other drug.

Fig. 36. Effect of Penicillin Concentration on Growth of PF-sensitive and -resistant E. coli B.

Control growth was, in A_{660} units; Resistant (1.0 mM), 0.70; Resistant (0.10 mM), 0.76; Sensitive, 0.80.



Growth (% of control)
100
80
60
40
20
Resistant (1.0 mM)
Resistant (0.1 mM)
Sensitive
25
50
75
100
125
150
Penicillin (units/ml)

Table 12

Cross-resistance between Penicillin and Proflavine in E coli B

Drug Selected on	Total Colonies Tested	Penicillin Resistance		Proflavine Resistance	
		No. of Colonies	% Total	No. of Colonies	% Total
None	57	0	0	2	3.8
Proflavine	64	54	83.9	64	100.0
Penicillin	13	13	100.0	12	92.3

Concentrations of Penicillin and Proflavine employed for selection and testing were 25 units/ml and 0.1mM respectively.

Discussion

As previously stated, permeability barriers have been implicated in bacterial resistance to acridines (130,134,167,220,223,278). Increased or decreased antibiotic resistance in some Gram negative organisms has also been attributed to permeability changes, in particular to modifications of the lipopolysaccharide (LPS) moiety of the cell envelope (206,243,244,267) as well as to properties of the cell membrane (181,253). Permeability barriers may also be involved in cross resistance between acridines and antibiotics (150,269).

Resistance to penicillin and related antibiotics has been attributed to the production of β-lactamases by resistant organisms (52,152,198,199,227). Several kinds of β-lactamases have been isolated from enteric bacteria and have been classified according to their substrate specificity and sensitivity to sulfhydryl reagents (152,198). These enzymes are active against penicillin and/or cephalosporin derivatives (198). The genes involved in their production are frequently carried and transfered by R factors (198).

Intrinsic resistance involving changes in cell envelope permeability is often responsible for resistance to β-lactam antibiotics. Penicillin-resistant

Salmonella sp. (174) as well as ampicillin-resistant mutants of E. coli (178,160) were shown to possess modifications in the LPS layer of the cell envelope. Removal of the LPS layer by EDTA treatment of several enteric bacteria reduced their effective resistance to β-lactam antibiotics (20,267). Strains of E. coli possessing a mutation in the env A gene controlling cell wall structure showed increased sensitivity to penicillin (35). Permeability barriers have also been implicated in penicillin resistance in Ps. aeruginosa (199,238,239). Resistance to β-lactam antibiotics in enteric bacteria is often a result of both β-lactamase activity and intrinsic resistance. Individual strains of bacteria have been classified according to the relative contribution of these two resistance mechanisms (52,152,227).

We have demonstrated a correlation between PF and penicillin resistance in E. coli. In the apparent absence of β-lactamase activity, and in view of the extensive evidence favoring permeability barriers as the mechanism involved in acridine and antibiotic resistance, we suggest that PF resistance in E. coli B/Pr is related to permeability changes in the cell envelope.

Previous work from our laboratory has suggested that gross permeability differences to PF do not exist

between PF-sensitive and -resistant strains (134) and that permeability is not involved in resistance (96). It is possible, however, that subtle permeability changes have taken place that were not detectable in PF binding studies. Nakamura has shown that AF-sensitive and -resistant E. coli K12 bind similar amounts of various acridines including PF (170) in spite of the fact that AF-resistant cell membranes are lacking a specific structural protein involved in AF uptake and binding (171).

Some of our studies have suggested that permeability barriers, if they exist at all, may be imposed by the cell membrane rather than the cell wall. PF affected ATP levels and amino acid transport in both B and B/Pr suggesting that the dye can penetrate as far as the membrane in both strains. It was also shown that rates of amino acid transport in B/Pr vesicles were up to four times faster than in B vesicles while B/Pr whole cells transported significantly slower than B cells. These results suggest that B/Pr membranes have been altered in some way, possibly due to the development of PF resistance. On the other hand, penicillin resistance has not been shown to involve modification of the cell membrane (87). Nakamura has also shown that penicillin resistance is not related to AF resistance

in E. coli K12 (171).

The study of PF resistance in B/Pr has been complicated by the diversity of binding sites available to PF(96) as well as by the energy-dependent release of bound dye (134). As a result, we suggest that penicillin may be useful as a probe to study PF resistance. It would be necessary, however, to first demonstrate that both PF and penicillin resistance are controlled by the same gene.

As was mentioned in the General Introduction, acridine dyes have been used to treat a wide variety of infections and diseases. Our results suggest that chemotheraputic use of these dyes may give rise to mutants cross resistant to other inhibitors including antibiotics. This phenomenon has recently been observed in N. gonorrhoeae (150).

Chapter 7

GENERAL DISCUSSION

GENERAL DISCUSSION

The original aim of this thesis was to determine the mechanism of PF inhibition of glucose utilization in *E. coli* B. We have shown that PKI, a key enzyme in the regulation of glucose utilization (148), was significantly inhibited in vitro in an allosteric manner by PF. In view of the absence of PF sensitivity in other processes related to glucose utilization, we propose that PKI constitutes the sensitive site. PKI activity could not be studied in vivo, and hence it was not possible to determine if PF inhibited it. However, it was suggested that sufficient unbound PF would be available, when glucose utilization was blocked, to upset the regulation of glycolysis.

It was previously proposed, as a working hypothesis, that glucose metabolism was the primary site of PF inhibition of *E. coli* B (153) since this process was affected by PF in sensitive but not resistant cells. A correlation was also observed between inhibition of growth and inhibition of glucose utilization (153). No attempt was made at that time to determine the PF-sensitivity of other essential cell processes. The data presented here shows that the original hypothesis was in error. Cells grown in glucose and glycerol

were equally sensitive to PF in spite of the fact that glycerol-grown cells contain low levels of PKI. This observation suggests that PKI (and, by implication, glucose utilization) may not be the primary site of PF inhibition. DNA replication was found to be more sensitive to PF than glucose utilization and, in fact, inhibition of growth could be better correlated to the former than to the latter.

Other PF-sensitive metabolic processes may exist in the cell. Cells grown in acetate or succinate as the sole source of carbon do not require PKI for growth, yet are highly sensitive to PF. DNA replication could be more sensitive to PF in these cells due to their low rate of metabolism; however, it is possible that other metabolic processes such as oxidative phosphorylation or certain biosynthetic enzymes are highly sensitive to PF.

Evidence has been presented to implicate PF as a membrane uncoupler. This dye was shown to stimulate respiration, inhibit amino acid transport, reduce ATP levels and stimulate membrane-bound ATPase. In studies involving amino acid transport however, PF affected sensitive and resistant cells in a similar fashion. It is therefore unlikely that the effects of PF on membrane function play an import-

ant role in inhibition of growth. A possible exception would be the dramatic effect of PF on intracellular ATP levels which may be partly indirect, due to blocking of metabolism, but which is probably partly a direct process, at the membrane level. Loss of ATP may have a significant effect on other cell processes such as DNA replication. We have proposed, however, that ATP levels in the presence of PF are controlled by the ability of the cells to metabolize glucose, and that this latter process is less sensitive to PF than DNA replication. Although the effects of PF on ATP levels in growing cells have yet to be investigated, it is unlikely that the ATP levels would drop sufficiently to block DNA replication while glucose utilization was only partially inhibited (Fig. 19).

It was previously thought that PF resistance in E. coli B existed at the metabolic level and that permeability played no role in determining resistance (96). None of the PF-sensitive metabolic processes studied in vitro, including PKI, ATPase and active transport, showed significant resistance to PF however, suggesting that resistance may not occur at the metabolic level. It was discovered that PF-resistant cells of E. coli were cross resistant to

a number of antibiotics including penicillin. Cross resistance between unrelated inhibitors may be attributed to permeability changes in the cell envelope. It appears likely, therefore, that PF resistance in _E. coli_ is due to permeability changes resulting in exclusion of the dye from some active site.

At present, the nature of these permeability changes are unknown. Modifications of the LPS layer of the cell envelope are usually responsible for intrinsic penicillin resistance (160,174,178); however, the observation that PF affects transport and ATP levels in PF resistant cells suggests that the dye penetrates to the cell membrane. Membranes from PF-resistant cells transport up to 5 times faster than membranes from sensitive cells and it is possible that resistant membranes have become modified in structure and/or function as a result of the development of PF resistance.

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