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TABLE OF CONTENTS

	Page
Table of Contents	i
Acknowledgements	v
List of Tables	vi
List of Figures	vii
Abstract	xi
Chapter I Introduction	1
Chemical properties and uses of proflavine and other acridine dyes	2
The physicochemical basis for the antibacterial properties of acridines	6
Some biological effects of acridines..	9
Mutagenic Activity	9
Antimutagenic activity	11
Episome elimination	11
Photosensitizing properties	12
Virus inhibition	13
Enzyme inhibition	14
Binding of acridines to receptor molecules	17
Nucleic acid sites	17
Non-nucleic acid sites	30
Uptake and resistance to acridine dyes	32

Chapter ll Materials and Methods 37

Cultures 38

Media 38

Culture conditions 38

Buffers 39

Reagents and chemicals 39

Analytical methods 40

Preparation and testing of spheroplasts 41

Treatment for inducing autodegradation of RNA 43

Treatment for lowering nucleic acid and protein content of spheroplasts and heated cells 45

Measurement of proflavine binding 46

Chapter lll Results 50

A. The effect of glucose on proflavine binding by cells and spheroplasts of proflavine -resistant and -sensitive Escherichia coli.. 51

i. Determination of optimal sucrose concentration for protection of spheroplasts 52

ii. Effect of sucrose on dye-binding capacity of cells and spheroplasts with and without glucose 55

iii. Effect of glucose on proflavine -binding - Time course study 57

B. Comparison of the dye-binding capacity of cells and spheroplasts 63

C. Studies on the relationship between nucleic acid content and proflavine binding 72

i. Effect of mercury-induced nucleic acid breakdown on proflavine-binding 73

ii. Effect of exposure of cells to PCMB+/EDTA 83

iii. Relation between proflavine binding and content of macro-molecules in heated cells and spheroplasts ... 84

D. Correlation of pH and dye-binding with metabolic activity 88

i. Effect of incubation with various metabolites in the presence or absence of proflavine 88

ii. Effect of pH changes and dye-binding with 0.5% and 5.0% glucose (stationary suspensions) 90

iii. Comparison of pH changes and dye-binding with glucose, ribose and glycerol 95

(a) Aerated suspensions 95

(b) Aerated or stationary suspensions in low buffer 98

iv. Effect of sodium cyanide on pH changes and dye release 108

E. Effect of proflavine on metabolic activity. 113

Chapter IV Discussion and Conclusions 124

Site of proflavine action 125

Mechanism of proflavine release 133

Literature cited 137

Appendix .....

Reprints .....

ACKNOWLEDGEMENTS

I am deeply grateful to Dr. D.J. Kushner for his guidance, patience and constant encouragement both during the course of this investigation and during the preparation of the dissertation. I am indebted to Dr. B.M.Mehta, who corroborated with me on some experiments, for valuable help and advice. I wish to thank Mr. Robin McKellar for communicating unpublished information. The technical assistance of Mrs. Carol Rossman in some experiments is also appreciated. The goodwill, interest, co-operation and enthusiastic discussions of my former fellow graduate students, Dr. Michael Forsyth, Dr. Jean-Yves D'Aoust, Mr. David Shindler and Mrs. Betty Hurst, and other members of Dr. Kushner's laboratory, Dr. M.B. Gochnauer and Mrs. Paulette Fejer, are warmly remembered. Special thanks are due to my husband, Ron, for his unfailing encouragement and diligence in proofreading the manuscript. I am much obliged to Mr. Jacques Helie for reproducing the figures, to Miss June Elizabeth Skinner for her enduring kindness and skill in typing the manuscript, and to Mrs. Maria Stein and Mrs. Edna Zuber for their aid in obtaining library materials in Toronto. The financial support of the Medical Research and National Research Councils of Canada is gratefully acknowledged.

LIST OF TABLES

TABLE		Page
1a	Release of E_{260} absorbing materials from proflavine-sensitive and -resistant spheroplasts of E. coli in different sucrose concentrations	53
1b	Release of E_{260} absorbing materials from spheroplasts in different sucrose concentrations after 15 and 30 minutes of incubation	54
2	Effect of glucose on proflavine binding by cells and spheroplasts	56
3	Macromolecular contents and proflavine binding in treated cells and spheroplasts of E. coli B	85
4	pH changes produced by cells incubated with various metabolites in the absence of proflavine	89

LIST OF FIGURES

FIGURE		Page
1	The chemical structure of some acridines and related compounds	4
a	Numbering methods for the acridine ring	4
b	Acridine and some important derivatives	4
c	Derivatives or related compounds of historical or present significance	4
2	Binding models for acridine dyes	23
a	Schematic representation of the tangential stacking model	23
b	The intercalation model	23
c	A modified intercalation model	24
d	Arrangements of the acridine molecule with respect to the base pair in intercalary binding	24
3a	Effect of glucose on dye binding by sensitive cells and spheroplasts	59
3b	Effect of glucose on dye binding by resistant cells and spheroplasts	61
4	Saturation of sensitive cells in the absence of sucrose at two different cell concentrations.	66
5a	Saturation of sensitive cells and spheroplasts with proflavine in the presence and absence of 10% sucrose	68

5b Saturation of resistant cells and spheroplasts with proflavine in the presence and absence of 10% sucrose 70

6a Proflavine binding and macromolecular contents of sensitive cells treated with mercuric ion for prolonged periods 76

6b Proflavine binding and macromolecular contents of sensitive cells treated with mercuric ion for shorter periods 78

6c Proflavine binding and macromolecular contents of resistant cells treated with mercuric ion for shorter periods 80

7 Proflavine binding and macromolecular contents of sensitive cells treated with PCMB with or without prior brief exposure to EDTA 82

8a pH changes and release of bound proflavine with glucose-treated resistant and sensitive cells 92

8b pH changes and release of bound proflavine with glucose-treated resistant and sensitive cells... 94

9a pH changes in aerated suspensions of resistant and sensitive cells treated with glucose, ribose, or glycerol 97

9b Release of bound proflavine from aerated suspensions of resistant or sensitive cells treated with glucose, ribose, or glycerol..... 100

10a pH changes in poorly buffered shaking or stationary suspensions of resistant or sensitive cells treated with glucose, ribose, or glycerol in the absence of proflavine 102

10b pH changes and release of bound proflavine from resistant cells treated with glucose, ribose,or glycerol in poorly buffered shaking or stationary suspensions 104

10c pH changes and release of bound proflavine from sensitive cells treated with glucose, ribose,or glycerol in poorly buffered shaking or stationary suspensions 106

11a Effect of sodium cyanide on pH changes and proflavine release from resistant cells treated with glucose or glycerol 110

11b Effect of sodium cyanide on pH changes and proflavine release from sensitive cells treated with glucose or glycerol 112

12a Glucose utilization and release of bound proflavine by resistant cells under aerobic conditions 115

12b Glucose utilization and release of bound proflavine by resistant cells under anaerobic conditions 117

13a Glucose utilization and release of bound proflavine by sensitive cells under aerobic conditions 120

13b Glucose utilization and release of bound proflavine by sensitive cells under anaerobic conditions 122

ABSTRACT

Proflavine-resistant and proflavine-sensitive Escherichia coli strains bound about the same amount of proflavine when suspended in buffer containing the dye. Resistant, but not sensitive, cells quickly released bound proflavine on the addition of glucose or certain other metabolites. Sensitive cells also released bound proflavine when the amount of dye bound per cell was low. Spheroplasts of resistant cells protected by 10% sucrose released bound proflavine, but osmotically lysed spheroplasts did not. Thus, intact membranes are not required for proflavine binding (which appears to be a passive process), but are required for proflavine release (which apparently is an enzymic process). The presence of 20% sucrose greatly reduced proflavine binding by resistant cells. Adding glucose to such cells did not cause a release of the residual proflavine.

Proflavine binding was compared in proflavine-sensitive and -resistant cells and spheroplasts. Cells and spheroplasts of the sensitive strain bound similar amounts of proflavine at saturation, whether or not the spheroplasts were osmotically protected by 10% sucrose. In the absence of sucrose, resistant cells and spheroplasts were saturated with the same amount of proflavine. In 10% sucrose, saturated resistant spheroplasts bound considerably less dye than the cells. This difference between the sensitive and resistant strains may be related to the greater instability of the resistant spheroplasts in 10% sucrose.

Treatment with Hg^{++} ions or with parachloromercuribenzoate (PCMB) led to degradation of RNA. Some losses in DNA and protein also occurred, depending upon the severity of the mercury treatment. Cells almost depleted of their nucleic acid contents and half their protein were still able to bind very considerable amounts of dye. With less severe treatment, Hg^{++} exposed cells bound as much dye as the untreated cells. Heating cells for 10 minutes at 100°C markedly increased their proflavine binding capacity. On treatment of heated cells or osmotically lysed spheroplasts with nucleases and Pronase, practically all the nucleic acids were removed. The proflavine binding ability of such preparations fell by only 30 to 50%. These experiments suggest that there are other important proflavine binding sites in cells besides nucleic acids. They also indicate that treatments which remove one class of binding site may open up others. For this reason it is extremely difficult to determine, by any selective degradation procedure, the quantitative binding sites of proflavine in the intact cell.

Proflavine inhibited the lowering of pH after glucose addition in sensitive cells but not in resistant cells. Glucose-treated sensitive cells gradually lost dye with prolonged incubation. The gradual decrease in the amount of cell-bound proflavine paralleled a gradual fall in pH during glucose utilization. More dye was released from both sensitive and resistant cells with 5.0% glucose than with 0.5% glucose. When glucose was added to resistant or sensitive cells in poorly buffered media, cells immediately released some dye. In vigorously aerated cell suspensions approximately one-quarter

of the dye was released from either strain. Both cell strains lost considerably more dye on further incubation. In stationary suspensions, cells first lost proflavine and later rebound it when glucose was exhausted.

Cyanide inhibited the pH changes produced by glucose in vigorously aerated cell suspensions. The inhibition was stronger with the sensitive cells. In the resistant cells, cyanide inhibited the glycerol-induced release of bound proflavine, but not that induced by glucose. This may reflect the fact that glucose can yield energy by both aerobic and anaerobic pathways, but glycerol only by the aerobic pathway. Cyanide also inhibited the release of proflavine from the vigorously aerated glucose-treated sensitive cells.

Proflavine inhibited glucose utilization by sensitive, but not by resistant, cells. The degree of inhibition was greater under anaerobic conditions. Aerobically, proflavine did not completely inhibit glucose utilization. In resistant cells that had bound proflavine, glucose utilization was accompanied by release of the dye. After glucose was used up, cells could again take up proflavine. If the amount of dye bound to sensitive cells was too low to inhibit glucose utilization, adding glucose to these cells caused them to release the dye. With higher proflavine concentrations, inhibitory to glucose utilization, the dye remained cell bound. Thus metabolic energy causes the release of proflavine by both sensitive and resistant cells. In the former energy production is inhibited by proflavine, and thus the dye prevents its own release.

I N T R O D U C T I O N

CHEMICAL PROPERTIES AND USES OF PROFLAVINE AND OTHER ACRIDINE DYES

In 1870 Graebe and Caro (cited by Albert, 1966) extracted a weakly basic, pale yellow constituent from the higher boiling fractions of coal tar. The highly crystalline solid emitted vapors which irritated the nose and throat; whence, the name "acridine". By appropriate substitution, properties such as basic strength and colour can be modified extensively. The acrid properties are lost when the substituents, such as the amino groups of aminoacridines, display hydrogen bonding (Albert, 1966).

Acridine may be regarded as an aza derivative of anthracene (Acheson, 1956). Eight years after its discovery Bernthsen found that the correct empirical formula was $C_{13}H_9N$, and in 1905 Hinsberg suggested an ordinary Kekulé representation of its almost planar structure. Various numbering methods for the acridine ring have been advanced in the years since its discovery (see Albert, 1966). The numbering system introduced by Graebe and Lagodzinski in 1893, recently adopted by the International Union of Pure and Applied Chemistry, is used throughout this thesis (Figure 1a). The chemical structures of acridine and some of its derivatives are shown in Figure 1b.

Interest in the acridines has arisen largely from the valuable chemotherapeutic properties of certain derivatives. Early work was reviewed by Albert (1966). In 1888 Auclert initiated clinical experiments with acridines in his attempt to find a medical use for chrysaniline (Figure 1c). Tappeiner's discovery in 1896 that the

FIGURE 1

THE CHEMICAL STRUCTURE OF SOME ACRIDINES AND RELATED COMPOUNDS.

FIGURE 1a

NUMBERING METHODS FOR THE ACRIDINE RING

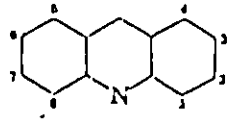
FIGURE 1b

ACRIDINE AND SOME IMPORTANT DERIVATIVES

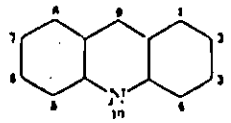
FIGURE 1c

DERIVATIVES OR RELATED COMPOUNDS OF HISTORICAL OR PRESENT SIGNIFICANCE.

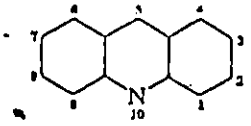
Figure 1A



Alternate Numbering
(After Schöpff, 1892).

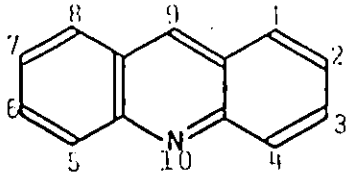


I.U.P.A.C. Numbering
(After Graebe and
Lagodzinski, 1893).

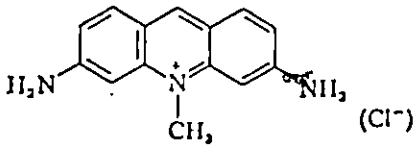


Alternate Numbering
(After Richter, 1900)

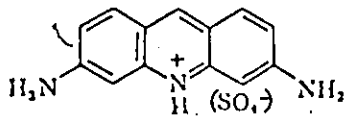
Figure 1B



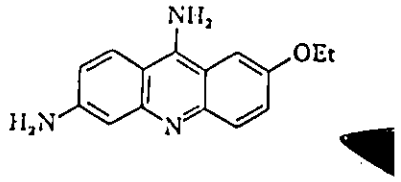
ACRIDINE



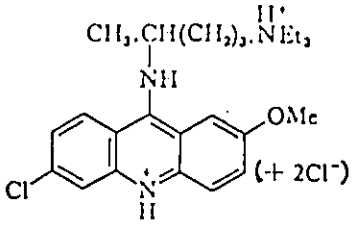
Euflavine, 'Trypaflavin'
(Purified acriflavine)
(3,6-diamino-10-methylacridinium
chloride)



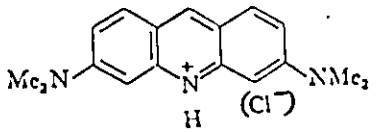
Proflavine
(3,6-diaminoacridinium
monohydrogen sulfate)



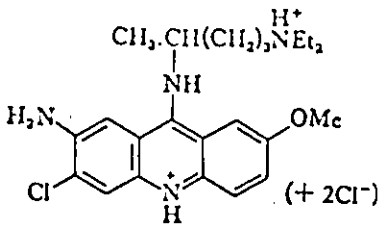
Ethacridine
(3,9-diamino-7-ethoxy
acridine)



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

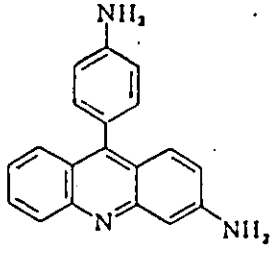


Acridine Orange
(3,6-Bis(dimethylamino)-
acridine hydrochloride)

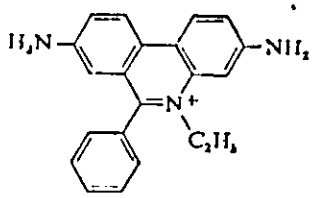


Aminoacrichine
dihydrochloride
(2-amino derivative of
mepacrine).

Figure 1C



Chrysaniline



Ethidium

derivatives, 7-methylchrysaniline and 2,7-dimethyl-chrysaniline, were more toxic than quinine towards several protozoa prompted the pharmacological studies of Jodlbauer in 1897. In the same year Mannaberg tried unsuccessfully to cure human malaria with these compounds.

Sixteen years later Ehrlich traced the trypanocidal properties of crude tryparosan (a triphenylmethane dye) to contaminating acridines. Under his direction Benda, in 1912, synthesized 3,6-diamino-N-methylacridinium chloride or 'Trypaflavin' which cured trypanosomiasis in the mouse but was inactive in man. Shiga's discovery in 1913 that 'Trypaflavin' was bactericidal for Vibrio cholera revived interest in the acridines. In 1913, Browning and Gilmor found that proflavine was unique among known antiseptics, being highly bacteriostatic in dilute solution for the majority of pathogenic bacteria, even in the presence of serum protein. Moreover, proflavine was relatively innocuous for human leucocytes. With the outbreak of World War I, largely through the work of Browning et al. (1917), proflavine and acriflavine rapidly established themselves as potent agents in the prevention and treatment of infection in wounds. During World War II neutral proflavine, and finally 9-aminoacridine, which is non-staining, were used. Although at present anti-biotics have largely replaced them, acridines still find limited use in casualty surgical procedures since (a) they can deal effectively with large innocula of almost all Gram-positive and Gram-negative organisms found in wounds; and (b) allergic sensitivity and drug resistance are relatively rare (Albert, 1966; Browning, 1964).

The chemotherapeutic value of acridines is not confined to their antibacterial action. In World War II with the loss of the Dutch East Indies and consequently the quinine supply, quinacrine (mepacrine or 'Atebrin', Figure 1b) was of major importance to the Allies as the only known shizontocidal antimalarial which could be synthesized at the time. It proved to be an efficient prophylactic with fewer side effects than quinine, and despite competition from newer antimalarials it is still widely used. Other protozoal and helminth infections commonly treated with acridines are theileriasis in cattle (by aminoacrichine), amoebiasis (by ethacridine), tapeworm (by mepacrine) and hookworm in man (by acriflavine).

'Autoimmune' diseases and 'petit mal' epilepsy are also relieved by mepacrine. Tacrine (9-amino-1,2,3,4-tetrahydroacridine) is a powerful analeptic and specific antidote for the nerve gas 'Ditran'.

Watanabe (1963) suggested that in the future, acridines may find clinical use in combatting multiple drug resistance in bacteria by curing cells of episomes.

THE PHYSICOCHEMICAL BASIS FOR THE ANTIBACTERIAL PROPERTIES OF ACRIDINES

The effects of chemical constitution on acridine antibacterial action were established largely through the research of Albert and his colleagues (Albert et al., 1938, 1941, 1945, 1948 and 1949; Rubbo et al., 1942; reviewed by Albert, 1966). The following overall picture has emerged:

(1) The antibacterial activity of acridines is dependent on their ionization as cations; anions, neutral molecules and zwitterions are ineffective.

(2) The most essential factor for a marked antibacterial action is a basicity sufficiently high to effect at least 50% ionization as cations at pH 7.3 and 37°C. Above 50% ionization there is little further increase in potency.

(3) Since the pH of the medium, as well as the pK_a of the acridine, controls the level of the bacteriostatic cationic species, both these parameters influence the antimicrobial activity.

(4) Plots of the least bacteriostatic concentration of cation versus pH reveal a direct competition between acridine cations and hydrogen ions. For bacteriostasis a ratio of about 500 acridine cations per hydrogen is necessary.

(5) A minimal planar surface area of between 28 and 38 square A° is an architectural prerequisite for marked antibacterial activity. Hydrogenation of even one of the rings which gives rise to the "buckled" tetrahydroderivative, produces a significant decline in biological potency. A planar polycyclic structure is also essential for the mutagenic properties of acridines (Greenstein, 1954; cited by Waring, 1966).

(6) As a rule the nature of the ring substituents is important only insofar as it influences either the ionization level or the area of the planar surface.

Albert (1966) suggested that the simplest interpretation of acridine inhibition is that acridines compete with hydrogen ions for vitally important anionic sites, possibly

nucleic acids, on the bacterium. The importance of interactions of acridines with nucleic acids will be considered in more detail below, but it should be mentioned here that high bonding affinities between nucleic acids and acridines have been demonstrated both *in vivo* and *in vitro* (e.g., Roth and Manjohn, 1967; Blears and Danyluk, 1967; Li and Crothers, 1969). In addition, acridine inhibition of DNA-primed DNA and RNA polymerase seemed to be related to interaction with the DNA-template (Hurwitz *et al.*, 1962). However, a number of enzymes including hexokinase of *Pl. gallinaceum* (Speck and Evans, 1945; cited by Albert, 1966), yeast phosphatase (Silverman, 1949) and yeast glyceraldehyde phosphate dehydrogenase (Witt *et al.*, 1968) are also inhibited by acridines.

Other lines of evidence suggest that acridines act on the surface of bacteria, namely (a) completely ionized derivatives are at least as active as those which are not entirely ionized; and (b) the introduction of aliphatic side chains on adequately ionized derivatives facilitates penetration of the cytoplasmic membrane, but leads to a reduction in bacteriostatic properties (Albert, 1968). In addition, an electron-microscopic examination of acriflavine-treated *E. coli* cells revealed that the dye injured the plasma membrane and the surface layer of the cytoplasm in acriflavine-sensitive cells, but not in acriflavine-resistant cells (Nakamura and Suganuma, 1972). Since both nucleic acids (Yudkin and Davis, 1965; Ryter, 1968) and enzymes (Russell, 1971) are associated with the plasma membrane, the two views on the site of acridine action may be compatible.

SOME BIOLOGICAL EFFECTS OF ACRIDINES

Acridines have many biological effects. Their mutagenic, antimutagenic, episome curing, and in some cases their photosensitizing and enzyme-inhibiting effects, depend upon their interaction with the genome.

Mutagenic Activity

With a few exceptions (e.g., Ames and Whitfield, 1966; Sesnowitz-Horn and Adelberg, 1969) acridines are not mutagenic for bacteria, but are mutagenic for viruses (DeMars, 1953; Brenner et al., 1958; Lerman, 1964b).

Brenner et al. (1961) proposed that acridines act as mutagens by the insertion or deletion of one or more nucleotides in the genome. Either of these acts would produce a reading frame-shift in the genetic code, creating an entirely new set of codons which would give rise to an inactive protein or none at all. They also proposed that the wild-type revertants (Orgel and Brenner, 1961) obtained after a second treatment with acridine were actually double mutants. The second mutational event would restore the normal reading frame except for an altered set of codons between the two mutant sites. If the amino acids coded for by these altered bases did not fully inactivate the final protein product, a revertant could be detected. By a genetic analysis of proflavine induced mutations in phage T_4, Crick et al. (1961) showed that these predictions were correct and also established the triplet nature of the genetic code. Streisinger and his collaborators (Streisinger et al., 1966; Okada et al., 1966) confirmed the frame-shift hypothesis by a study of the amino acid sequences in normal and altered lysozymes produced

by pseudo-wild T_4 strains carrying (a) a mutually suppressing pair of acridine mutations, and (b) three "insertion" mutations.

The accumulated research of Streisinger's group (including Imada et al., 1970; Ocada et al., 1970) established that most proflavine-induced mutations arise by insertion, generally of two or more base pairs. The insertions usually duplicate the immediately neighbouring base-pair sequences in the wild-type DNA. These workers also suggested that regions of base-pair redundancy would be the most susceptible to frame-shift mutagenesis.

Waring (1972) gives a comprehensive review of the various mutational mechanisms that have been proposed. Sesnowitz-Horn and Adelberg (1969) showed that proflavine was an efficient mutagen for conjugational zygotes of E. coli in which recombination was occurring. Their findings support Lerman's (1963) hypothesis that the frame-shifts result from the crossing over between DNA molecules which, because of the presence of an intercalated acridine, have short regions of non-homologous pairing. More recently Lerman (1968) has suggested that the "recombination" could involve reunion between newly synthesizing DNA and template strands as a means of circumventing a terminal one-strand block (i.e. an acridine) to DNA synthesis. In the recombinational event, short regions of the blocked template would be excised. Such a mutational mechanism would account for the copious production of low molecular weight DNA found when T_4- infected E. coli cells were treated with 9-aminoacridine (Lerman and Altman, 1968; Altman and Lerman, 1970). Acridine inhibition of ligase action (an enzyme

involved in the repair of single strand breaks) could also give rise to such fragments (Altman and Lerman, 1970). Sarabhai and Lamfrom (1969) found that frame-shifts occurred more frequently in mutants with a defective polynucleotide ligase. This finding favours the mutational mode suggested by Lindstrom and Drake (1970): frame-shifts arise by proflavine-induced anomalies of DNA repair.

Antimutagenic Activity

Acridines and other nitrogen-containing heterocyclic compounds reduce the rate of emergence of resistant populations in bacteria and cancer cells (Sevag and Ashton, 1964). Although the relationship between structure and activity has not been completely clarified, the activity of certain compounds appears to be related to their intercalary properties (Heller and Sevag, 1966; Johnson and Bach, 1967).

Episome Elimination

Acridine dyes in low concentration cure cells of a number of cytoplasmically inherited genetic factors including the sex factor (Hirota, 1960) and drug resistance (R) factors (Watanabe, 1963; Kontomichalou, 1967; Mitsuhashi, 1971). Yamagata and Uchide (1969) showed that acridines act by decreasing the relative multiplication of the sex factor, possibly by a selective interference with the initiation of F^+ factor replication (Nishimura et al., 1971). Acridines also eliminate other plasmids, such as the respiratory factors of yeasts (De Deken, 1966), moulds, and staphylococci (Arlett, 1957; Browning and Adamson, 1950; cited by Albert, 1966) and the kinetoplast of trypanosomes (Simpson, 1968). Similar phenomena

have been reported with ethidium compounds (Bouanchaud et al., 1968).

Extensive investigations of the loss of respiratory competence in yeast induced by these compounds revealed that the mutation is associated with a rapid halt in the synthesis of certain respiratory enzymes, and with the inhibition of mitochondrial DNA synthesis and transcription (Perlman and Mahler, 1971; cited by Waring, 1972; Fukuhara and Kujawa, 1970). Similar effects indicating interference with mitochondria have been reported for trypanosomes (Simpson, 1968), Acetabularia (Heilporn and Limbosch, 1971) and the cells of higher organisms (Milner, 1972).

One feature that the numerous effects noted above have in common is that they involve an attack on extra-chromosomal DNAs, many examples of which have been shown to exist as closed, circular, duplex molecules (Waring, 1970; Mitsuhashi, 1971). In 1968 Bauer and Vinograd found that at low dye concentrations, the binding of ethidium (in vitro) to supercoiled DNA was enhanced over its binding to non-supercoiled DNA, whereas the reverse was true at high dye concentrations. Waring (1972) suggested that supercoiling of episomes may permit low dye concentrations to eliminate these without harming the cell.

Photosensitizing Properties

In vitro, visible light damaged the acridine-orange DNA complex (Freifelder et al., 1960). Acridines photoinactivated infectious polio virus RNA (Schaffer, 1962). Mature TMV was also photoinactivated (Oster and McLaren, 1950).

Mature enterovirus was relatively resistant to such photo-inactivation, presumably because it did not bind acridines under ordinary conditions (Schaffer, 1962). Dye present at the time of virus propagation, however, permanently photo-sensitized the virus, as shown by the fact that dark-propagated virus was fully infectious until exposed to light (Schaffer, 1962). Mutations, chromosome abnormalities in plants (Webb and Kubitscheck, 1963; Kihlman, 1961; cited by Albert, 1966) and chromosome breakage in human cells (Ostertag and Kersten, 1965) have been shown to result from the photosensitizing effects of acridines.

Virus Inhibition

It is generally considered that acridines inhibit the formation of infectious bacteriophage or virus by interfering with some process late in the latent phase concerned with virus assembly from its subunits (Foster, 1948; Susman et al., 1965; Piechowski and Susman, 1967). This view originated from the findings that (a) in the presence of acridines large amounts of phage specific protein and nucleic acid, but few or no intact progeny, were synthesized; and (b) an increase in phage titre occurred immediately after removal of the acridine (De Mars, 1955; Iosson and Fry, 1969).

The lighting conditions employed, the virus system studied, and the acridine used (and its concentration) influenced the experimental results. Under dark conditions (with proflavine) Brown and Stewart (1960) found that infectious, ribonuclease-sensitive RNA and viral protein of the foot-and-mouth virus accumulated in about 50% of their normal amounts, yet only 1-10% of the normal virus yield was obtained. In the

light no infectious RNA was found.

In the presence of proflavine T-phage infected E. coli lysed at the expected time, although few (T_2) or no (T_4 and T_6) infectious phage were produced (Foster, 1948). In contrast, 9-aminoacridine was found to retard or prevent lysis by phage T_4 but not by phage λ (Susman et al., 1965; Iosson and Fry, 1969). Lerman and Altman (1968) reported that although a normal amount of T_4 DNA was synthesized in the presence of 9-aminoacridine, almost none of the usual replicative forms of phage DNA were found. They also showed that further DNA synthesis was necessary for the production of viable T_4 phage after 9-aminoacridine (at optimal mutagenic concentration) was removed from the culture media. On the basis of these and other results, they (1970) suggested that acridine interference in virus production is not completely characterized as a block in the packaging of normal viral precursors.

Enzyme Inhibition

Bacteria exposed to bacteriostatic concentrations of acridines exhibit unbalanced growth and frequently grow into unusually large or filamentous cells (Ciak and Hahn, 1967; Wolfe et al., 1971). The appearance of these cells suggests that acridines interfere in macromolecular synthesis.

In vivo studies showed that acridines rapidly suppressed the uptake of radioactive precursors into the nucleic acids of bacteria (Woese et al., 1963), yeasts (Nago and Sugimura, 1965), plasmodia (Schellenberg and Coatney, 1961), and mammalian cells (Scholtissek, 1965). However, it should be emphasized that considerable variation in the effects of acridines on macro-

molecular synthesis has been observed as follows. Bacteriostatic concentrations of proflavine inhibited the de novo synthesis of mRNA in E. coli cells (Woese et al., 1963) without influencing translation (Hurwitz and Rosano, 1965). In contrast, bacteriostatic concentrations of quinacrine, which inhibited DNA and protein synthesis up to 40% in E. coli, had no effect on RNA synthesis; bactericidal concentrations completely inhibited DNA synthesis and greatly reduced RNA and protein synthesis (Ciak and Hahn, 1967). In the dark, protein synthesis by acridine orange-treated amnion cells (Zelenin and Liapunova, 1964) was strongly inhibited though RNA synthesis was not. Under similar conditions Watts and Davis (1966) studied the effects of proflavine on macromolecular processes of Hela cells. In this system, RNA, then DNA, and finally protein synthesis (in that order) were inhibited by increasing concentrations of proflavine.

Acridine inhibition of nucleic acid synthesis has also been demonstrated in vitro with purified DNA-dependent DNA-and RNA-synthesizing enzymes (Hurwitz et al., 1962; Goldberg and Rabinowitz, 1962; Hochester and Chang, 1963; Waring, 1966; O'Brien et al., 1966). Proflavine interfered with both the initiation and elongation processes of RNA synthesis (Maitra et al., 1967; Sentenac et al., 1968). Hurwitz et al. (1962) showed that concentrations of proflavine which inhibited RNA-polymerase by 30%, produced an 85% inhibition of DNA-polymerase. Since the inhibition of either enzyme was relieved by increasing the amount of DNA present in the reaction mixture, it was concluded that acridines inhibit

by binding to the DNA template. Acridine inhibition of DNAase is considered to occur in a similar fashion (Kurnick and Radcliffe, 1962; Leith, 1963).

On the basis of these *in vitro* studies it has been suggested that many of the effects of proflavine *in vivo* are due to the inhibition of DNA-dependent nucleic acid synthesis (Hurwitz *et al.*, 1962). However, Nicholson and Peacocke (1966) have shown that the decreased activity of RNA polymerase appears to involve a direct inhibition of the enzyme by aminoacridines. Moreover, as has already been mentioned (p. 8), acridines inhibit a variety of other enzymes (e.g. cytochrome c reductase, glucose-6-phosphate dehydrogenase, etc., reviewed by Albert (1966)) and readily bind to non-nucleic acid sites (p. 31). Acridines have also been found to activate the catalytic action of certain proteases (Hall *et al.*, 1972). The variable sensitivity of macromolecular synthesis to acridines was noted above. The damaging effects of acridines on ribosomes (Soffer and Gros, 1964) and their combination with transfer RNA (Weinstein and Finkelstein, 1967) are likely to contribute to reduced protein synthesis.

This broad spectrum of action makes it unlikely that the growth inhibitory and chemotherapeutic effects of acridines result solely from their ability to interact with DNA.

BINDING OF ACRIDINES TO RECEPTOR MOLECULES

Nucleic Acid Sites

Many of the biological effects of acridines do suggest that they act by combining with DNA. Acridine dyes are, in fact, widely used as histological stains both *in vivo* and *in vitro* for nucleic acids (Smiles and Taylor, 1957; cited by Albert, 1966; Bradley, 1965; Jasper and Jain, 1966; MacInnes and McClintock, 1970). DeBruyn *et al.* (1953) demonstrated that the extraordinary *in vivo* affinity of diaminoacridines for intranuclear deoxyribose- and ribose-nucleoproteins, in contrast to that of other basic fluorochromes, did not depend upon deleterious experimental conditions. They also found that acridines had little affinity for extranuclear nucleoproteins *in vivo*, but that *in vitro* they had affinity for all such proteins regardless of their localization in the cell.

Acridine orange is a particularly useful stain because of its distinctive polychromatic fluorescence. In dilute solution or when bound to polyanions in such a fashion as to be isolated from one another, acridine orange molecules exhibit green orthochromatic fluorescence. In concentrated solutions or when bound as aggregates on polyanions, they exhibit red metachromatic fluorescence (Zanker, 1952; Bradley and Wolf, 1959).

In unfixed tissue these fluorescent variations form the basis of the "Strugger effect" (Strugger, 1948); that is, that unfixed live protoplasts treated with acridine fluoresce green; dead protoplasts take up more dye and fluoresce red.

Numerous conflicting reports of red metachromatic staining in apparent in vivo preparations led to much speculation on the validity of the Strugger effect (von Bertalanffy and Bickis, 1956; Korgaonkar and Ranade, 1966). Wolf and Aronson (1961) treated cells cultured in perfusion chambers with acridine orange, then correlated the fluorescent image obtained with the degree of toxic and photodynamic injury sustained by the cells upon introducing the dye. They found healthy cells stained orthochromatically (fluoresced green); injured cells had red granules in their cytoplasm. In irreversibly damaged cells all the cytoplasm, the nucleoli, and occasionally the nucleus, stained metachromatically (fluoresced red). Degenerate cells reverted gradually to orthochromatic staining. Thus, they concluded that the Strugger effect is an insufficient vitality test.

Wolf and Aronson attributed the different colours obtained to a variable stacking of bound dye molecules, as follows. In living cells only a few scattered binding sites were considered to be available on the nucleic acid-protein complexes. Thus, since no aggregation would be possible, dye, bound mainly to DNA, would fluoresce orthochromatically. With cellular injury, binding sites on cytoplasmic polymers would become available. Initially only mucopolysaccharides, which promote dye aggregation, and subsequently RNA, would fluoresce metachromatically. Upon denaturation the stacking coefficient of DNA would increase (Bradley and Felsenfeld, 1959) and so nuclear metachromasia could also occur. Aggregation (and hence metachromasia) would be suppressed by degeneration of macromolecules (Wolf and Aronson, 1961).

Armstrong (1956) investigated the polychromatic fluorescence induced by acridine orange in fixed sections under certain conditions. He found that preincubation with deoxyribonuclease (DNAase) selectively abolished the greenish fluorescence associated with the nucleus; RNAase similarly prevented the red fluorescence of the cytoplasmic basophilic granules. Thus, he was able to identify the distribution of nucleic acids within the tissue and to distinguish between DNA and RNA. Red fluorescence of mast cell granules and cartilage matrix, and yellow fluorescence of vascular elastic laminae could be distinguished by their insensitivity to nuclease treatments and their stability at lower pH values (Armstrong, 1956).

Fluorescence variations have been used to follow viral infection in Tipula larvae and animal tissues, and DNA development in phage infected bacteria (Armstrong and Niven, 1957; Anderson, 1957).

The evidence from a variety of sophisticated physicochemical techniques also indicates that a strong interaction occurs between nucleic acids and acridine dyes (Peacocke and Skerrett, 1956; Steiner and Beers, 1959; Bradley and Wolf, 1959; Lerman, 1961; Neville and Bradley, 1961; Isenberg et al., 1964; Gardner and Mason, 1967; Yamaoka, 1968; Li and Crothers, 1969; Zama and Ichimura, 1970). Oster (1951) observed that small amounts of nucleic acid quenched the fluorescence of acriflavine solutions and changed the absorption spectrum. He attributed these effects to a complex formation. Possibly the flat acridine structure could slip between

successive planes of the DNA rings with the dye nitrogen in close proximity to a phosphoric acid group of the helix backbone. This configuration would permit strong van der Waals' bonding as well as electrostatic interaction, without requiring much change in the DNA structure.

Binding isotherms for cationic dyes on nucleic acids have been determined by fluorescence quenching and partition analysis (Heilweil and Van Winkle, 1955), spectrophotometric analysis (Peacocke and Skerrett, 1956; Blake and Peacocke, 1967), and equilibrium dialysis (Peacocke and Skerrett, 1956). From plots of r against c, where r is the number of moles of dye bound per mole of DNA phosphorous, and c is the concentration of free dye, it has been concluded that there are two distinct stages in the binding process which are dependent upon the relative dye to nucleic acid concentration (Peacocke and Skerrett, 1956). At high nucleotide to dye ratios a strong, primary binding of individual dye cations takes place, which results in the formation of a plateau (Process I). With the progressive lowering in the nucleotide-to-dye ratio a much weaker secondary binding (Process II) occurs, which proceeds until one dye molecule is bound for every nucleotide (that is, $r = 1.0$). The value that r attains in either process may vary with the dye, the polymer, the ionic strength and the temperature (Peacocke and Skerrett, 1956; Drummond et al., 1965; Chambron et al., 1966 a and b; Jordan, 1968; Blake and Peacocke, 1968).

Electrostatic forces contribute to the stability of both types of binding, but are more important in Process II than in Process I. In Process I the attractive forces between the

acridine rings and base rings of DNA, and in Process II mutual interaction between different dye cations, further stabilize the complex (Peacocke and Skerrett, 1956).

Various interpretations of the binding process have been proposed (Beers et al., 1958; Peacocke and Skerrett, 1956; Bradley and Wolf, 1959; Lerman, 1961; Mason and McCaffery, 1964; Blake and Peacocke, 1966). The models proposed by Bradley (1959) and Lerman (1961) present widely different concepts of the nature of the dye binding process at high DNA to dye ratios. Because of the controversy they have engendered and their impact on further research, [e.g. the use of the K value of Bradley and Wolf (1959) for characterizing polymers (Stone et al., 1963)] and their importance in understanding DNA structure [e.g. code cracking (Crick et al., 1961; Berger, 1968) and supercoiling (Bauer and Vinograd, 1968)], these two models are reviewed here (Figure 2, a to d).

In the tangential stacking model of Bradley and Wolf (1959) (Figure 2a) with acridine orange as the complexing dye, the acridine is held by an ionic bond between the dimethylamino group and a phosphate residue of the DNA with the molecular plane of the dye orientated perpendicular to the helix axis of the DNA. With this type of external binding the dimensions of the DNA helix itself remain essentially unchanged. Binding of monomers constitutes Process I($r \ll 1$); aggregation of the dye upon the polymer surface gives rise to Process II($r \rightarrow 1$). Relaxation kinetic measurements have confirmed that at low dye to phosphate ratios, a minor portion of the dye binds to the outside of the double helix in monomeric form (Li and Crothers, 1969). The external stacking process is cooperative, with the affinity of the polymer

FIGURE 2

BINDING MODELS FOR ACRIDINE DYES

FIGURE 2a

SCHEMATIC REPRESENTATION OF THE TANGENTIAL STACKING MODEL.

FIGURE 2b

THE INTERCALATION MODEL

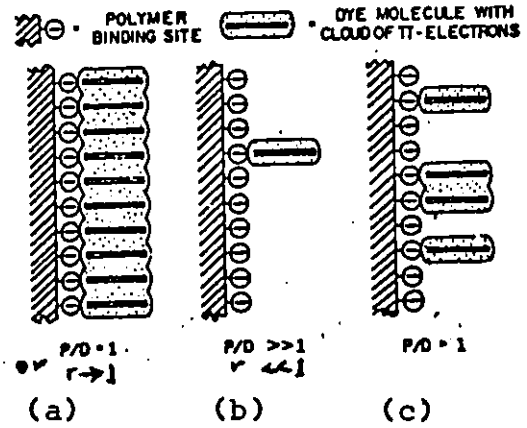
FIGURE 2c

A MODIFIED INTERCALATION MODEL

FIGURE 2d

ARRANGEMENTS OF THE ACRIDINE MOLECULE WITH RESPECT TO THE BASE PAIRS IN INTERCALARY BINDING.

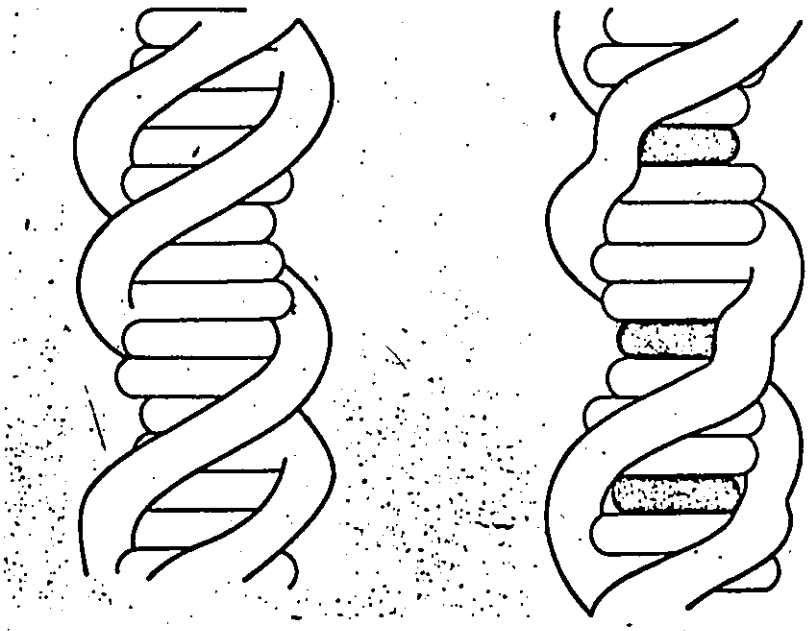
Figure 2.a



a) Complete Stacking Process II. (b) No Stacking Process I. (c) Partial Stacking Intermediate.

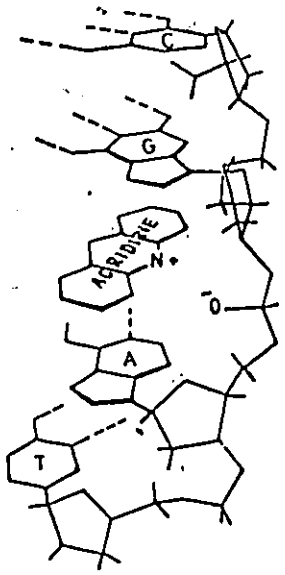
chematic representation of the aggregation of dye molecules bound to the urface of the polyelectrolytes (after Bradley and Wolf, 1959).

Figure 2b



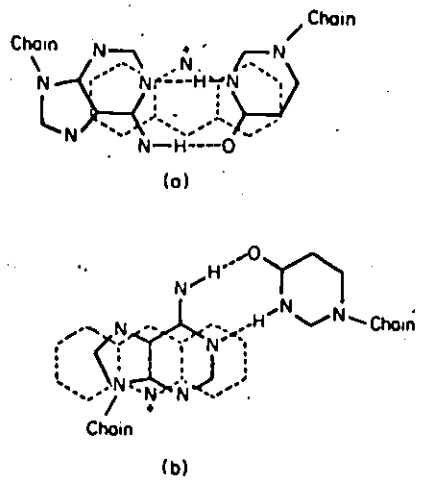
ketches representing the secondary structure of normal DNA (left) and DNA ontaining intercalated proflavine molecules (right). The helix is drawn s viewed from a remote point, so that the base-pairs and the intercalated roflavine appear only in edgewise projection, and the phosphate-deoxyribose ackbone appears as a smooth coil (after Lerman, 1964 b).

Figure 2c



Perspective view of the modified intercalation model showing an acridine molecule oriented between adjacent bases on a single polynucleotide chain; the other chain has been omitted for clarity. In this diagram the spacing between A and G represents 6.8A, although this distance is not critical to the model. The actual bases shown are examples only (from Pritchard et al., 1966).

Figure 2d



(a) The relative position of an acridine nucleus and a base pair in the intercalation model according to Lerman (1961).
(b) The relative position of the acridine nucleus and the purine of a base pair in the intercalation model of Pritchard et al. (1966) (from Jordan, 1968).

for the dye increasing upon binding of the isolated monomer (Bradley and Lifson, 1968). It is generally accepted that Process II binding occurs according to this model.

Bradley and Wolf based their interpretation upon the spectral changes which occur when certain heterocyclic compounds combine with aqueous solutions of polymers. Depending on the relative amounts and the particular polymer and dye involved, dissolving the polymer in the dye shifts the absorption maximum of the chromaphore to either longer or shorter wave lengths (Michaelis, 1947; Morthland et al., 1954; Peacocke and Skerrett, 1956). Bradley and Wolf (1959) attributed the slight red spectral shift to binding of monomeric dye molecules to the polymer site; to explain the blue optical shift they proposed that metachromatic dyes aggregate and interact when bound to the surface of a polyanion just as they do in solution. Thus when the polymer to dye ratio is high (Process I binding), only the monomeric alpha band colour (red shift) is produced. Progressive lowering of the polymer to dye ratio constrains the molecules to a smaller surface giving rise to aggregation (Process II binding) and the accompanying beta band (blue spectral shift).

Bradley and Wolf (1959) observed that the number of excess polymer sites required to develop the alpha band colour varied greatly from polymer to polymer. They expressed this property in terms of a numerical parameter, K, the dye stacking coefficient. Single-stranded, flexible polymers exhibited high K values. In contrast, the stacking tendency of native DNA preparations was found to be uniformly low (Stone and Bradley, 1961). Heat denaturation of DNA markedly increased the value

of K (Bradley and Felsenfeld, 1959). These results suggest that the degree of stacking is largely a function of the disorder of the polymeric material.

An alternative explanation for the Process I binding was advanced by Lerman (1961) (Figures 2b and c). On the basis of enhanced viscosity, reduced sedimentation coefficient, and altered x-ray diffraction pattern of the proflavine-DNA complex, he postulated that the strong binding ensues from the intercalation of planar dye molecules between successive base pairs. The insertion occurs in such a way that the flat ring system of the dye is bound by van der Waals' forces to the DNA-base pair above and below. Hydrogen bonding, where possible, between amino groups of the ligand and the charged oxygen in the phosphate groups of both the polynucleotide strands would provide additional stabilization.

The changes in the hydrodynamic properties indicated that as proflavine was bound the helix lengthened, such that in the complex the helix behaved as a stiffer, more slender rod with a diminished mass per unit length. It should be noted here that an intercalated acridine would contribute the same length increment as a base pair but less than half the mass. Using a small angle x-ray scattering technique, Luzzati et al. (1961) confirmed the decrease in both the length-specific mass and the helix radius.

The elongation of the double helix (confirmed by Cairns, 1962; Cohen and Eisenberg, 1969; Jaffe-Brachet and Ruysschaert, 1970), achieved by a local untwisting, would give rise to the space of 3.4 Angstroms between the stacked base pairs.

This space would accommodate the acridine skeleton in a plane perpendicular to the helix axis and is equivalent to that required for a normal DNA base pair. It is significant in view of the mutagenic, and possibly the bactericidal properties of these drugs. Lerman (1961) originally proposed that an uncoiling of 45° was required, but later estimated that 36° was adequate. Fuller and Waring (1964, cited by Waring, 1966) maintained that for an identical intercalation with ethidium bromide, 12° of uncoiling sufficed. Experiments with proflavine and supercoiled DNA have provided additional evidence for the uncoiling (Waring, 1970; Smith et al., 1971).

Using the sophisticated technique of flow polarized fluorescence and flow dichroism, Lerman (1964a) confirmed that the acridines were bound approximately perpendicular to the helix axis and that in the complex the perpendicularity of the helix base pairs was not significantly altered.

Lerman predicted that intercalated dyes would lie in the space bounded by the external van der Waals' contour of the helix base pairs. Thus protected, they would be inaccessible to elecrophilic agents and hence exhibit reduced reactivity. An investigation of the diazotization rates for dyes (Lerman, 1964a and b) clearly showed that this was so: the reactivity of DNA-bound proflavine, for example, was reduced twentyfold.

The binding of various cationic dyes to DNA has been found to produce a significant increase in the melting or thermal denaturation temperature (Tm) of DNA (Freifelder et al., 1961;

Kleinwächter et al., 1969; Waring, 1966; Chambron et al., 1966a and b). Kleinwächter and Koudelka (1964) found that the Tm increased as the dye/DNA ratio increased up to a limiting value; at this value the amount of dye bound by the strong binding process was maximal. According to Waring (1972) this effect is consistent with, but does not specifically indicate, intercalary binding, since any agent which binds to helical DNA at elevated temperatures stabilizes the structure against thermal denaturation.

Lerman (1964b) anticipated that certain base pairs in DNA would be more conducive to intercalation than others. Theoretical calculations (Jordan, 1968; Gilbert and Claverie, 1968) appear to suggest that the strong binding sites are heterogeneous. On the basis of their early investigations of the fluorescence of the DNA-acriflavine complex, Tubbs et al. (1964) proposed that acriflavine binds more readily to AT-containing sites. Studies by Thomes et al. (1969) and Roth and Manjohn (1968) support this concept. Other experimental evidence on this point is conflicting (O'Brien et al., 1966; Chan and Van Winkle, 1969; Chan and McCarter, 1970; Ellerton and Isenberg, 1969).

A modification of the intercalation model has been proposed (Pritchard et al., 1966; Blake and Peacocke, 1968) (Figure 2d) to satisfy several experimental findings (especially the fact that an extensive helical structure was not required for strong binding) that were considered incompatible with Lerman's model (Drummond et al., 1965 and 1966; Liersch and Hartman, 1965; Blake and Peacocke, 1965, 1966 and 1967; Dalgleish et al., 1969).

In the modified model the acridine molecule interacts in a stacked configuration with two adjacent nucleotide bases on the same polynucleotide chain. Thus the ring nitrogen of the acridine could interact with the negatively charged oxygen atom on the phosphate group between the two bases (Pritchard et al., 1966). Such interaction would occur even if these bases are not hydrogen bonded. Since strong binding was absent at extremes of pH and temperature, it was concluded that a relatively stable configuration of the macromolecule is required (Blake and Peacocke, 1968).

Löber and Achtert (1968 and 1969) observed that the binding constant for acridine base compounds decreased with ring-N-alkylation, while alkylation of the ring nitrogen of aminoacridines enhanced the binding constant. They also found that alkylation of the amino groups decreased the binding constant in proportion to the bulkiness of the substituting group. These observations are consistent with the idea of different binding conformations for intercalated acridines without amino groups (according to Pritchard's model (1966) and the aminoacridines (according to Lerman's model (1961)).

Data from a variety of sources indicates that in the strong binding range some dye is externally bound as monomers (Drummond et al., 1966; Neville and Davies, 1966; Blears and Danyluk, 1967; Roth and Manjohn, 1968). The kinetic studies

of Li and Crothers (1969) and Schmechel and Crothers (1971) have shown that intercalation is preceded by an external electrostatic binding of the dye to the helix. At low salt concentrations, external monomeric binding increases in importance and at equilibrium may account for up to 30% of the ligand.

In toto the data indicate that several possible binding modes are available at low dye to phosphate group ratios. In any given interaction, the actual nature of the complex is determined by the dye, the polymer involved and the environment.

Non-nucleic Acid Sites

Silver (1967 and 1968) showed that acriflavine bound more effectively to whole cells than proflavine, which in turn bound more effectively than 9-aminoacridine or quinacrine and that this order of relative binding efficiencies stayed the same after treatment with various membrane disrupting agents. Drummond et al. (1965) examined a limited series of acridines including proflavine, 9-aminoacridine and quinacrine and found that the binding efficiencies of the dyes to DNA were the same, but that the dyes differed in the total number of binding sites available on DNA. Of the acridines mentioned, quinacrine, considered the least biologically active (Hessler, 1963) was bound in the greatest amount by DNA. These findings are consistent with the view that binding to non-nucleic acid sites may play an important role in acridine inhibitory phenomena (Silver, 1966).

Although not as well documented as the polynucleotide-dye interaction, binding to other types of cellular polyanions is known to occur (Bradley and Wolf, 1959; Loeser et al., 1960;

Scott and Willet, 1966). Dye complexes formed with heparin (Bradley and Wolf, 1959; Stone, 1964), polyphosphate (Bradley and Wolf, 1959; Weill and Calvin, 1963), chondroitin sulphate (Stone, 1967), poly ∝,L-glutamic acid (Yamaoka and Resnik, 1966 and 1969), and various other plant and animal polysaccharides (Stone, 1963 and 1967), have been investigated spectrophotometrically. Acridine binding by protein has also been demonstrated (Bernhard et al., 1966; Feinstein and Feeney, 1967; Jori et al., 1973; Wermuth and Brodbeck, 1973).

In fixed tissues, mast cell granules, cartilage matrix and vascular elastic laminae fluoresced metachromatically with acridine orange (Armstrong, 1956). Metachromatic staining of mitochondria has also been observed (Borchert and Helmke, 1950; cited by Wolf and Aronson, 1961). Staining procedures such as that developed by Saunders (1964) employ acridine orange to distinguish between hyaluronic acid,chondroitin sulfate and heparin.

The "acridine orange particles" in the cytoplasm of vitally stained mammalian cells have been identified as lysosymes (Robbins et al., 1964). Dye uptake by the cells was selective (Allison and Young, 1964): at non-toxic concentrations acridine orange stained both the nucleus and cytoplasm, while acriflavine stained only the nucleus and mepacrine only the lysosymes. Some of the toxic and photosensitizing effects observed when mammalian cells were exposed to acridines have been attributed to an increased permeability of cell or lysosomal membranes as a result of acridine binding (Allison et al., 1966). Azzi et al.

(1971a and b) showed that the acridines, mepacrine and acridine orange, and the related compound ethidium, were strongly bound by mitochondrial membrane preparations.

Acridines exhibit specific binding to bacterial surface antigens; thus, acriflavine-induced clumping has been used to distinguish between rough and smooth bacterial strains (Pampana, 1931; Sertic and Boulgakov, 1937; Hirsch, 1937; cited by Albert, 1966). This use implies that acridines bind to the exterior of the cell. Beers (1964) suggested that the bulk of the acridine orange taken up by living bacteria was bound to cell wall mucopolysaccharides. Various degradative studies, including some to be reported in this thesis, indicate that in bacteria there is no set stoichiometric relationship between macromolecular content and acridine dye binding capacity. For example, cholate extraction of Clostridium welchii, which not only depleted the organism of its nucleic acid content but also rendered it gram-negative, reduced but did not eliminate proflavine binding (Peacocke, 1954). These experiments indicate that nucleic acids, though important, are not the only binding sites for proflavine.

UPTAKE AND RESISTANCE TO ACRIDINE DYES

Acridine resistance developes as a result of mutation and indirect selection (Thornley and Yudkin, 1959; Sinai and Yudkin, 1959), rather than by adaptation (Baskett, 1952). The gene (acr+) which controls resistance to acriflavine and other basic dyes in E. coli, is located near the determinant for lactose utilization (Nakamura, 1965). In E. coli K-12, but not in a proflavine-resistant mutant of E. coli B (Rosenkranz et al., 1965),

acr+ also determines resistance to phenethyl alcohol (Nakamura 1967 and 1968; Silver and Wendt, 1967). A number of acridine-resistant mutants have been isolated from the sensitive wild types of the T-even phages (De Mars, 1955; Silver, 1965). Hessler (1963) has shown that there are two unlinked phage genes each of which confers partial resistance to acridines. The wild-type function (sensitive) is dominant (Edgar and Epstein, 1963).

In the hemoflagellate Trypanosoma rhodesiense (Hawking, 1934), in yeasts (Johnson and Brown, 1972), in Serratia marcescens (Woods et al., 1973), in E. coli (Nakamura 1965 and 1966) and in T. phages of E. coli (Silver, 1967), acridine resistance appeared to correlate with a reduced uptake of dye by the cell or host. In contrast, Peacocke and Hinshelwood (1948) reported that proflavine-resistant Aerobacter aerogenes suspended in buffer took up slightly more dye than sensitive cells. Previous work from this laboratory has been concerned with the entry of proflavine into intact cells of proflavine-sensitive and -resistant Bacillus subtilis (Barabas et al., 1970) and Escherichia coli (Kushner and Khan, 1968). This showed that both organisms bound proflavine very rapidly by a passive process and that in buffer, sensitive and resistant cells of a given strain could bind equal amounts of proflavine. When suspended in growth media, proflavine-resistant E. coli B/Pr cells bound considerably less dye than proflavine-sensitive E. coli B cells (Kushner and Khan, 1968).

The addition of growth media, or any one of its constituents, to resting E. coli B/Pr cells that had bound proflavine caused them to lose the dye, but had less or no effect on E. coli B cells. Sugars such as galactose and lactose caused the release of bound dye in E. coli cells grown under conditions which would be expected to induce enzymes capable of attacking these sugars. Certain salts, presumably by displacing the positively charged dye molecule from its binding site, were equally effective in releasing bound proflavine from sensitive and resistant cells in both E. coli and B. subtilis (Kushner and Khan, 1968; Barabas et al., 1970). More proflavine was bound at 0°C than at 37°C by resting E. coli cells. When glucose was added more dye was released at the higher temperature. Metabolic inhibitors, including iodoacetate did not affect dye uptake, but did inhibit the glucose-induced release of bound proflavine from B/Pr cells.

The effects outlined above suggest that dye uptake is a passive process, but that the release of bound proflavine from E. coli cells is dependent upon metabolism (Kushner and Khan, 1968). Voll and Leive (1970) described an actinomycin-resistant mutant of E. coli in which the resistance appears to be associated with a similar energy-dependent expulsion of bound drug. No such energy-dependent mechanism for expelling bound proflavine was found to be associated with acridine resistance in B. subtilis (Barabas et al., 1970) or in Serratia marcescens (Woods et al., 1973). In the latter strain there is some evidence to suggest that sensitivity to acridines is linked with the presence of a cell envelope protein (complexed to pigment) which is present in

red, sensitive Serratia and absent in the orange, resistant variant (Woods et al., 1971).

OBJECTIVES OF PRESENT STUDY

Although a number of workers (Normark and Westling, 1971; Silver, 1966 and 1967; Silver et al., 1968) have used the uptake of acridines and other basic dyes as an index of envelope permeability, the experiments reported above (Kushner and Khan, 1968) indicate that this usage may be open to question. Thus, the first objective of this thesis was to investigate further the relationship between permeability and proflavine binding and release in E. coli. Therefore, these processes were compared in experiments, reported herein, in intact cells and spheroplasts that had undergone different degrees of osmotic stress. The binding potential of resting sensitive and resistant cells and spheroplasts at saturating proflavine concentrations was also examined.

A second aim of this study was to assess the relative importance of nucleic acids and other cellular compounds as binding sites for acridine dyes. This question was studied by means of experiments, reported here, in which (a) the proflavine binding of intact cells and of cells in which autodegradation of nucleic acids had occurred, was compared; and (b) the proflavine binding of intact cells and of spheroplasts and heat-treated cells freed from nucleic acids, was compared.

The remainder of the experiments reported in this thesis are concerned with the mechanism whereby bound proflavine is released and its relation to acridine resistance. In order to determine to what extent, if any, the pH of the suspending medium influenced dye release, the pH changes (after the addition of certain metabolites) and dye release in both sensitive and resistant cells were investigated under various conditions. The effect of proflavine on glucose utilization by E. coli B and B/Pr, and the relationship between glucose utilization and dye release, were also investigated.

Some of the results from this thesis have been published (Gravelle et al., 1972; Mehta et al., 1973).

MATERIALS AND METHODS

CULTURES

The previously described proflavine-sensitive and -resistant strains of Escherichia coli, Escherichia coli B and B/Pr were used in this study (Kushner and Kahn, 1968). Stock cultures were maintained with biweekly transfers on Trypticase Soy Agar slants (containing 10^{-3}M proflavine in the case of the resistant cells).

MEDIA

Cells were generally cultured in Trypticase Soy Broth (TSB, Baltimore Biological Laboratories). Trypticase Soy Agar was prepared by adding 2.0% Bacto-Agar to this broth.

In experiments involving RNA degradation, cells were grown in either the synthetic minimal medium of Beppu and Arima (1969) or in the synthetic minimal medium of Davis and Mingioli (1950), modified to contain 0.4% glucose.

CULTURE CONDITIONS

The young stationary cells used in the experiments were obtained by growing cultures for 4-7 hours with aeration at 37°C in conical flasks (screw-capped Erlenmyer or cotton-plugged Fernbach, depending upon the volume of cells required) containing 1/5 volume of growth medium. Overnight cultures were used as the innocula in a 1:100 dilution. Aeration was provided by shaking cultures on a reciprocal Eberbach shaker operating at approximately 100 strokes per minute. Growth was measured by reading the optical density (O.D.) of cultures at 660 nm in a Coleman Junior Spectrophotometer, with the use of 18 mm diameter tubes and a medium blank.

For experiments involving RNA degradation, the overnight culture was diluted 1:20 into fresh, prewarmed medium and grown aerobically at 37°C until the optical density was approximately 0.225 at 660 nm.

BUFFERS

For most experiments 0.01M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.4, plus 0.001M $MgCl_2$ was used. This buffer is referred to as solution A. For one experiment only, solution A was diluted tenfold. Solution B (solution A containing 20% sucrose) was used to wash and resuspend spheroplasts. Higher concentrations of Tris buffer, 0.3M or 0.12M, pH 8.0, were used in the preparation of spheroplasts and ethylenediaminetetraacetic acid(EDTA)-treated cells respectively.

REAGENTS AND CHEMICALS

Proflavine sulphate (3,6-diaminoacridine monohydrogen sulfate) produced by the National Aniline Division of the Allied Chemical Corporation, New York, was used without further purification or treatment. Dye stocks were stored in red Kimax brand "Ray Sorb" Erlenmeyer flasks (Canlab) since it has been shown that proflavine solutions are rapidly photoreduced (Millich and Oster, 1959). During the experiments tubes or flasks were covered with black cloth to exclude light as much as possible. Only tubes of hard glass (Pyrex or Corex) were used in the experiments as soft glass readily adsorbs the dye (Gersch and Jordan, 1965). Fresh dye solutions were prepared frequently. Proflavine was measured quantitatively by its absorbance at 445 nm on a Coleman Junior spectrophotometer

(for experiments investigating the glucose effect in spheroplasts), or on a Beckman D.U. spectrophotometer (for saturation experiments), or on a Beckman D.B. spectrophotometer (for experiments correlating pH changes and dye binding).

Fisher "certified" (Fisher Scientific Company) reagents were used with the following exceptions: bovine serum albumin (Sigma, Fraction V), orcinol (5-methyl resorcinol monohydrate, Sigma), ribose (d(-)NRC, General Biochemicals), diphenylamine (Baker, A.C.S. specifications), perchloric acid (Analar analytical reagent, British Drug House), DNA (Nutritional Biochemicals, not highly polymerized), acetaldehyde (Eastman Organic Chemicals), para-nitro phenylhydrazine in 95% alcohol (Eastman Organic Chemicals), amidol (2,4-diaminophenol dihydrochloride, practical, filtered), (Matheson, Coleman and Bell), acetic acid and hydrochloric acid (Mallinckrodt, U.S.P. specified) DNAase [(Deoxyribonuclease 1,2x crystalline (Beef Pancreas)](Nutritional Biochemicals), RNAase[Ribonuclease (Beef Pancreas)](Nutritional Biochemicals) Pronase, B grade, (Calbiochem) and lysozyme [(3x crystalline (egg white)] (Nutritional Biochemicals).

ANALYTICAL METHODS

Inorganic phosphorous, was determined by Bartlett's (1959) modification of Allen's (1940) method.

For determining nucleic acid and protein contents of E. coli the shortened trichloroacetic acid (TCA) extraction procedure of Beppu and Arima (1969) was followed. Samples (3ml) of the cell suspension at an O.D. of 1.0 in solution A were mixed with an equal volume of cold 10% TCA. The precipitates were

washed twice in cold 5% TCA, then extracted with 6 ml of 5% TCA for 30 minutes at 100°C. The supernatants from the hot extract were diluted, generally to 10 ml with cold 5% TCA and then used for determining nucleic acids: RNA as ribose by the orcinol reaction (Albaum and Umbreit, 1947), and DNA as deoxyribose by the method of Webb and Levy (1955), or by the diphenylamine reaction (Burton, 1956). The sediment remaining after hot TCA extraction was dissolved in 0.5 N NaOH and the protein content determined by the method of Lowry et al. (1951) with bovine serum albumin (Sigma) as the standard.

Glucose was measured by the method of Park and Johnson (1949).

PREPARATION AND TESTING OF SPHEROPLASTS

Spheroplasts of E. coli B and E. coli B/Pr were prepared according to the EDTA- lysozyme method essentially as described by Murray et al. (1965). One ml of an overnight culture was added to 100 ml of TSB[1] and incubated with shaking for 4-7 hours to an O.D. of 0.5-0.7. Fifty ml of culture were centrifuged down (8,000 x g for 10 minutes), washed once in solution A and resuspended in solution A to an O.D. of 1.0 (equivalent to 1.85 mg(dry weight)/ml for E. coli B and 2.0 mg(dry weight)/ml for E. coli B/Pr). The other half of

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1. In some experiments this process was scaled up to give a greater yield of spheroplasts.

the culture was centrifuged down, resuspended in 100 ml of lysozyme (68 to 100 µg/ml) contained in 0.3 M Tris, 143 µg/ml of EDTA and 10% sucrose at a final pH of 8.0. After 5-8 minutes, when all cells appeared to be transformed into spheroplasts under phase contrast, 5 ml of 1.0M $MgSO_4$ and 50 ml of 40% sucrose were added.

Spheroplasts centrifuged out of the EDTA- lysozyme mixture (12,000 x g for 10 minutes) were washed once in solution B and resuspended in solution B to the same volume as the cell suspension made up from the other half of the culture. Thus, spheroplasts of E. coli B or B/Pr were made up as equivalent to 1.85 or 2.0 mg/ml of cells respectively. Subsequent calculations were based on the weight of cells from which spheroplasts were derived.

The completeness of spheroplast formation was checked by measurement of osmotic fragility. The extent of leakage of inorganic phosphate from the cells into their incubation media can be considered an index of the osmotic fragility of the cells. It was suggested (Korngold and Kushner, 1968) that spheroplasts exposed to solution A (of low osmotic pressure) should release as much inorganic phosphate as could be released by a complete disruption of the cells with 5% cold TCA. (See Korngold and Kushner (1968) for a discussion of this method of testing completeness of lysis). The phosphorous level found in the latter cases is taken as 100%.

For the determination, equal volumes of spheroplasts in solution B were spun down. One pellet was resuspended in 10 ml of glass-distilled water and 1.0 ml of 50% cold trichloracetic acid (TCA) and centrifuged to yield supernatant 1. The other pellet was resuspended in 10 ml of solution A and centrifuged. To 9 ml of its supernatant was added 0.9 ml of 50% cold TCA and the precipitated material centrifuged to yield supernatant 2. In all experiments shown, the inorganic phosphate contents of supernatants 1 and 2 (determined by Bartlett's (1959) modification of Allen's (1948) method) were identical to within ±5%. When the osmotic fragility of intact cells was tested by this method, only 5% of their inorganic phosphate was released. When spheroplasts were suspended in solution B, only about 30% of their inorganic phosphate was released. To assess the osmotic protection given by different sucrose concentrations, leakage of cell constituents was measured as loss of ultraviolet absorbing substance (optical density (O.D.) at 260 nm). The loss of ultraviolet absorbing substances from spheroplasts derived from cells at an O.D. of 1.0 (at 660 nm) after 30 minutes incubation in solution A at 37°C was taken as 100%.

TREATMENT FOR INDUCING AUTODEGRADATION OF RNA

Techniques developed by Beppu and Arima (1969) were used to cause selective autodegradation of cellular RNA. Cells were grown in the synthetic medium of Beppu and Arima (1969) or in the synthetic medium of Davis and Mingioli (1950), modified to contain 0.4% glucose, in Fernbach flasks shaken at 37°C. An

overnight culture was diluted twentyfold with fresh, prewarmed medium and grown to an O.D. of 0.225. The culture was then diluted with an equal volume of fresh, prewarmed medium and a control sample (0 time sample) immediately removed. One one-hundredth volume of mercuric chloride was added to the remaining culture (to a final concentration of 2 or 5 x 10^{-5} M $HgCl_2$). The incubation was continued and the treated cells were sampled at later time intervals.

The control sample was centrifuged (10,000 x g for 10 minutes), washed once in solution A and resuspended in solution A to an O.D. of 1.0. Samples of the treated culture were centrifuged (10,000 x g for 10 minutes), washed once in solution A, and diluted with this solution to the same extent as the control sample. Their final optical density was lower than the control (.8-.9).

Measurements of proflavine binding were made on the control and treated cells as quickly as possible. Samples of the control and treated cells, adjusted to an O.D. of 1.0 in solution A (or the equivalent volume), were diluted immediately with an equal volume of cold 10% TCA for subsequent extraction and determination of macromolecular contents.

In experiments in which para-chloromercuribenzoate (PCMB) and ethylenediaminetetraacetic acid (EDTA) (Leive,1968) were used to induce RNA breakdown, an overnight culture in the synthetic medium of Beppu and Arima (1969) was diluted 20-fold in the same medium, grown to an optical density of 0.25 and divided into two equal portions. These were centrifuged down

and washed twice in 0.12M Tris buffer, pH 8.0. The pellets were then resuspended in 1/5th volume of this buffer, with or without EDTA (10^{-3}M final concentration). After shaking for 5 minutes at 37°C, both the untreated and the EDTA-treated cells were diluted to twice their original volume in fresh medium. Half of each sample was centrifuged down immediately (0 time controls); PCMB (to a final concentration of 5×10^{-4}M), was added to the other half of each sample and the incubation, with shaking, was continued at 37°C for one hour before centrifuging (10,000 x g for 10 minutes). All centrifuged cells were washed once and re-suspended in solution A. The 0 time controls were adjusted to an optical density of 1.0. The samples incubated for one hour with PCMB alone, or PCMB and EDTA, were taken up in the same volume of solution A as the control samples. As the values for protein show (Fig. 7) some growth had taken place during the time of incubation. Proflavine binding ability and content of macromolecules were determined on samples of control and treated cells.

TREATMENT FOR LOWERING NUCLEIC ACID AND PROTEIN CONTENT OF SPHEROPLASTS AND HEATED CELLS

Suspensions of E. coli B spheroplasts in solution B were divided into three 25-ml lots, sedimented, and lysed by resuspension in 20 ml of solution A. One lot was treated with DNAase (Deoxyribonuclease) and RNAase (Ribonuclease), 125 µg of each per ml, for 30 minutes at 37°C, centrifuged, and

then washed three times with solution A. The second lot of spheroplasts was treated with DNAase and RNAase as above, followed by a further digestion with Pronase (125 µg/ml), for 90 minutes at 37°C, and then was washed three times with solution A. The third lot of spheroplasts was treated with Pronase alone and washed. Cells heated to 100°C for 10 minutes were also treated in a similar manner to reduce nucleic acids and proteins.

The macromolecular contents of control and treated cells were determined as outlined above. Proflavine binding was determined at 10^{-4}M proflavine as described below for saturation experiments.

MEASUREMENT OF PROFLAVINE BINDING

In the initial experiments showing the effect of glucose on proflavine binding of cells or spheroplasts, the following procedure was used. 1.0 ml aliquots of cells at an O.D. of 1.0 (1.85 mg(dry weight)/ml for E. coli B and 2.0 mg (dry weight)/ml for E. coli B/Pr) in solution A or spheroplasts (derived from cells at the above concentrations) in solution B were centrifuged (12,000 x g for 10 minutes at 5°C). The pellets were taken up in 10 ml of 2 x 10^{-5}M proflavine in solution A containing sucrose, [0, 5, 10 or 20% (w/v) final concentration] with or without glucose (0.5% final concentration). Following 30 minutes incubation in stationary tubes at 37°C in the dark, the tubes were centrifuged. The supernatants were decanted, the tubes drained and the lips of the tubes wiped dry. The pellets were then extracted with 10 ml of 2% sodium lauryl sulfate (SLS)

in water, for 20 minutes at 60°C (Kushner and Khan, 1968). Proflavine bound to the cell pellet was determined colorimetrically by its absorbance at 445 nm. Values were corrected for cellular material absorbing light at 445 nm. As before (Kusner and Khan, 1968) the degree of concentration of proflavine in the sedimented material was so high that correction for the very small amounts in any adhering supernatant fluid was unnecessary.

Standard curves were routinely run. Free dye in the supernatant was found by subtracting the dye bound by the pellet from that originally present.

In subsequent time course experiments showing the effect of glucose or other metabolites on proflavine binding, cells (at an optical density of 1.0) in solution A or spheroplasts (from cells at this concentration) in solution B, were spun down and the pellet was taken up in 10 volumes of pre-warmed 2 x 10^{-5} M buffered proflavine with or without sucrose (10% or 20% w/v). The suspensions were incubated for 30 minutes in the dark at 37°C and then samples were removed for measuring proflavine binding. Each remaining suspension was then divided as required and treated with either water (as a control) or glucose (0.5 or 5.0% final concentration, as indicated) or other metabolite (0.5% final concentration) and the incubation continued.

The suspensions were sampled at subsequent time intervals. In experiments correlating pH changes with the release of bound dye, samples ("0" time) were also taken immediately after the addition of water or metabolite. All samples were centrifuged and bound proflavine measured in the pellet as described above. The pH of the supernatant was recorded where indicated. The amount of dye bound by the control cells (i.e. water-treated) at a given sampling time was taken as 100%.

For experiments in which the effect of proflavine on glucose utilization was measured directly, the following procedure was used. Cells were incubated for 30 minutes at 37°C in the presence of different proflavine concentrations (0.1,2,5 or 10 x 10^{-5}M), either aerobically (shaking in air) or anaerobically (standing under N_2). After measuring bound proflavine, (100% binding) glucose (0.01% final concentration) was added. A sample was centrifuged immediately and others were centrifuged at intervals, for measuring proflavine bound to the cell pellet (as above), and glucose in the supernatant.

For experiments where proflavine binding under conditions of saturation was measured, 0.1 ml of cells, in duplicate or triplicate at an O.D. of 1.0 in solution A or spheroplasts (from cells at these concentrations) in solution B, or treated cells were pipetted into 10 ml of Solution A containing different proflavine concentrations (in the range of 2, 5, 8, 10, 15, 20, 30 and 40 x 10^{-5}M) with or without 10% sucrose. The contents were mixed and, after incubation,

in standing tubes in the dark for one hour at 37°C, cells or spheroplasts were centrifuged (12,000 x g for 10 minutes at 5°C). Control tubes, without cells or spheroplasts, were prepared, incubated, centrifuged, extracted and read in the same manner. These readings were subtracted from the experimental tube readings to give the actual amount of dye bound by the cells. The correction for cellular materials absorbing light at 445 nm was negligible in the saturation experiments (i.e., 0.001 O.D. units at 445 nm).

RESULTS

THE EFFECT OF GLUCOSE ON PROFLAVINE BINDING BY CELLS AND SPHEROPLASTS OF PROFLAVINE-RESISTANT AND -SENSTIVE ESCHERICHIA COLI

Earlier work had shown that proflavine-resistant (*E. coli* B/Pr) and -sensitive (*E. coli* B) strains bound about the same amount of proflavine when suspended in buffer containing the dye, and that the addition of glucose or certain other metabolites caused resistant but not sensitive cells to release bound proflavine (Kushner and Khan, 1968). The effects of temperature and other metabolic inhibitors indicated that dye uptake was a passive process, but that dye release was dependent upon metabolism (Kushner and Khan, 1968). In order to further explore factors controlling dye uptake and release, these processes were studied in spheroplasts. Possibly wall components or structural wall rigidity inhibit the glucose-induced release of bound proflavine in the sensitive cells. If so, we might expect glucose to effect removal of bound dye in spheroplasts of these cells. The effect of glucose on dye binding by cells and spheroplasts of both sensitive and resistant strains with and without the osmotic protection of sucrose in the incubation media was examined.

Cells and spheroplasts (prepared as indicated in 'Materials and Methods') in the presence of different sucrose concentrations, with or without glucose, were permitted to bind proflavine. Following incubation the dye bound by the cell pellet was determined. Tubes without dye were prepared and treated in the same manner. Supernatants from these tubes were saved for optical density readings at 260 nm (See below).

1. Determination of Optimal Sucrose Concentration for Protection of Spheroplasts

Sucrose was used to protect spheroplasts in these and subsequent experiments since it neither interfered with the colorimetric determination of proflavine nor altered the pH of dye solutions. In order to compare dye binding by cells and spheroplasts and the effect of glucose on such binding, it was necessary to find that sucrose concentration which afforded the best protection to the spheroplasts without affecting proflavine uptake by the cells or spheroplasts.

To assess the osmotic protection given by different sucrose concentrations, leakage of cell constituents was measured as the loss of ultraviolet (UV) -absorbing substances (O.D. at 260nm) (Table 1a). It was found that the lower the external sucrose concentration, the greater the loss of UV-absorbing material from the spheroplasts. Losses from whole cells (10%) generally showed less variation and were much lower than losses from the spheroplasts.

Sensitive spheroplasts were found to be more stable in these experiments than the resistant spheroplasts, releasing less UV-absorbing material at all sucrose concentrations; this difference was observed in the presence and absence of glucose. Greater losses generally occurred when glucose was present. Changes with time are shown in Table 1b. Somewhat more material was lost with the longer incubation period.

These results indicate that 5% sucrose offers little osmotic protection to spheroplasts of the resistant strain, but gives somewhat greater protection to the sensitive spheroplasts which are about equally stable in 10% and 20% sucrose.

Because of the greater stability of the resistant spheroplasts in 20% sucrose, this sucrose concentration was originally used to protect spheroplasts during the time course experiments. However, as reported in the next section, 20% sucrose was found to interfere with both dye uptake and dye release and so subsequent time course experiments, and later dye saturation experiments, employed 10% sucrose despite the instability of resistant spheroplasts. In 5% sucrose too much leakage occurred.

TABLE 1a

RELEASE OF E_{260} ABSORBING MATERIALS FROM PROFLAVINE SENSITIVE AND -RESISTANT SPHEROPLASTS OF E. COLI IN DIFFERENT SUCROSE CONCENTRATIONS

Material	+/- 0.5% Glucose	% of E_{260} ABSORBING MATERIAL RELEASED			
		% Sucrose (w/v): 0	5	10	20
Sensitive Spheroplasts E. coli B	-	100	29.2 ± 6.4	13.1 ± 3.7	12.7 ± 1.9
Resistant Spheroplasts E. coli B/Pr	-	100	55.0 ± 7.0	36.2 ± 15	17.1 ± 6.0
Sensitive spheroplasts E. coli B	+	100	35.2 ± 6.4	18.7 ± 4.3	16.6 ± 4.2
Resistant Spheroplasts E. coli B/Pr	+	100	70.7 ± 7.8	43.1 ± 5.1	24.8 ± 8.8

The loss of ultraviolet absorbing substances from spheroplasts in 0% sucrose after 30 minutes of incubation was taken as 100%, i.e., 0.396 and 0.415 (without and with added glucose, respectively) in E. coli B for 0.185 mg/ml spheroplasts and 0.319 and 0.429 (without and with added glucose, respectively) in E. coli B/Pr for 0.2 mg/ml spheroplasts. Losses from whole cells (compared to spheroplasts as 100%) were about 10%. Figures show mean values from 3 experiments ± S.E.

TABLE 1b

RELEASE OF E_{260} ABSORBING MATERIAL FROM SPHEROPLASTS IN DIFFERENT SUCROSE CONCENTRATIONS AFTER 15 and 30 MINUTES OF INCUBATION

Spheroplasts	% Sucrose (w/v)	% OF E_{260} ABSORBING MATERIAL RELEASED	
		Time in minutes: 15	30
E. coli B	0	85	100*
(Sensitive)	5	32	42
	10	16	18.1
	20	5.5	12.5
E. coli B/Pr	0	94.5	100
(Resistant)	5	42.6	63.0
	10	39.3	35.0
	20	25.2	30.1

* 100% as in Table 1a, here equivalent to 0.371 for *E. coli* B at 0.185 mg/ml spheroplasts and 0.467 for *E. coli* B/Pr at 0.2 mg/ml. Incubation media contained 0.5% glucose.

ii. Effect of Sucrose on Dye-binding Capacity of Cells and Spheroplasts with and without Glucose

As reported earlier (Kushner and Khan, 1968) in buffer with no sucrose added, resistant cells incubated with glucose bound about half as much dye as cells without glucose (Table 2). As the sucrose concentration in the incubation medium increased, the effect of glucose decreased. Sucrose concentrations of 5 and 10% had little effect on the amount of dye taken up by the cells. At 20% sucrose, the intact cells (resistant and sensitive) in the absence of glucose bound only 66-69% as much proflavine as cells without sucrose; glucose had little effect on binding. Thus, 20% sucrose appeared to inhibit dye uptake by either strain. Glucose had little or no effect on the amount of dye bound by sensitive cells at any external sucrose concentration.

The amount of dye bound by the resistant spheroplasts varied with the external sucrose concentration. Osmotically lysed spheroplasts bound less than cells while osmotically protected spheroplasts bound the same as, or more than cells. E.coli B/Pr spheroplast binding was maximal at 5% sucrose and decreased with increasing sucrose concentration. Glucose had little or no effect on the amount of dye bound by resistant spheroplasts in buffer or protected by 20% sucrose. But at 5 and 10% sucrose, glucose treated resistant spheroplasts bound about 21% less dye than the controls.

Sensitive spheroplasts bound more dye than the cells at 20% sucrose and less dye than cells at lower sucrose concentrations. No glucose-induced release of bound dye

TABLE 2

EFFECT OF GLUCOSE ON PROFLAVINE BINDING BY CELLS AND SPHEROPLASTS

MATERIAL	+/-0.5% Glucose	PROFLAVINE BOUND (x 10^{-2} µmoles/mg dry weight)			
		Sucrose Concn (%) 0	5	10	20
Resistant Cells	-	2.71 ± 0.13	2.74 ± 0.14	2.60 ± 0.12	1.78 ± 0.07
Resistant Cells	+	1.28 ± 0.02	1.80 ± 0.06	1.72 ± 0.16	1.51 ± 0.11
Resistant Spheroplasts	-	2.11 ± 0.29	2.80 ± 0.20	2.61 ± 0.21	1.94 ± 0.20
Resistant Spheroplasts	+	1.85 ± 0.23	2.21 ± 0.14	2.08 ± 0.23	1.97 ± 0.14
Sensitive Cells	-	3.55 ± 0.06	3.58 ± 0.03	3.43 ± 0.06	2.44 ± 0.01
Sensitive Cells	+	3.37 ± 0.10	3.41 ± 0.28	3.37 ± 0.08	2.38 ± 0.05
Sensitive Spheroplasts	-	1.87 ± 0.24	2.95 ± 0.17	3.25 ± 0.14	2.84 ± 0.01
Sensitive Spheroplasts	+	2.01 ± 0.18	2.99 ± 0.19	3.23 ± 0.14	2.95 ± 0.02

Cells and spheroplasts (at 0.185 mg(dry weight)/ml for *E. coli* B and 0.200 mg(dry weight)/ml for *E. Coli* B/Pr) were incubated in buffered 2 x 10^{-5} M proflavine in the presence of various sucrose concentrations (0, 5, 10, or 20% w/v) with or without glucose (0.5% final concentration). After 30 minutes incubation at 37°C the tubes were centrifuged and bound dye was determined in the cell pellet. Figures show mean values from 3 experiments ± S.E.

could be demonstrated for spheroplasts of the E. coli B proflavine-sensitive strain. For the loss of UV-absorbing substances in these experiments see Table 1.

iii. Effect of Glucose on Proflavine-binding — Time Course Study

The experiments outlined above suggested that the "glucose effect" was present in resistant spheroplasts but not in sensitive spheroplasts. In order to confirm these results a time course study was conducted on the effect of glucose on spheroplasts of E. coli B and E. coli B/Pr.

Cells or spheroplasts in the presence of different sucrose concentrations were permitted to bind proflavine and treated with water (as a control) or glucose (0.5% final concentration) and then the incubation continued, with samples being removed at intervals. Bound proflavine was determined as described in 'Methods' and the pH of the supernatants recorded.

Figures 3a and b show how the proflavine contents of cells and spheroplasts (sensitive and resistant) changed after glucose addition in the presence of various sucrose concentrations.

As shown earlier, the presence of 20% sucrose greatly reduced dye uptake by both sensitive and resistant intact cells, whereas 10% sucrose had only a slight inhibitory effect on dye uptake (at 2×10^{-5}M proflavine) by intact cells.

As before (Kushner and Khan, 1958) glucose caused no release of proflavine from sensitive cells. In fact, after the

FIGURE 3a

EFFECT OF GLUCOSE ON DYE BINDING BY SENSITIVE CELLS AND SPHEROPLASTS

Cells and spheroplasts (at 0.185 mg(dry weight)/ml) were permitted to bind proflavine during 30 minutes of incubation at 37° C in solution A in the presence of various sucrose concentrations (0, 10, or 20% w/v). [redacted]es were removed and proflavine binding was measured. Each remaining suspension was divided into two equal parts and then treated with water or glucose (final concentration 0.5%). Incubation was continued at 37° C. At the intervals shown samples were removed for measurement of proflavine binding. The pH of the supernatants was recorded. Cells in 0 (●), 10% (▲) or 20% (■) sucrose; spheroplasts in 0 (o), 10% (△) or 20% (□) sucrose; plus water (———) or glucose (----).

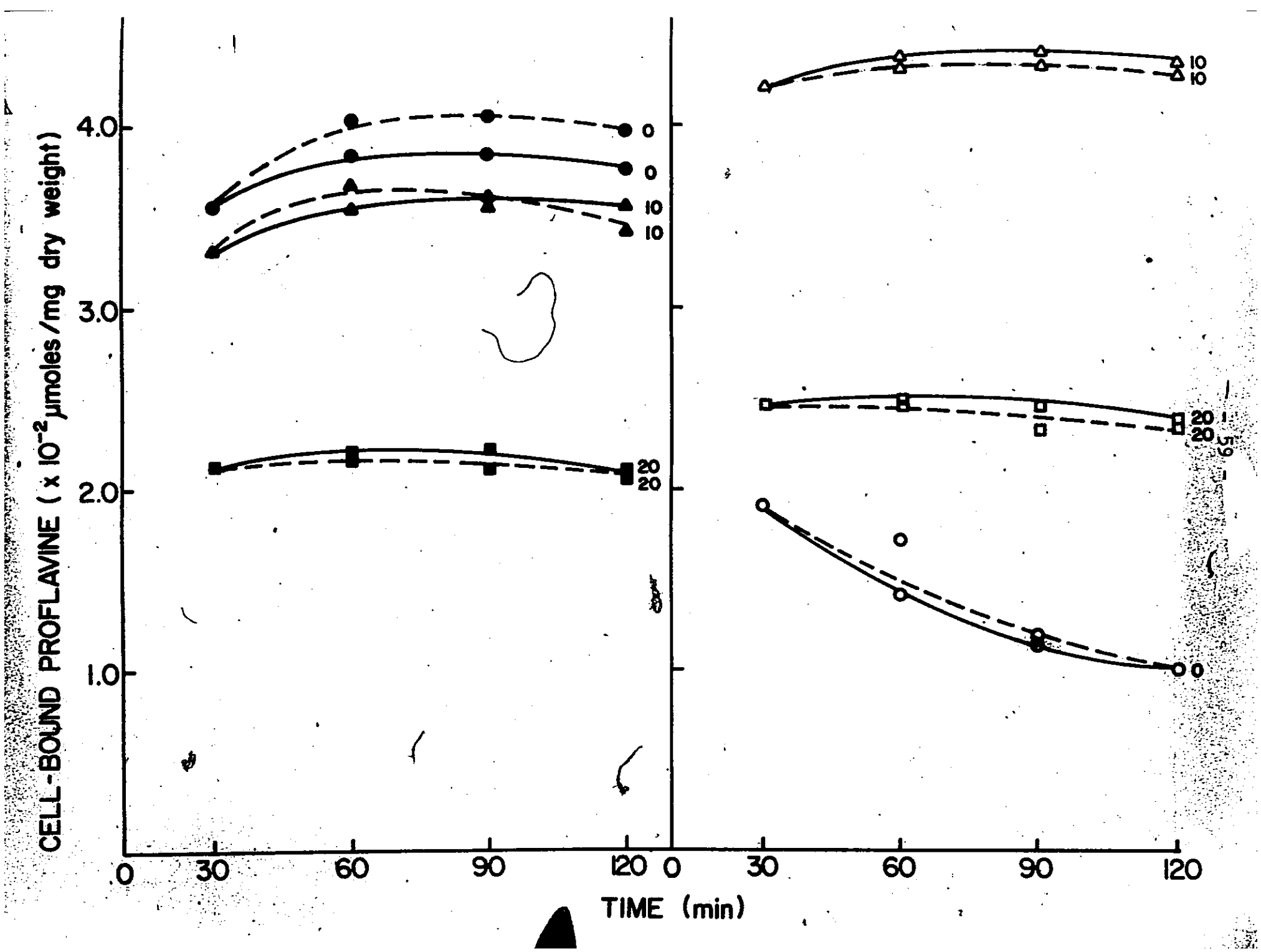
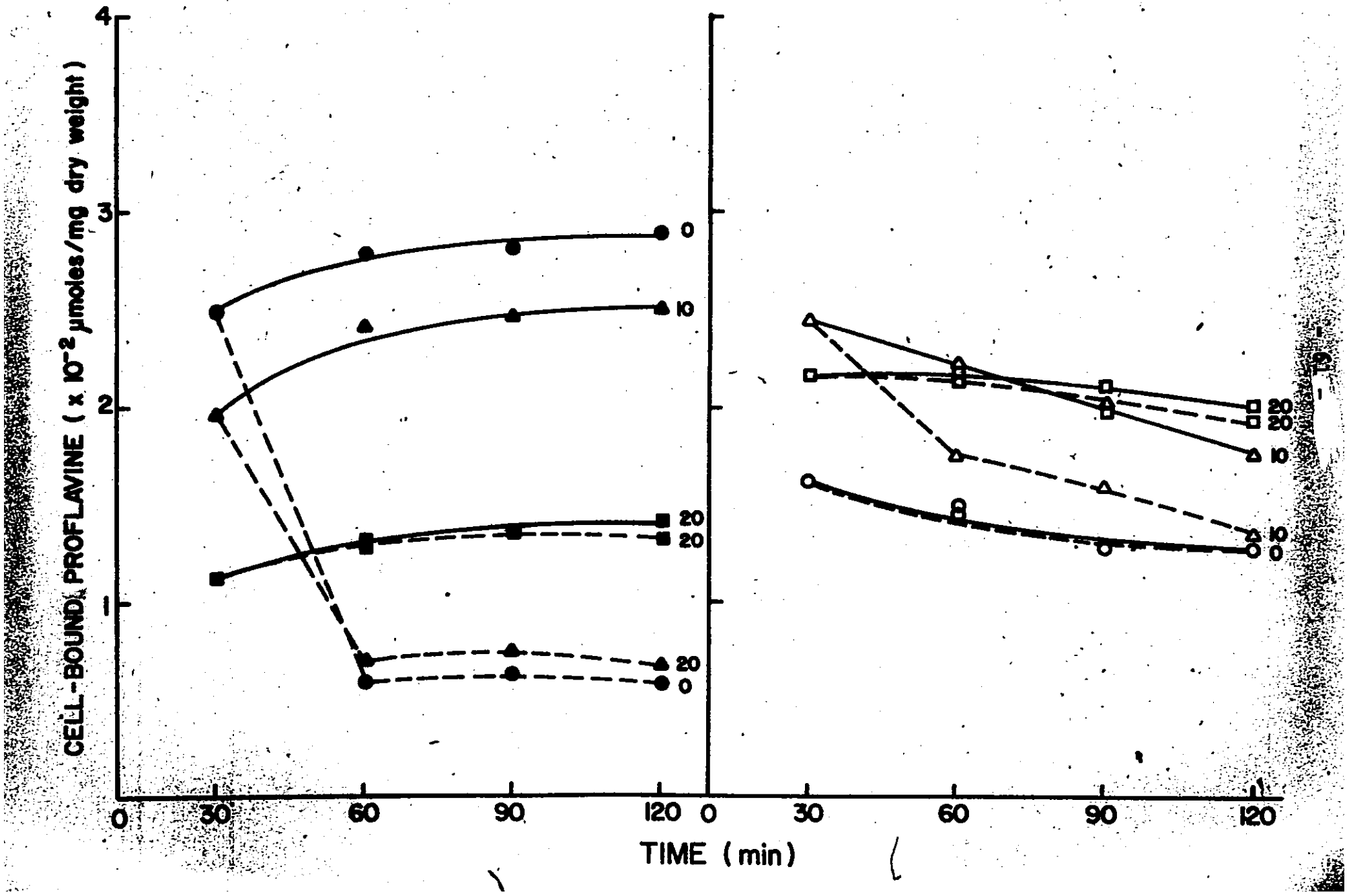

CELL-BOUND PROFLAVINE (x 10⁻² µmoles/mg dry weight)
4.0
3.0
2.0
1.0
0
30
60
90
120
TIME (min)
0
0
10
10
20
20
10
10
20
20
5°
0

FIGURE 3b

EFFECT OF GLUCOSE ON DYE BINDING BY RESISTANT CELLS AND SPHEROPLASTS

Conditions were as in Figure 3a. Cells were at 0.200 mg(dry weight)/ml. Cells in 0(●), 10% (▲), or 20% (■) sucrose; spheroplasts in 0(○), 10% (△), or 20% (□) sucrose; plus water (——) or glucose(– – –).


CELL-BOUND PROFLAVINE (x 10⁻² µmoles/mg dry weight)
4
3
2
1
0
30
60
90
120
0
30
60
90
120
TIME (min)
0
10
20
20
20
10
0

addition of glucose, sensitive cells generally bound slightly more dye than the water-treated controls over most of the incubation period. In numerous other cell systems it has been observed that the addition of a growth stimulus is associated with an enhanced dye binding (Auer et al., 1973). Glucose also caused no release of proflavine either initially or with prolonged incubation from sensitive spheroplasts, whether or not sucrose (10% or 20%) was present.

The pH of sensitive cells treated with glucose and proflavine fell by only 0.4 units over the whole incubation period. No pH changes occurred with glucose-treated cells in 10 or 20% sucrose or spheroplasts in any sucrose concentration.

As shown by Kushner and Khan (1968), the addition of glucose to intact resistant cells in buffered proflavine caused a profound decrease in the amount of cell-bound dye. A similar, though slightly smaller release was observed with glucose-treated resistant cells in 10% sucrose. The release occurred quickly and preceded any detectable pH change. No further loss or rebinding occurred over the remainder of the incubation period. In 20% sucrose resistant cells bound about half as much dye as in the absence of sucrose. Adding glucose to cells suspended in 20% sucrose caused little or no release of proflavine even after prolonged incubation.

When glucose was added to lysed resistant spheroplasts that had bound proflavine in the absence of sucrose, no more dye was released than when water was added. Adding glucose to

resistant spheroplasts suspended in 20% sucrose also caused little or no release of proflavine. But addition of glucose to spheroplasts of resistant cells protected by 10% sucrose let to a small but detectable decrease in the amount of bound proflavine.

As the sucrose concentration of the suspension increased, the pH drop following glucose addition to resistant cells decreased. At the end of the incubation without sucrose the pH had fallen by 1.2 units, but by only 0.5 units in 10% sucrose. No pH drop, and presumably little or no glucose utilization, occurred in 20% sucrose. With glucose-treated resistant spheroplasts the pH decreased 0.2 units or less at any sucrose concentration.

These experiments suggest that, although the presence of an intact (osmotically protected) membrane is not essential for proflavine binding, it is essential for metabolically induced proflavine release. Since glucose did not induce release of bound dye from spheroplasts of sensitive cells, the results also indicate that wall rigidity probably does not inhibit the glucose-induced release of the bound dye in these cells.

COMPARISON OF THE DYE-BINDING CAPACITY OF CELLS AND SPHEROPLASTS

The uptake of acridines has been used as an index of cell permeability (Silver et al., 1968). As already stated (p.35) this usage is open to question. To examine further the relationship between dye binding and permeability I compared the dye binding capacities, at saturating proflavine concentrations,

of cells and of spheroplasts whose permeability was altered by the presence or absence of sucrose.

Cells or spheroplasts were incubated with different proflavine concentrations in the presence and absence of 10% sucrose. Binding was determined as described in 'Methods'. The results of a preliminary experiment at two different cell concentrations are shown in Figure 4. As would be expected, cells at 37.0 µg (dry weight)/ml bound very nearly twice as much dye at all external proflavine concentrations as cells at 18.5 µg (dry weight)/ml. At both cell concentrations saturation was achieved when the external proflavine after binding, was 20 x 10^{-5}M. In order to determine the dye bound by the cells at 37.0 µg (dry weight)/ml, the extracted cell pellet solution centrifuged down from the higher proflavine concentrations had to be diluted. Using the lower cell concentration, no dilutions were necessary, and this concentration was chosen for further experiments.

Representative experiments showing the dye-binding capacities of sensitive and resistant cells and spheroplasts in the presence and absence of 10% sucrose are illustrated in Figures 5a and b.

In a typical experiment, sensitive cells saturated with proflavine in the absence of sucrose bound only 5% more dye than osmotically lysed spheroplasts. Intact cells in 10% sucrose bound about 17% less dye at saturation than cells in the absence of sucrose. Osmotically protected spheroplasts usually bound less dye than intact cells in buffer but more dye

FIGURE 4

SATURATION OF SENSITIVE CELLS IN THE ABSENCE OF SUCROSE AT TWO DIFFERENT CELL CONCENTRATIONS

Cells at 18.5 µg(dry weight)/ml (●) and 37.0 µg(dry weight)/ml (■) were incubated in solution A with various concentrations of proflavine. After 1 hour proflavine was determined in the supernatant and in the resuspended pellet solution.

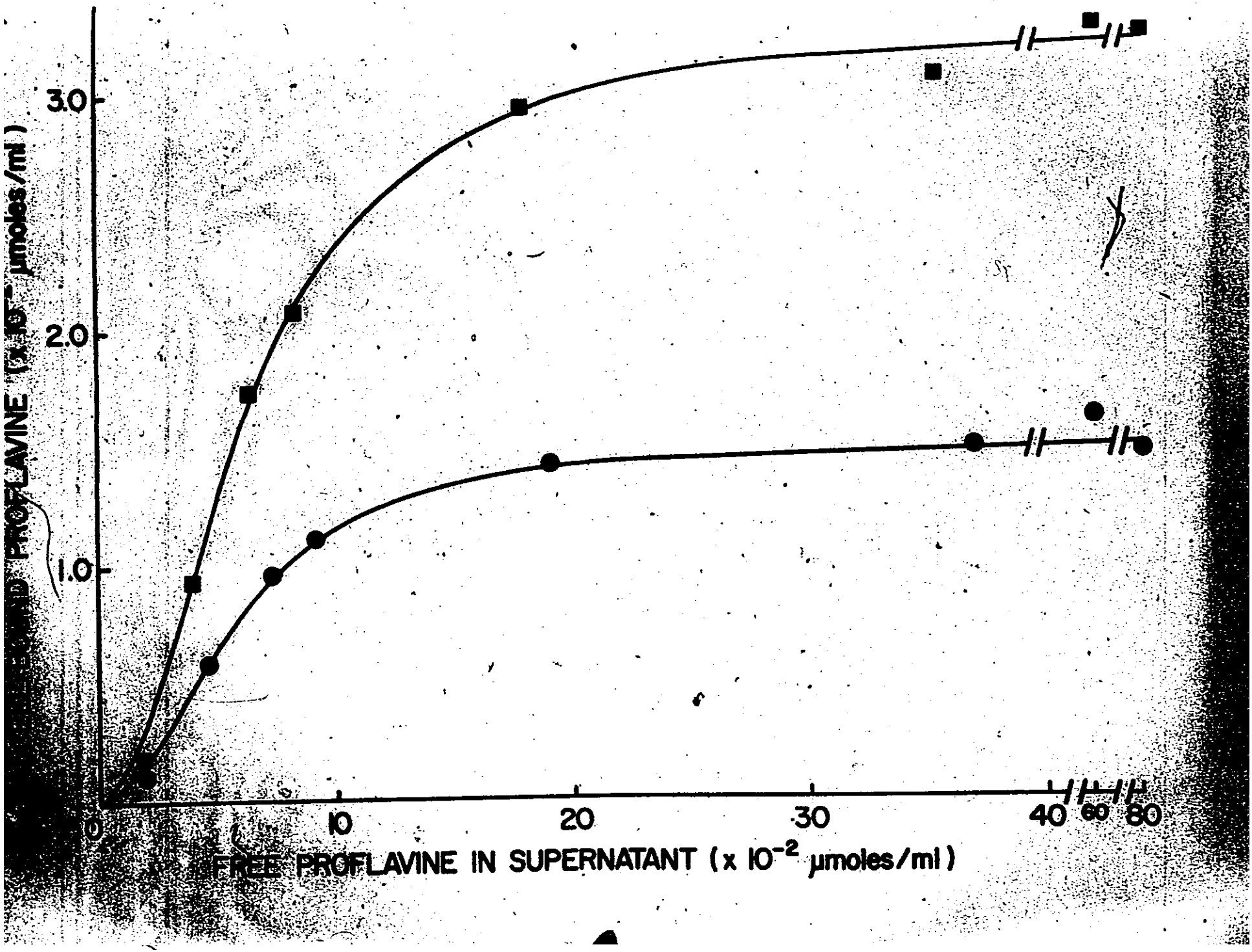

3.0
2.0
1.0
0
10
20
30
40
60
80
FREE PROFLAVINE IN SUPERNATANT (x 10^{-2} µmoles/ml)

FIGURE 5a

SATURATION OF SENSITIVE CELLS AND SPHEROPLASTS WITH PROFLAVINE IN THE PRESENCE AND ABSENCE OF 10% SUCROSE

Cells and spheroplasts (at 18.5 µg(dry weight)/ml) were incubated in solution A containing different proflavine concentrations. After 1 hour of incubation 10 ml samples were centrifuged and proflavine was determined in the pellet and in the supernatant fluid. Cells (●) and spheroplasts (o) in the absence of sucrose (———); cells (▲) and spheroplasts (△) in 10% sucrose (----).

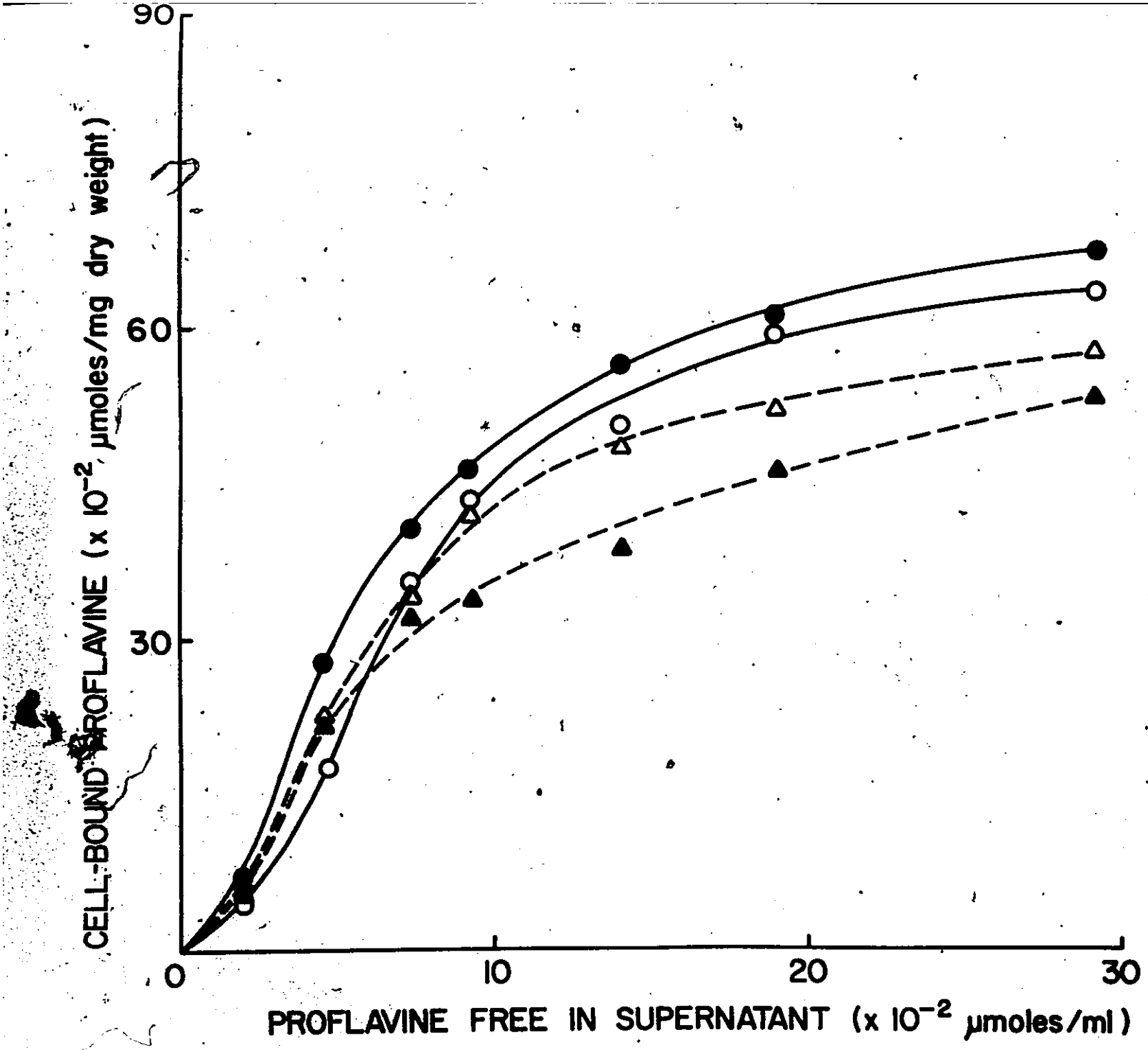
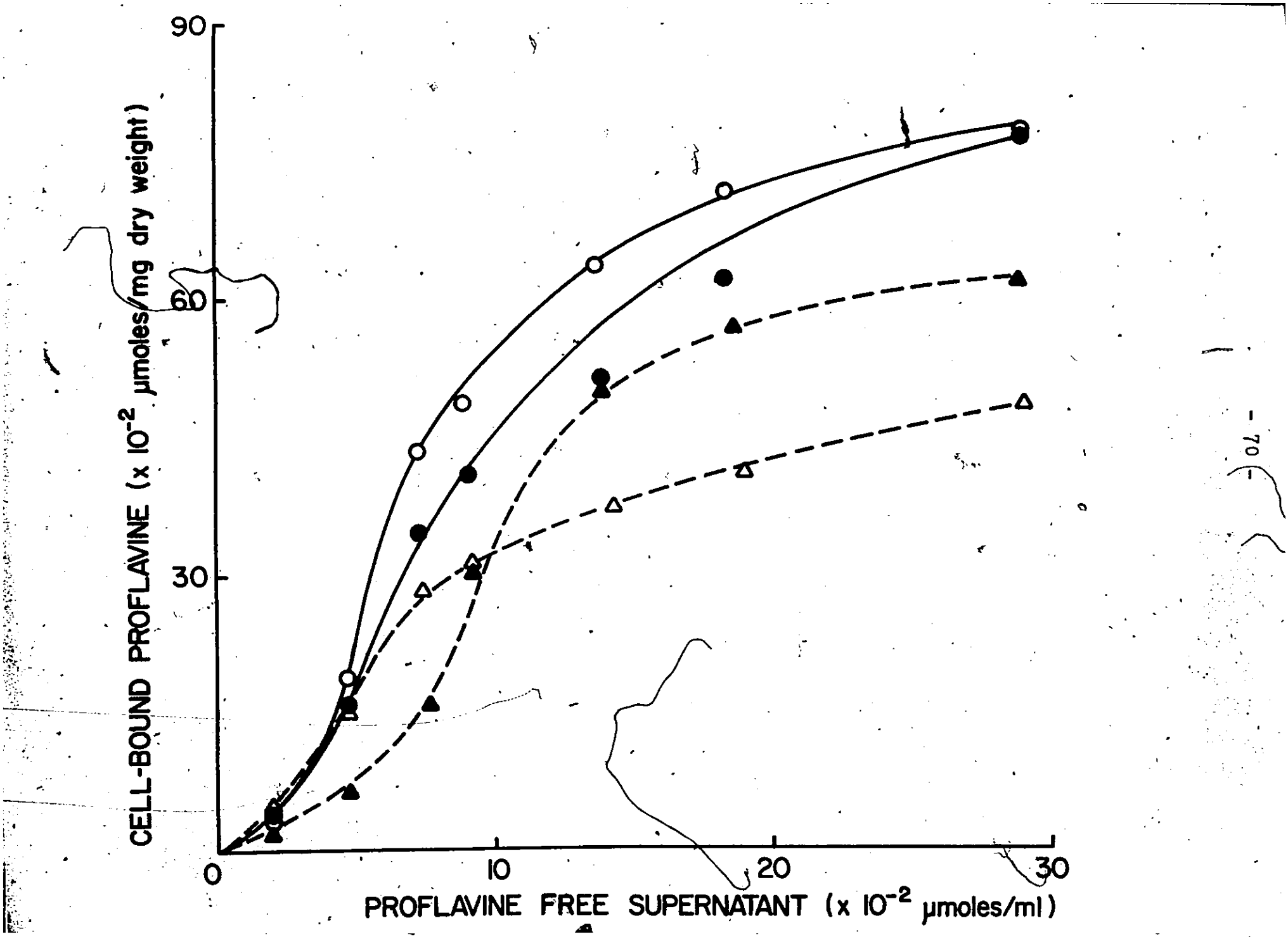

90
60
30
0
10
20
30
CELL-BOUND PROFLAVINE (x 10^{-2} µmoles/mg dry weight)
PROFLAVINE FREE IN SUPERNATANT (x 10^{-2} µmoles/ml)

Figure 5b

SATURATION OF RESISTANT CELLS AND SPHEROPLASTS WITH PROFLAVINE IN THE PRESENCE AND ABSENCE OF 10% SUCROSE

Conditions were as in Figure 5a. Cells were at 20.0 µg(dry weight)/ml. Cells (●) and spheroplasts (o) in the absence of sucrose (——); cells (▲) and spheroplasts (Δ) in 10% sucrose (---).


90
60
30
0
10
20
30
CELL-BOUND PROFLAVINE (x 10⁻² μmoles/mg dry weight)
PROFLAVINE FREE SUPERNATANT (x 10⁻² μmoles/ml)

than intact cells in sucrose. When saturated with proflavine, protected spheroplasts took up only 6% more dye than cells in 10% sucrose. At low dye concentrations, osmotically protected spheroplasts bound more dye than lysed spheroplasts; at high dye concentrations, spheroplasts in the absence of sucrose bound more proflavine.

Resistant cells and spheroplasts had somewhat different binding capacities. In the absence of sucrose under non-saturating conditions, spheroplasts generally bound more dye than cells. At saturation, cells and spheroplasts bound about the same amount of dye. Spheroplasts in sucrose had a greater dye binding capacity than cells in sucrose at low proflavine concentrations. At high dye concentrations cells took up considerably more dye than protected spheroplasts, and at saturation bound about 25% more proflavine. Lysed spheroplasts saturated with proflavine bound about 40% more dye than protected spheroplasts. At all dye concentrations, cells in buffer bound more dye than cells in sucrose, and when saturated, bound about 20% more dye than cells in sucrose.

These experiments show that an osmotically protected membrane is not essential for proflavine binding since saturated cells and spheroplasts in the absence of sucrose have approximately equal dye binding capacities. In the presence of sucrose, saturated sensitive, but not resistant, cells and spheroplasts also bound about equal amounts of proflavine. Dye uptake by both cells and spheroplasts was generally reduced by 10% sucrose.

This effect was most pronounced with spheroplasts of the resistant strain at high dye concentrations and may be related to the greater instability of these spheroplasts in 10% sucrose.

Kushner and Khan (1968) showed previously that in buffer, sensitive and resistant cells bound the same amounts of proflavine. Their experiments were conducted in 0.01 M phosphate buffer under non-saturating conditions. At saturation, in our experiments sensitive cells bound less, though possibly not significantly less, dye than resistant cells. At lower dye concentrations, sensitive cells bound somewhat more dye than the resistant cells; this may be due to the presence of 0.001 M Mg++ in the buffer used in these saturation studies. Kushner and Khan (1968) found that Mg++ can displace dye bound by both sensitive and resistant cells, presumably by competing for negatively charged groups in the cells; moreover, concentrations of Mg++ above 10^{-4}M were found to cause a slightly greater release of proflavine from resistant cells. At the higher dye concentrations used in our experiments, Mg++ would not be able to compete effectively with proflavine.

STUDIES ON THE RELATIONSHIP BETWEEN NUCLEIC ACID CONTENT AND PROFLAVINE BINDING

The experiments discussed above showed that proflavine binding was changed little by transforming cells to spheroplasts or by altering spheroplast permeability. This raised some questions concerning the nature of the cellular binding sites for proflavine.

The techniques for inducing autodegradation of nucleic acid contents and for enzymically lowering the macromolecular contents of spheroplasts and heated cells are given in the 'Methods' section. The trichloroacetic acid extraction procedures (Beppu and Arima, 1969) and the determination of macromolecular contents are also outlined in the 'Methods' section. The dye binding capacities of control and treated cells or spheroplasts were determined as described for the saturation experiments.

1. Effect of Mercury-Induced Nucleic Acid Breakdown on Proflavine Binding

Beppu and Arima (1969) found that low concentrations of $HgCl_2$ induced extensive degradation of RNA and inhibition of RNA synthesis in exponentially growing Escherichia coli cells, but not in stationary phase cells. Other organic mercurials including parachloromercuribenzoate (PCMB) also triggered degradation of RNA. On adding $HgCl_2$ to E. coli K-12 W2252 thy$^-$, they observed that synthesis of RNA, DNA and protein ceased and degradation of RNA occurred without a detectable decrease in the DNA or protein content. A limited decrease (about 10%) of the RNA content occurred immediately. After 30 minutes, rapid degradation began and by 120 minutes more than 80% of the cellular RNA was degraded. Significant strain differences were observed: with E. coli B the period of limited decrease (15%) persisted for about 90 minutes and the rate of the rapid degradation was somewhat slower (50% decrease after 150 minutes incubation with 2×10^{-5} M $HgCl_2$) (Beppu and

Arima, 1969) . Changes in the DNA and protein content for $HgCl_2$-treated E. coli B were not mentioned.

The results of several experiments at 5.0×10^{-5} and 2.0×10^{-5}M $HgCl_2$ and 5×10^{-4}M PCMB concentrations are shown in Figures 6 and 7.

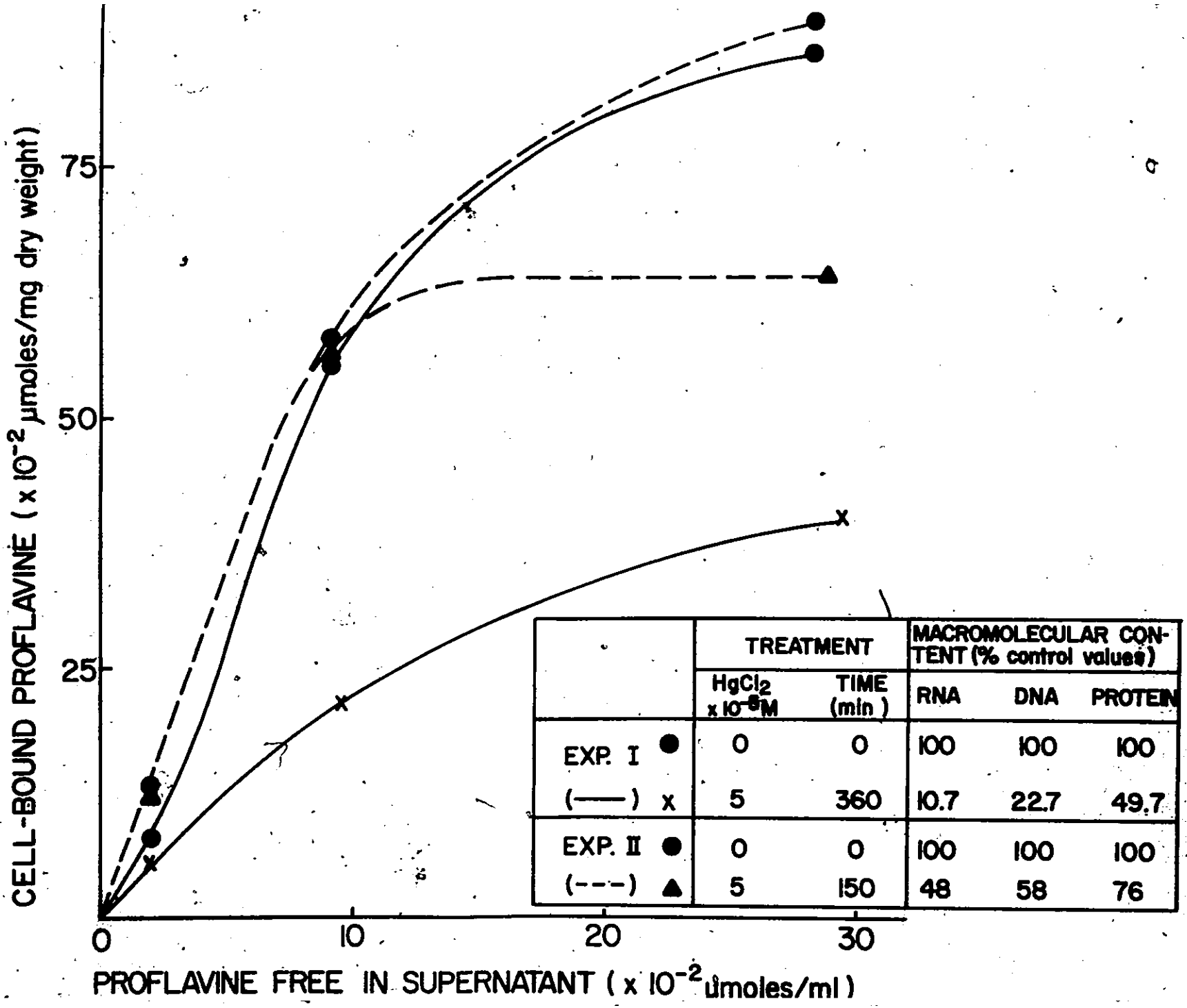
To increase RNA degradation, 5×10^{-5}M $HgCl_2$ was used initially (Figure 6a). A prolonged incubation period of 6 hours depleted the cells of most of their RNA and DNA and about half their protein. The optical density of the treated cells was 20% lower than the control cells. The losses in DNA and protein complicate the interpretation of the results since they indicate that some cell lysis occurred. Nevertheless, the decrease in nucleic acid content was considerably greater than the decrease in protein content. Despite these losses, treated cells were able to bind about 50% as much dye as the control cells at saturation. Cells which had lost about half their nucleic acid content and one-quarter of their protein during 150 minutes treatment with 5×10^{-5}M $HgCl_2$ bound 78% as much dye as the untreated cells at saturation. At subsaturating concentrations of proflavine, the binding capacity of the treated cells equalled that of the control.. If the nucleic acids were the main binding sites, much less binding would be expected in treated cells than was found in these experiments.

At 2×10^{-5}M $HgCl_2$, the RNA degradation pattern (for both sensitive and resistant cells) was very similar to that reported by Beppu and Arima (1969) for E. coli B. Some typical experiments

FIGURE 6a

PROFLAVINE BINDING AND MACROMOLECULAR CONTENTS OF SENSITIVE CELLS TREATED WITH MERCURIC ION FOR PROLONGED PERIODS

Cells (O.D.~0.23) were diluted with an equal volume of fresh medium and a control sample immediately removed. The remaining culture was treated with $HgCl_2$ (final concentration as indicated). The incubation was continued; treated cells were sampled at later time intervals as shown. All samples were washed once in solution A and resuspended in that volume of solution A required to bring the control cells to an O.D. of 1.0. Proflavine binding abilities of control and treated cells were determined as outlined for Figure 5a. The RNA, DNA and protein contents of the control cells were [in µg/mg(dry weight)] 70.7, 40.6 and 517 respectively, for experiment I; and 92.2, 43.5 and 611 respectively, for experiment II. DNA was determined by the method of Webb and Levy (1955).

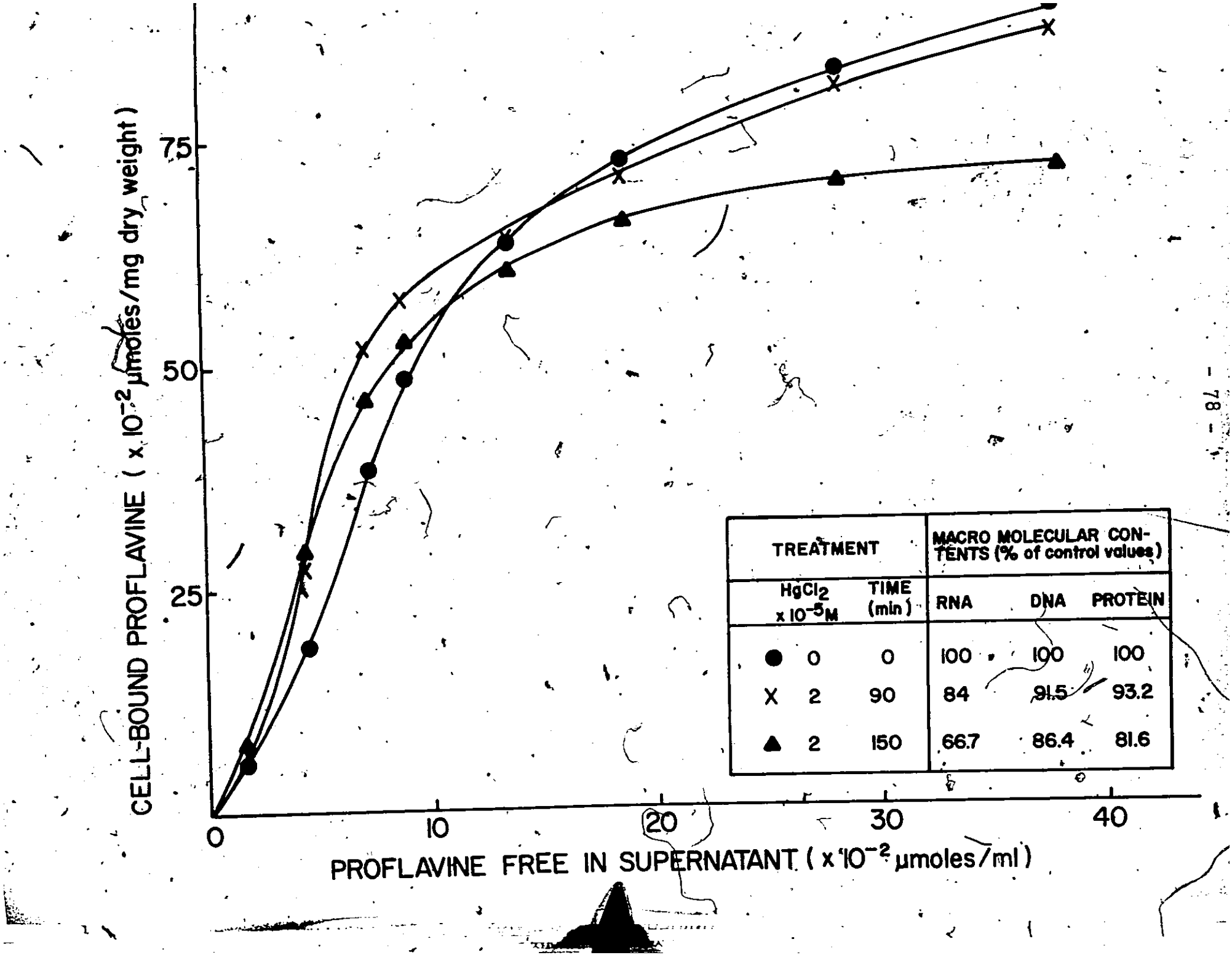


	TREATMENT		MACROMOLECULAR CONTENT (% control values)		
	$HgCl_2$ x 10^{-5}M	TIME (min)	RNA	DNA	PROTEIN
EXP. I ●	0	0	100	100	100
(——) x	5	360	10.7	22.7	49.7
EXP. II ●	0	0	100	100	100
(---) ▲	5	150	48	58	76

FIGURE 6b

PROFLAVINE BINDING AND MACROMOLECULAR CONTENTS OF SENSITIVE CELLS TREATED WITH MERCURIC ION FOR SHORTER PERIODS

Conditions wene as described in Figure 6a. The RNA, DNA, and protein contents of the control cells were [in µg/ml(dry weight)] 95.8, 28.3 and 632 respectively. DNA was determined by Burton's (1965) method..



TREATMENT		MACRO MOLECULAR CONTENTS (% of control values)		
$HgCl_2$ x 10^{-5}M	TIME (min)	RNA	DNA	PROTEIN
● 0	0	100	100	100
X 2	90	84	91.5	93.2
▲ 2	150	66.7	86.4	81.6

FIGURE 6c

PROFLAVINE BINDING AND MACROMOLECULAR CONTENTS OF RESISTANT CELLS TREATED WITH MERCURIC ION FOR SHORTER PERIODS

Conditions were as described in Figure 6a. The RNA, DNA and protein contents of the control cells were [in µg/mg(dry weight)] 102.6, 28.3 and 632 respectively. DNA was determined by Burton's (1965) method.

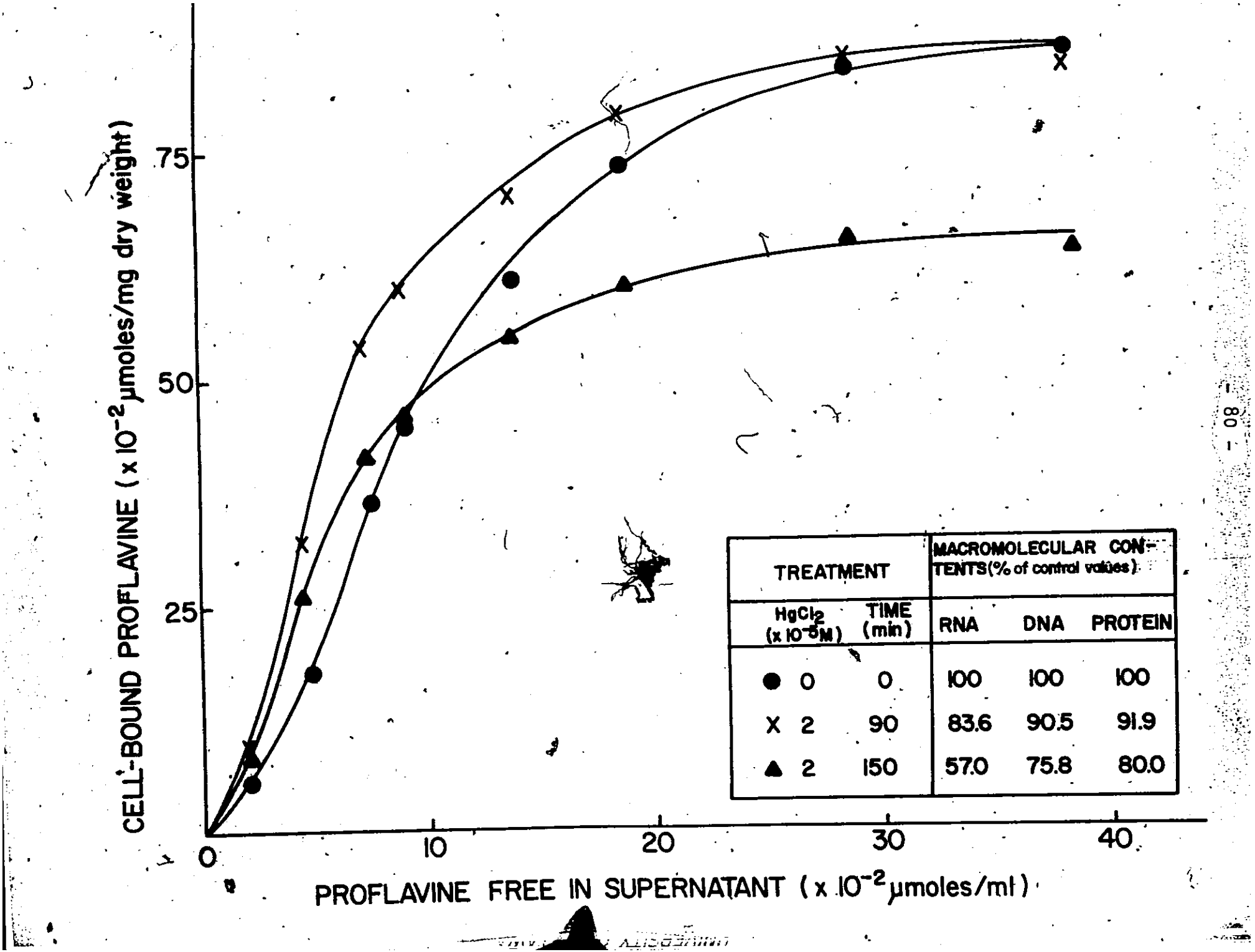
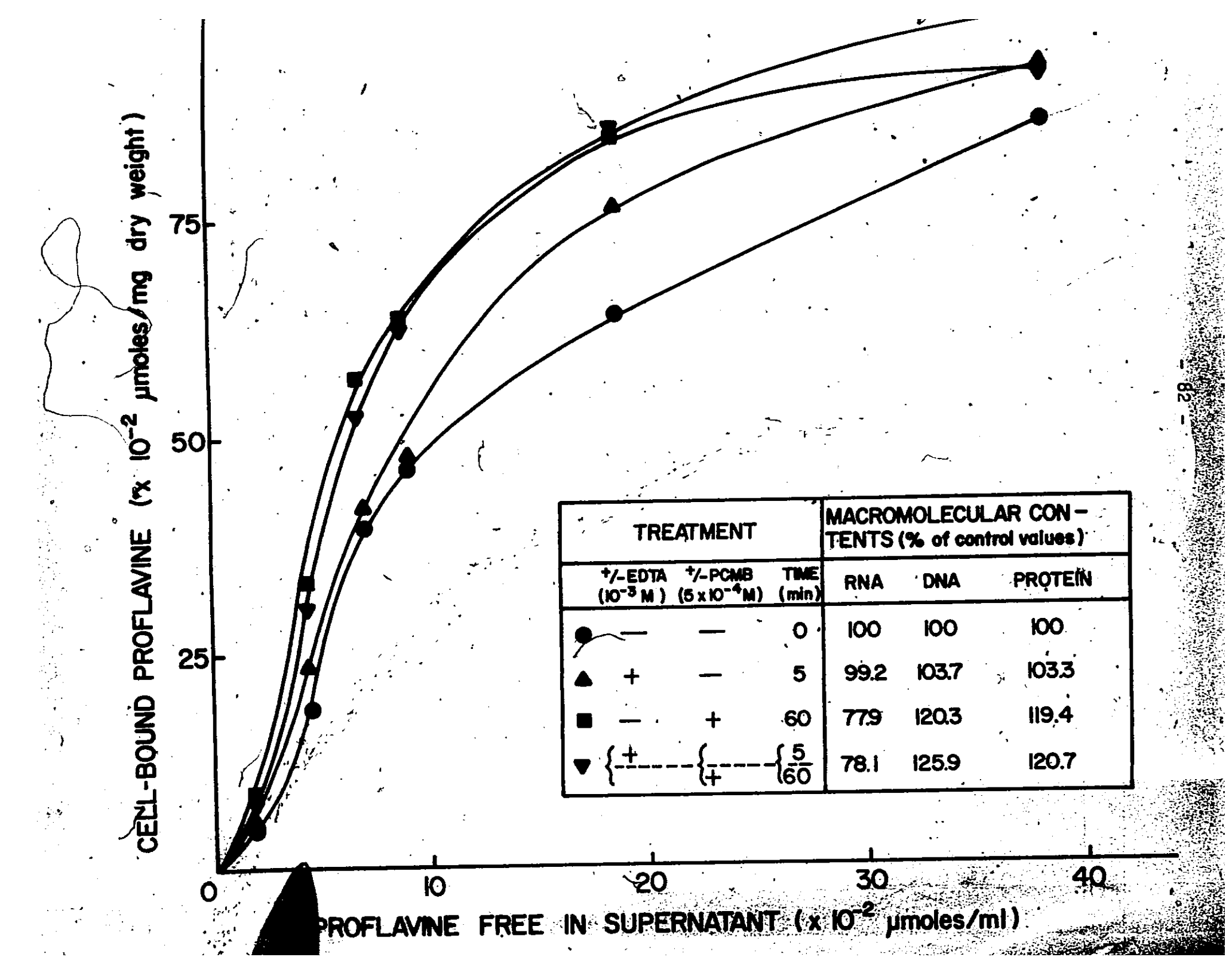

CELL-BOUND PROFLAVINE (x 10^{-2} µmoles/mg dry weight)
75
50
25
0
10
20
30
40
PROFLAVINE FREE IN SUPERNATANT (x 10^{-2} µmoles/ml)
TREATMENT
MACROMOLECULAR CONTENTS (% of control values)
$HgCl_2$ (x 10^{-5}M)
TIME (min)
RNA
DNA
PROTEIN
● 0 0 100 100 100
X 2 90 83.6 90.5 91.9
▲ 2 150 57.0 75.8 80.0

FIGURE 7

PROFLAVINE BINDING AND MACROMOLECULAR CONTENTS OF SENSITIVE CELLS TREATED WITH PCMB WITH OR WITHOUT PRIOR BRIEF EXPOSURE TO EDTA

Conditions were as described for Figure 6a except that prior to being diluted in fresh medium, the cells were washed twice in 0.12M Tris buffer, pH 8.0, then resuspended in 1/5 volume of this buffer, with or without EDTA (final concentration 10^{-3}M), and incubated 5 minutes at 37°C. The RNA, DNA and protein contents of the control cells were [in µg/mg(dry weight)] 68.8, 40.5, and 605 respectively. DNA was determined by the method of Webb and Levy (1955).



TREATMENT			MACROMOLECULAR CONTENTS (% of control values)		
+/- EDTA (10^{-3} M)	+/- PCMB (5×10^{-4} M)	TIME (min)	RNA	DNA	PROTEIN
—	—	0	100	100	100
+	—	5	99.2	103.7	103.3
—	+	60	77.9	120.3	119.4
+	+	5/60	78.1	125.9	120.7

are shown in Figures 6b and c. The decrease in RNA content was generally attended by smaller losses in DNA and protein. At subsaturating concentrations of proflavine both sensitive and resistant cells treated with $HgCl_2$ were able to bind more dye than the control cells. At saturation, resistant cells treated with 2.0×10^{-5} M $HgCl_2$ for 90 minutes bound as much as control cells. Sensitive cells bound very slightly less. Exposure of 150 minutes to 2.0×10^{-5} M $HgCl_2$ reduced the binding capacity of both sensitive and resistant cells at saturation by approximately 20%. As before, the decrease in dye binding capacity did not parallel the decrease in nucleic acid content.

ii. Effect of Exposure of Cells to PCMB+/-EDTA

Figure 7 shows the results of a binding experiment with sensitive cells in which PCMB (5×10^{-4} M) was used as the agent to induce RNA degradation with or without a brief pre-incubation in EDTA(10^{-3} M). Unlike $HgCl_2$, PCMB(5×10^{-4} M) did not completely inhibit growth of E. coli B. Both protein and DNA increased by about 20% in the treated cells (+/-EDTA), while the RNA content fell by about 22%. In the experiments shown, cells treated with PCMB alone or with EDTA and PCMB lost about equal amounts of RNA. In a preliminary experiment it was found, in agreement with Beppu and Arima (1969), that cells pretreated with EDTA were more susceptible to RNA degradation by PCMB.

Cells which had been treated with PCMB alone generally bound more dye than the control cells. EDTA-PCMB treated cells behaved similarly, but were saturated with less proflavine than

were cells that had been treated with PCMB alone. In PCMB treated cells (+/-EDTA-treatment) there was no net increase in nucleic acid content. If nucleic acids were the only sites available for proflavine binding, the treated cells would be expected to bind no more dye than the control cells.

At saturating concentrations of proflavine, cells treated with EDTA alone bound about 8% more than control cells. Possibly, pretreatment with EDTA reduces the Mg^{++} content of the cells, and so enhances their affinity for proflavine (Weiser, 1968).

Since large changes in nucleic acid content only slightly changed the ability to bind proflvine, these experiments suggest that nucleic acids are not the only binding sites for proflavine. Support for this conclusion was sought using other degrading techniques such as heat.

iii. Relation between Proflavine Binding and Content of Macromolecules in Heated Cells and Spheroplasts

A comparison of DNA, RNA, and protein contents and proflavine-binding abilities of intact cells and of cells and spheroplasts subjected to various treatments is shown in Table 3.

The most clearcut results were obtained with cells heated at 100°C for 10 minutes. When these were suspended in buffer, they lost 25% of their RNA, only 6% of their protein, and none of their DNA. The proflavine-binding capacity of these cells increased by 49%. Such increase of proflavine binding on heating has been previously demonstrated for a number of acridines in *E. coli* and for sensitive (but not resistant) *Bacillus subtilis* cells (Silver *et al.*, 1968; Barabas *et al.*, 1970).

TABLE 3

MACROMOLECULAR CONTENTS AND PROFLAVINE BINDING IN TREATED CELLS AND SPHEROPLASTS OF *E. COLI* B

Treatment	Heated cells (100° C for 10 min)				Spheroplasts			
	Macromolecular contents (% of control values)			Bound proflavine (μmoles/mg)	Macromolecular contents (% of control values)			Bound proflavine (μmoles/mg)
	DNA	RNA	Protein		DNA	RNA	Protein	
Untreated cells (control).....	100	100	100	38.0×10^{-2}	100	100	100	30.0×10^{-2}
Heated cells or spheroplasts In solution A.....	111	75	94	56.5×10^{-2}	61	70	68	32.0×10^{-2}
In solution A + 10% sucrose.....	---	---	---	---	94	102	100	19.9×10^{-2}
Treated with nucleases ..	13	1.4	80	20.5×10^{-2}	.3	11	43	9.5×10^{-2}
Treated with nuclease and Pronase.....	8.4	1.1	15	16.7×10^{-2}	0	10	29	14.4×10^{-2}
Treated with Pronase alone.....	67	27	12	39.2×10^{-2}	22	25	19	22.6×10^{-2}
Washed 10 times with solution A.....	---	---	---	---	13	24	39	22.6×10^{-2}
Washed 3 times with 95% ethanol.....	94	28	72	56.0×10^{-2}	---	---	---	---

Treatment of heated cells with nucleases or with nucleases plus Pronase reduced DNA by about 90%, and RNA by about 99%, and lowered proflavine binding to approximately half that of the intact cells (or to one-third that of the heated cells not treated with enzymes). Heated cells treated with Pronase alone lost more protein (88%) than nucleic acids; their proflavine-binding capacity was reduced by about one-third. Finally, washing heated cells prewashed with 95% ethanol, a procedure that would be expected to remove membrane lipids, reduced RNA by 72% and had much less effect on DNA and protein, but did not reduce proflavine binding at all.

These experiments show that nucleic acids, though important, are by no means the only binding sites for proflavine.

Similar conclusions as to the importance of nucleic acids were reached by using spheroplasts. Transforming cells to osmotically protected spheroplasts (in 10% sucrose) did not significantly lower their DNA, RNA, or protein, but did lower their proflavine-binding power. In the absence of sucrose, spheroplasts lost 30 to 40% of their macromolecules, but proflavine binding was at least as great as that of intact cells. Treatment with nucleases reduced the nucleic acid content of spheroplasts by 90% or more and their protein content by about 70%. Treatment with Pronase alone, however, caused a substantial reduction in nucleic acid as well as protein content, and these macromolecules could also be reduced by washing spheroplasts several times in solution A without any enzyme treatment. This suggests that lysis of spheroplasts caused much of the loss of macromolecules. Such lysis complicates interpretation of these

experiments, but the results do show that the fall in proflavine binding did not parallel the fall of either nucleic acid or protein. Spheroplasts with only 10% nucleic acid remaining bound almost half as much proflavine as intact cells.

In general, the results of the various degradative studies illustrate the difficulties in quantitatively relating changes in macromolecular content with the amount of proflavine bound. The enhanced dye binding capacity of heat-treated cells indicates that a given degradative process, while destroying one class of site may open up new ones. Brief treatment with EDTA may also open up new binding sites. The generally steeper slopes of the binding curves of cells treated with 2.0×10^{-5} M $HgCl_2$ compared to those of control cells, may indicate that the binding sites in the mercury-exposed cells differ in their affinity for proflavine.

In experiments using mercury-treated cells the changes in the amount of cell-bound dye appear to correlate with the changes in the protein content. The enzymic studies (Table 3) show that protein is not a major determinant of proflavine binding. Since some lysis was occurring in cells treated with $HgCl_2$ and some growth took place with PCMB, the changes in protein content might be taken as an index of other available cell constituents (excluding nucleic acids) such as mucopolysaccharides, phospholipids etc. which might also influence dye binding.

CORRELATION OF pH AND DYE-BINDING WITH METABOLIC ACTIVITY

i. Effect of Incubation with Various Metabolites in the Presence and Absence of Proflavine

Kushner and Khan (1968) showed that a number of metabolic substances caused the release of bound proflavine from resistant but not sensitive cells (see P. 34). Organic salts, such as sodium-acetate, sodium-formate and mono-sodium glutamate (presumably because of simple ionic competition) were about equally effective in causing the release of bound dye from both strains.

It was of interest to determine to what extent if any, the release of proflavine was due to changes of pH in the suspending medium. Moreover, since the pH indicated the extent of metabolic activity these experiments also introduced more extensive work on the metabolic effects of proflavine. The experimental conditions, i.e. shaking or non-shaking, buffer concentration and the presence or absence of an inhibitor, were modified to produce conditions which favour or impede pH changes.

Table 4 shows the results of a survey of the external pH changes produced by a number of compounds in sensitive and resistant cells in the absence of proflavine. Only glucose produced a marked lowering of the pH in both the sensitive and resistant strain in 210 minutes. The ph fell more slowly with ribose, galactose and lactose. At 210 minutes of incubation only a slight drop in pH was observed with ribose and galactose. In 24 hours the ph had fallen to 5.2 with ribose, and less than

TABLE 4

pH CHANGES PRODUCED BY CELLS INCUBATED WITH VARIOUS METABOLITES IN THE ABSENCE OF PROFLAVINE

Time of Incubation (min. or hr.*)	Cell Strain	Water	Glucose (0.5%)	Ribose (0.5%)	Sodium Pyruvate (0.1%)	Sodium Succinate (0.1%)	Sodium Acetate (0.1%)	Monosodium Glutamate (0.45%)	L. Proline (0.45%)	Galactose (0.5%)	Lactose (0.5%)	Glycerol (0.5%)	Lysine (0.45%)	Glycine (0.45%)	Alanine (0.45%)	Serine (0.45%)
0	B	7.35	7.20	7.25	7.32	7.38	7.58	7.40	7.42	7.22	7.28	7.30	7.25	7.25	7.20	7.15
	B/Pr	7.30	7.10	7.30	7.25	7.35	7.28	7.32	7.22	7.28	7.25	7.22	7.20	7.20	7.20	7.20
60	B	7.30	5.88	7.20	7.32	7.40	7.55	7.40	7.40	7.10	7.23	7.25	7.23	7.30	7.25	7.20
	B/Pr	7.30	5.68	7.20	7.25	7.38	7.30	7.38	7.30	7.15	7.25	7.20	7.20	7.23	7.23	7.18
90	B	7.30	4.53	7.18	7.30	7.35	7.50	7.40	7.35	7.10	7.22	7.23	7.23	7.32	7.28	7.22
	B/Pr	7.30	4.31	7.25	7.32	7.38	7.30	7.40	7.30	7.02	7.22	7.20	7.20	7.63	7.73	7.20
210	B	7.31	3.98	7.10	7.35	7.42	7.55	7.52	7.38	6.98	7.08	7.18	7.20	7.40	7.30	7.25
	B/Pr	7.38	3.88	7.12	7.38	7.50	7.40	7.50	7.28	6.90	7.20	7.20	7.20	7.32	7.25	7.35
24 Hr.*	B	7.35	4.00	5.20	8.35	8.08	8.22	8.30	7.70	4.90	5.40	7.12	7.32	7.65	7.95	7.75
	B/Pr	7.50	4.00	5.22	8.25	8.18	8.05	8.30	7.60	4.38	7.30	7.28	7.35	7.70	7.82	7.70

Cells (at 0.185 mg(dry weight)/ml for *E. coli* B and 0.200 mg(dry weight)/ml for *E. coli* B/Pr) were preincubated in solution A for 30 minutes at 37°C, then treated with metabolite (0.5% final concentration) or water. A "0" time sample was taken and the incubation continued. Samples were taken at later time intervals as indicated. After centrifuging, the pH of the supernatant was recorded.

5 with galactose, for both sensitive and resistant cells. Lactose effected a drop in pH with the sensitive cells only. Under the same conditions a pH decrease of less than 0.2 units was observed with glycerol. All other compounds produced an alkaline pH change after 24 hours of incubation. Only glucose, ribose and glycerol were investigated further.

ii. Effect of pH Changes and Dye-Binding with 0.5% and 5% Glucose (Stationary Suspensions)

Figures 8a and b show the results of experiments correlating dye-binding and pH changes when 0.5% glucose or 5.0% glucose (final concentrations) were added to cells that had been preincubated with or without proflavine.

In the absence of proflavine, approximately the same decrease in pH occurred with both sensitive and resistant glucose-treated cells. Proflavine markedly reduced the rate of lowering of pH following glucose addition to sensitive cells. With resistant cells the pH changes were the same in the presence and absence of proflavine.

When glucose was added to resistant cells, approximately half of the cell-bound proflavine was released. The initial release occurred quickly and preceded any detectable pH change. Cells treated with a lower glucose concentration (0.5%) then began to rebind proflavine. With a higher glucose concentration (5.0%) resistant cells lost further dye as the pH gradually dropped. Cells treated with 5.0% glucose also rebound proflavine. At the end of the incubation, in a pH of 4.4, they bound 60% as much dye as the controls.

FIGURE 8a

pH CHANGES AND RELEASE OF BOUND PROFLAVINE WITH GLUCOSE-TREATED RESISTANT AND SENSITIVE CELLS

Cells (at 0.185 mg(dry weight)/ml for *E. coli* B and 0.200 mg(dry weight)/ml for *E. coli* B/Pr) were preincubated in standing flasks at 37°C for 30 minutes with buffered 2×10^{-5} M proflavine. The suspension was then divided as required and one-tenth volume of water or glucose (0.5% final concentration) was added to each flask. A '0' time sample was taken and the incubation continued. Samples were taken at later time intervals as indicated. After centrifuging, the pH of the supernatants was recorded. Bound proflavine was determined in the pellet. Cells treated with water (○) or glucose (△) in the absence of proflavine; cells treated with water (●) or glucose (▲) in the presence of proflavine. Arrows show levels of pH or bound proflavine before additions.

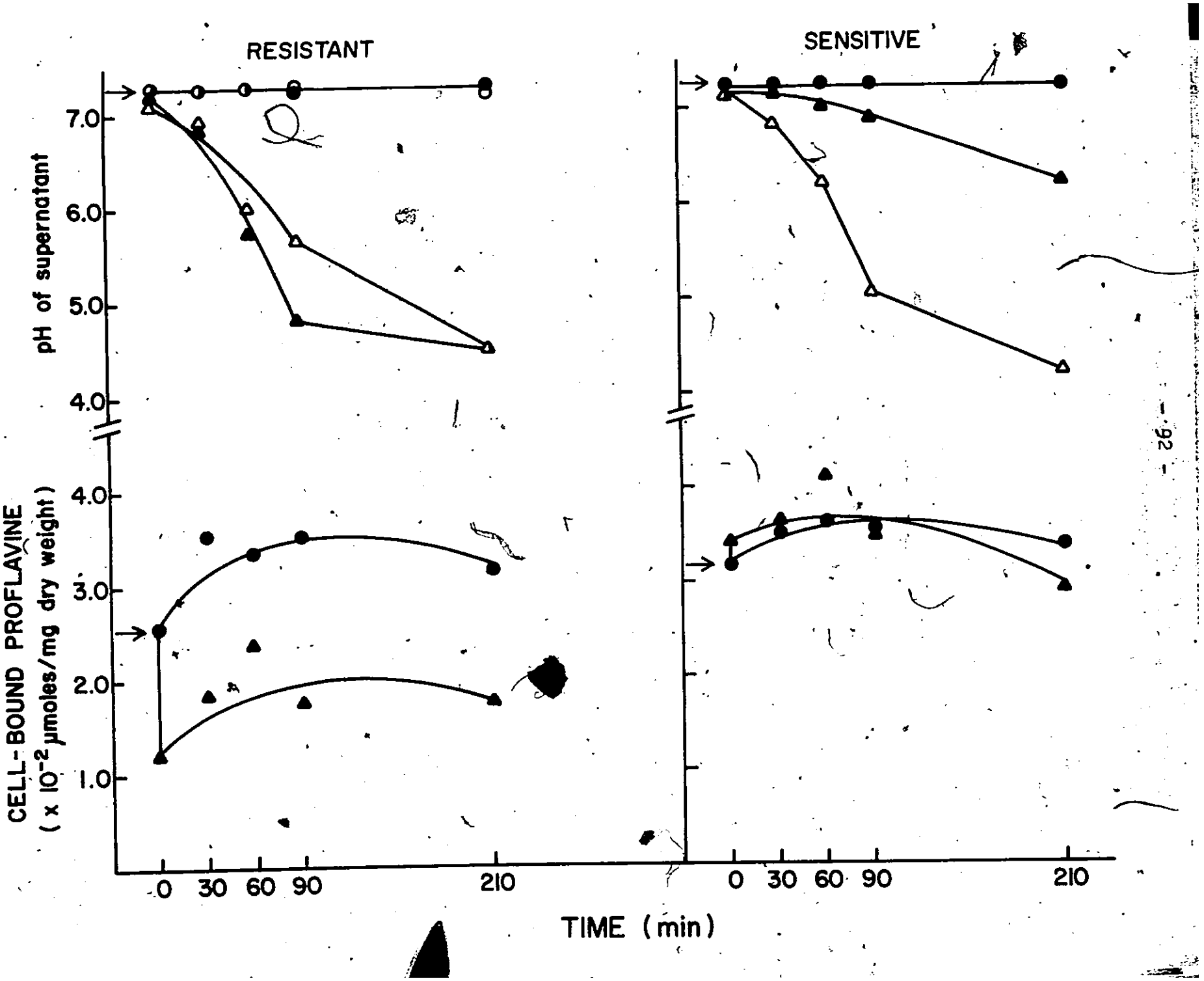
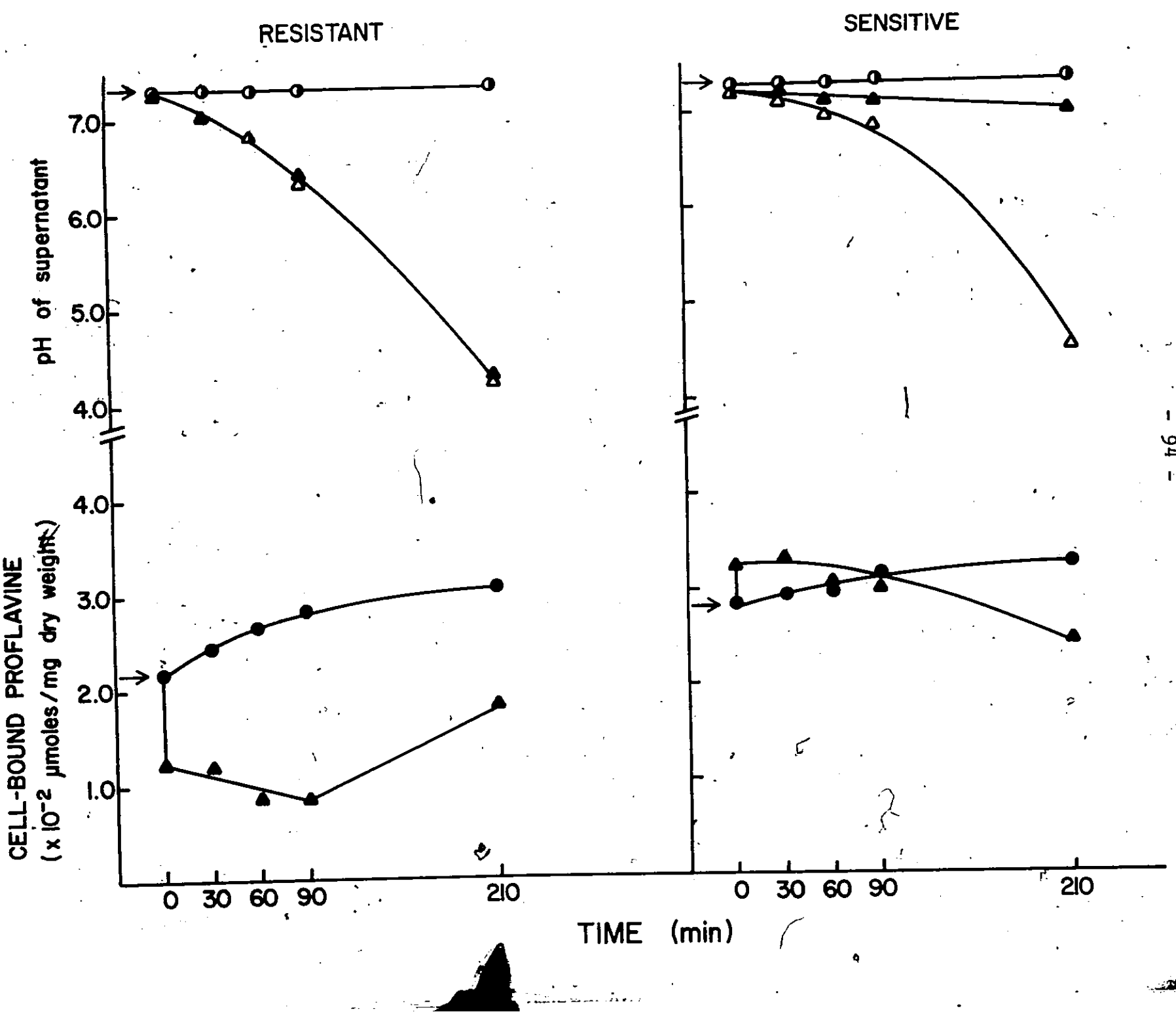

RESISTANT
SENSITIVE
pH of supernatant
7.0
6.0
5.0
4.0
CELL-BOUND PROFLAVINE
(x 10⁻² μmoles/mg dry weight)
4.0
3.0
2.0
1.0
0
30
60
90
210
TIME (min)

FIGURE 8b

pH CHANGES AND RELEASE OF BOUND PROFLAVINE WITH GLUCOSE-TREATED RESISTANT AND SENSITIVE CELLS

Conditions were as in Figure 8a except that final glucose concentration was 5.0%. Cells treated with water (○) or glucose (△) in the absence of proflavine; cells treated with water (●) or glucose (▲) in the presence of proflavine. Arrows show levels of pH or bound proflavine before additions.


RESISTANT
SENSITIVE
pH of supernatant
7.0
6.0
5.0
4.0
CELL-BOUND PROFLAVINE
(x 10⁻² μmoles/mg dry weight)
4.0
3.0
2.0
1.0
0 30 60 90 210
0 30 60 90 210
TIME (min)

Glucose-treated sensitive cells initially bound slightly more dye than the control cells. Approximately one hour after the addition of glucose, the amount of dye bound by the cells began to decrease gradually. The gradual decrease in the amount of dye bound paralleled the gradual fall in pH in the presence of proflavine. Glucose (5.0%) also decreased the amount of dye bound by the sensitive cells. At the end of the incubation, sensitive cells treated with 5.0% glucose had lost 25% of their bound dye, more than twice the amount lost with 0.5% glucose.

iii. Comparison of pH Changes and Dye-binding with Glucose, Ribose and Glycerol

The effects of glucose, ribose and glycerol on bound proflavine were investigated in similar experiments, with the following modifications: (a) cells were aerated during the incubation period by shaking vigorously on an Eberbach shaker at about 100 r.p.m.; (b) cells were incubated in 1/10 dilution of solution A (0.001M tris(hydroxymethyl) aminomethane buffer plus 0.0001M $MgCl_2$) with or without proflavine, in either shaken (as above) or stationary flasks and the metabolite added as before.

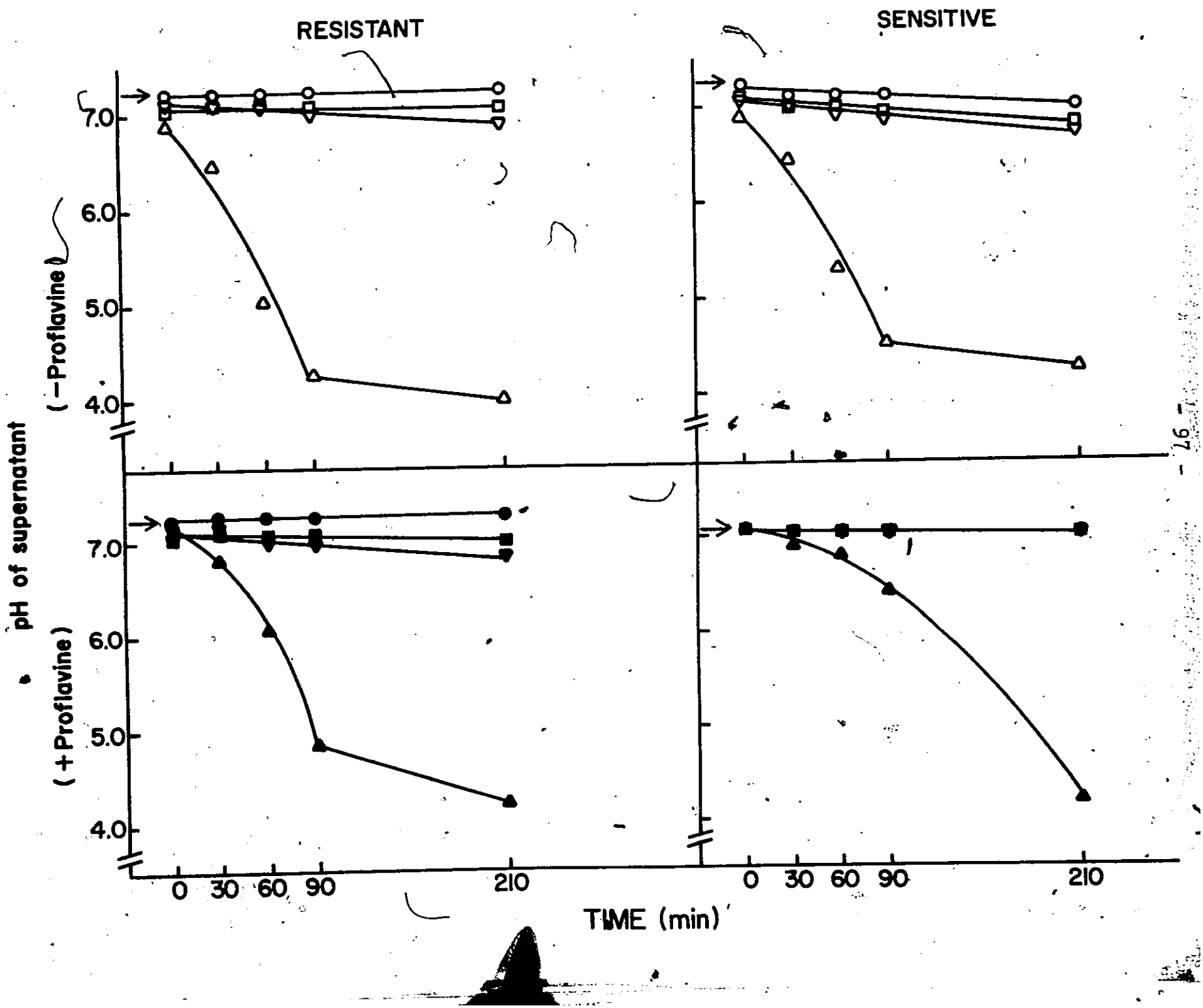
(a) Aerated Suspensions

In the absence of proflavine, the large pH changes produced by glucose, and the small pH changes produced by ribose and glycerol were about the same in both aerated sensitive and resistant cells (Figure 9a; see also Table 4).

FIGURE 9a

pH CHANGES IN AERATED SUSPENSIONS OF RESISTANT AND SENSITIVE CELLS TREATED WITH GLUCOSE, RIBOSE, OR GLYCEROL

Conditions were as in Figure 8a except that cells were vigorously aerated during the incubation period. The final concentration of all metabolites was 0.5%. Cells treated with water (o), glucose (Δ), ribose (▽), or glycerol (□) in the absence of proflavine; cells treated with water (●), glucose (▲), ribose (▼), or glycerol (■) in the presence of proflavine. Arrows show levels of pH before additions.


RESISTANT
SENSITIVE
pH of supernatant
(−Proflavine)
(+Proflavine)
7.0
6.0
5.0
4.0
0
30
60
90
210
TIME (min)

Proflavine had essentially no effect on the pH lowering produced when these compounds were added to resistant cells. Approximately the same amount of dye (51%) was released initially from resistant cells by all three compounds and no further loss or rebinding occurred during the remainder of the incubation (Figure 9b).

Proflavine abolished the small decrease in pH observed when aerated sensitive cells were incubated with glycerol or ribose. Very little dye was released with these compounds even after prolonged incubation (Figure 9b). The rate of pH lowering in the presence of glucose was decreased by proflavine [though not as much as in standing suspensions (see Figure 8a)]. By 210 minutes, however, the pH had fallen to the same level as in the absence of proflavine; and about 50% of the cell-bound dye was released. This release was equivalent to that from resistant cells under similar conditions.

(b) Aerated or Stationary Suspensions in Low Buffer

In the poorly buffered suspensions an immediate decrease in pH occurred following glucose addition to both sensitive and resistant cells (Figure 10a). The decrease was larger with resistant cells and was the same under aerated and stationary conditions both in the absence and presence of proflavine (Figures 10a and b). Without proflavine, the initial fall in pH with glucose-treated sensitive cells was somewhat larger in the shaken suspensions. Proflavine slightly inhibited the pH lowering following glucose addition to the sensitive cells (Figure 10c).

FIGURE 9b

RELEASE OF BOUND PROFLAVINE FROM AERATED SUSPENSIONS OF RESISTANT OR SENSITIVE CELLS TREATED WITH GLUCOSE, RIBOSE, OR GLYCEROL

Conditions were as in Figure 9a. Cells treated with water (●), glucose (▲), ribose (▼), or glycerol (■) in the presence of proflavine. Arrows show levels of proflavine before additions.

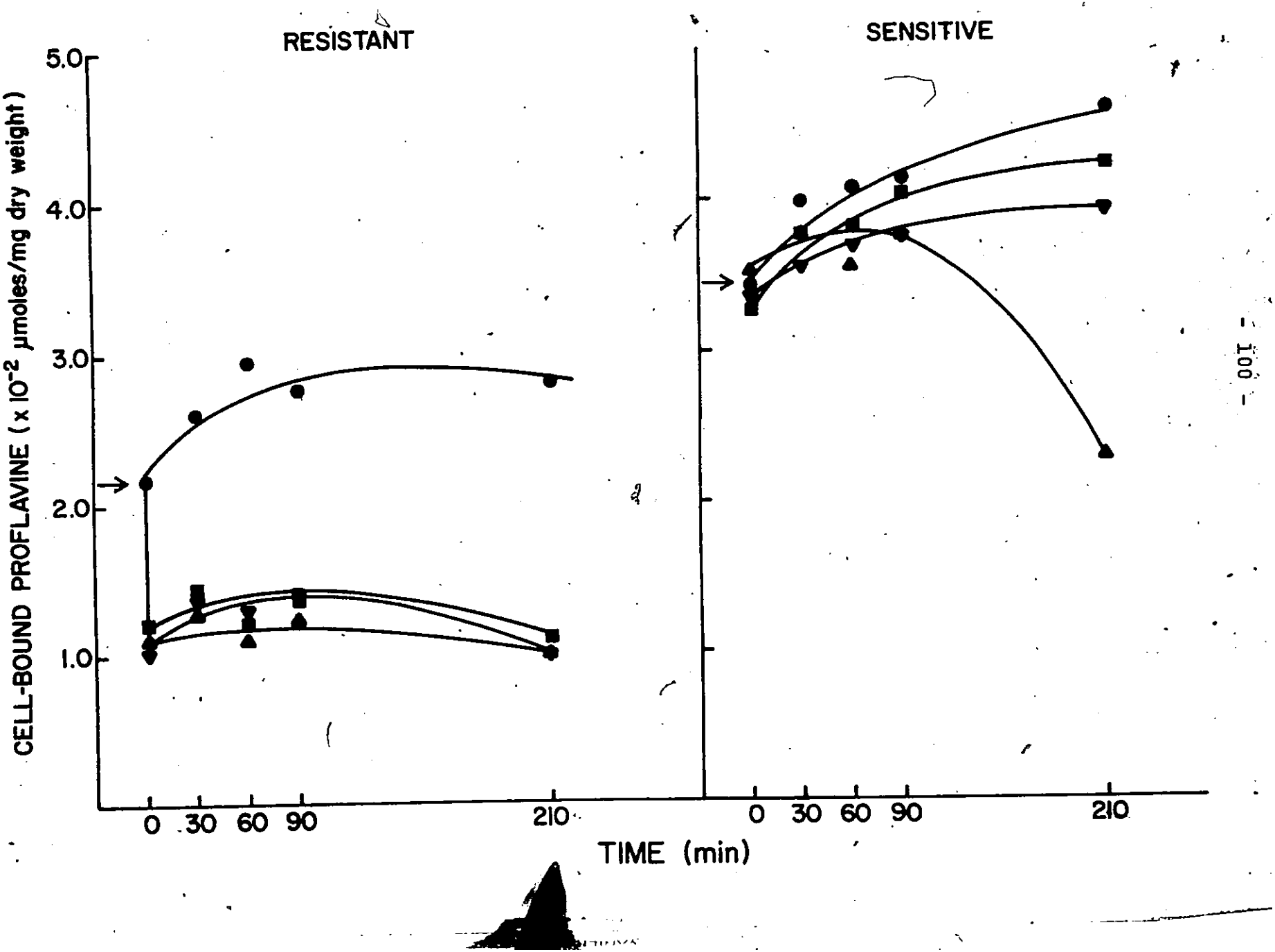

RESISTANT
SENSITIVE
CELL-BOUND PROFLAVINE (x 10⁻² µmoles/mg dry weight)
5.0
4.0
3.0
2.0
1.0
0 30 60 90 210
0 30 60 90 210
TIME (min)

FIGURE 10a

pH CHANGES IN POORLY BUFFERED SHAKING OR STATIONARY SUSPENSIONS OF RESISTANT OR SENSITIVE CELLS TREATED WITH GLUCOSE, RIBOSE, OR GLYCEROL IN THE ABSENCE OF PROFLAVINE

Conditions were as in Figure 8a except the buffer (solution A) was diluted tenfold. Cells were incubated in aerated or standing flasks as indicated. Final concentration of all metabolites was 0.5%. Cells treated with water (o), glucose (Δ), ribose (∇) or glycerol (□) in the absence of proflavine. Arrows show levels of pH before additions.

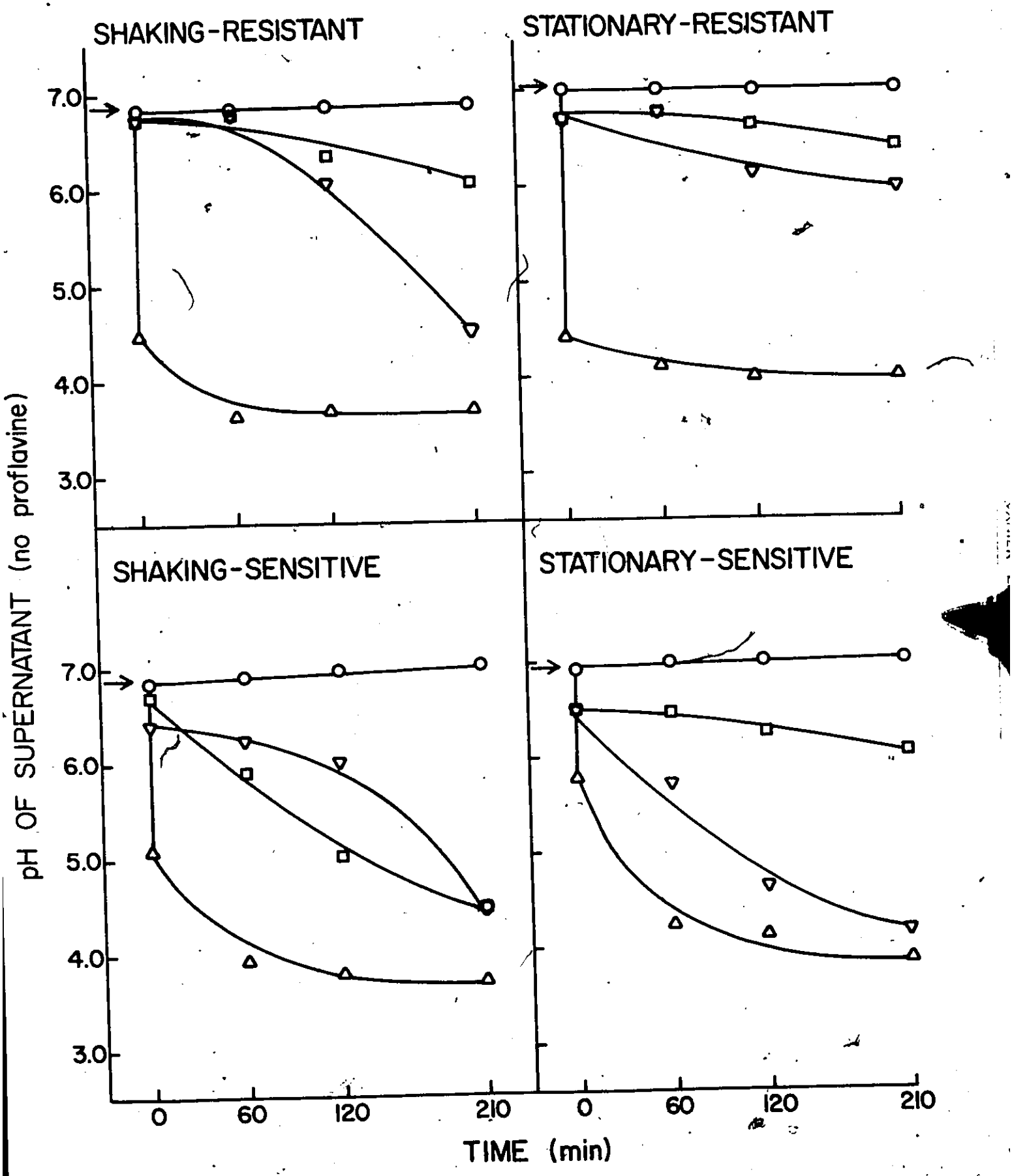

SHAKING-RESISTANT
STATIONARY-RESISTANT
SHAKING-SENSITIVE
STATIONARY-SENSITIVE
pH OF SUPERNATANT (no proflavine)
7.0
6.0
5.0
4.0
3.0
0
60
120
210
TIME (min)

FIGURE 10b

pH CHANGES AND RELEASE OF BOUND PROFLAVINE FROM RESISTANT CELLS TREATED WITH GLUCOSE, RIBOSE, OR GLYCEROL IN POORLY BUFFERED SHAKING OR STATIONARY SUSPENSIONS

Conditions were as in Figure 10a. Cells treated with water (●), glucose (▲), ribose (▼) or glycerol (■) in the presence of proflavine. Arrows show levels of pH or bound proflavine before additions.

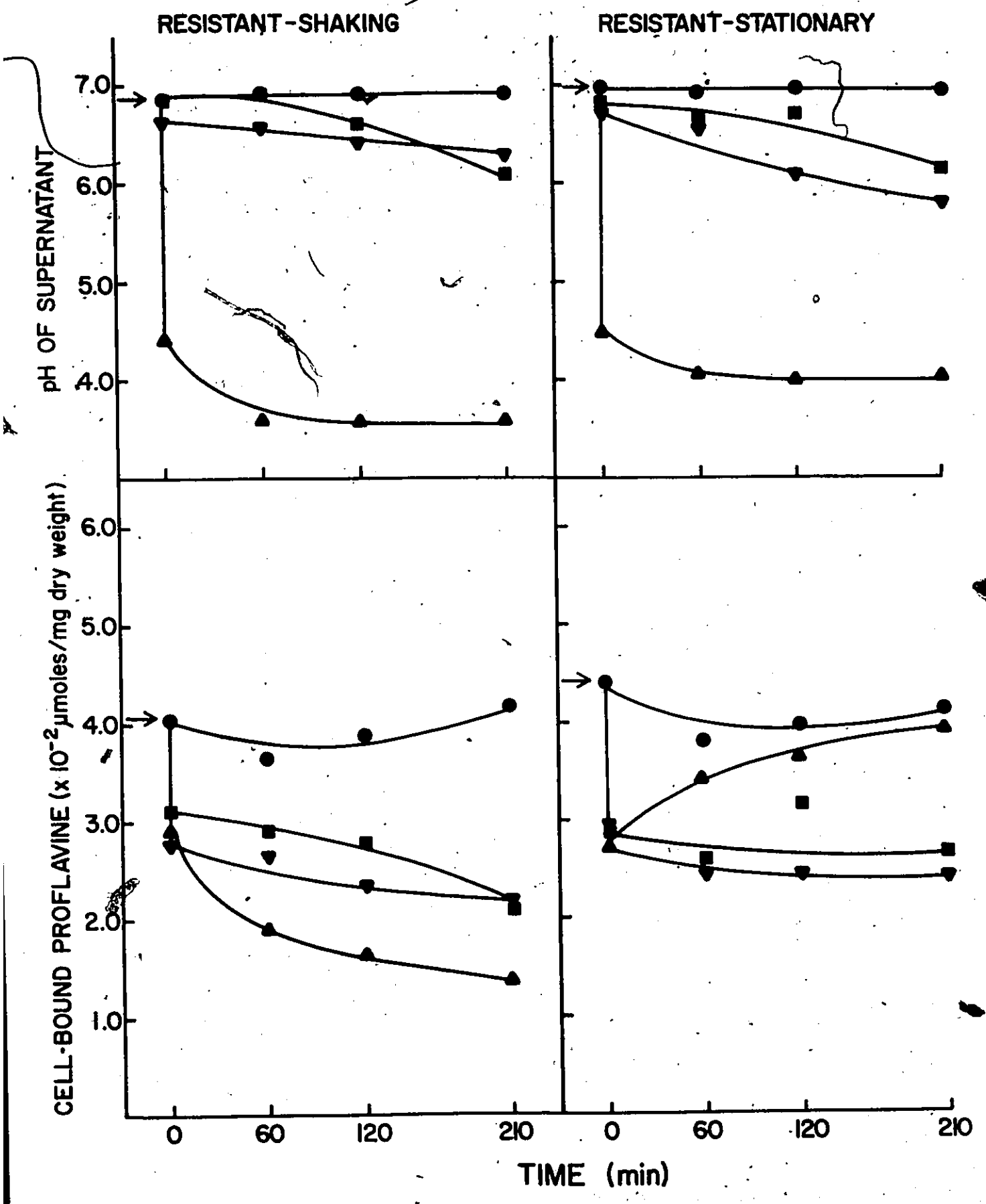
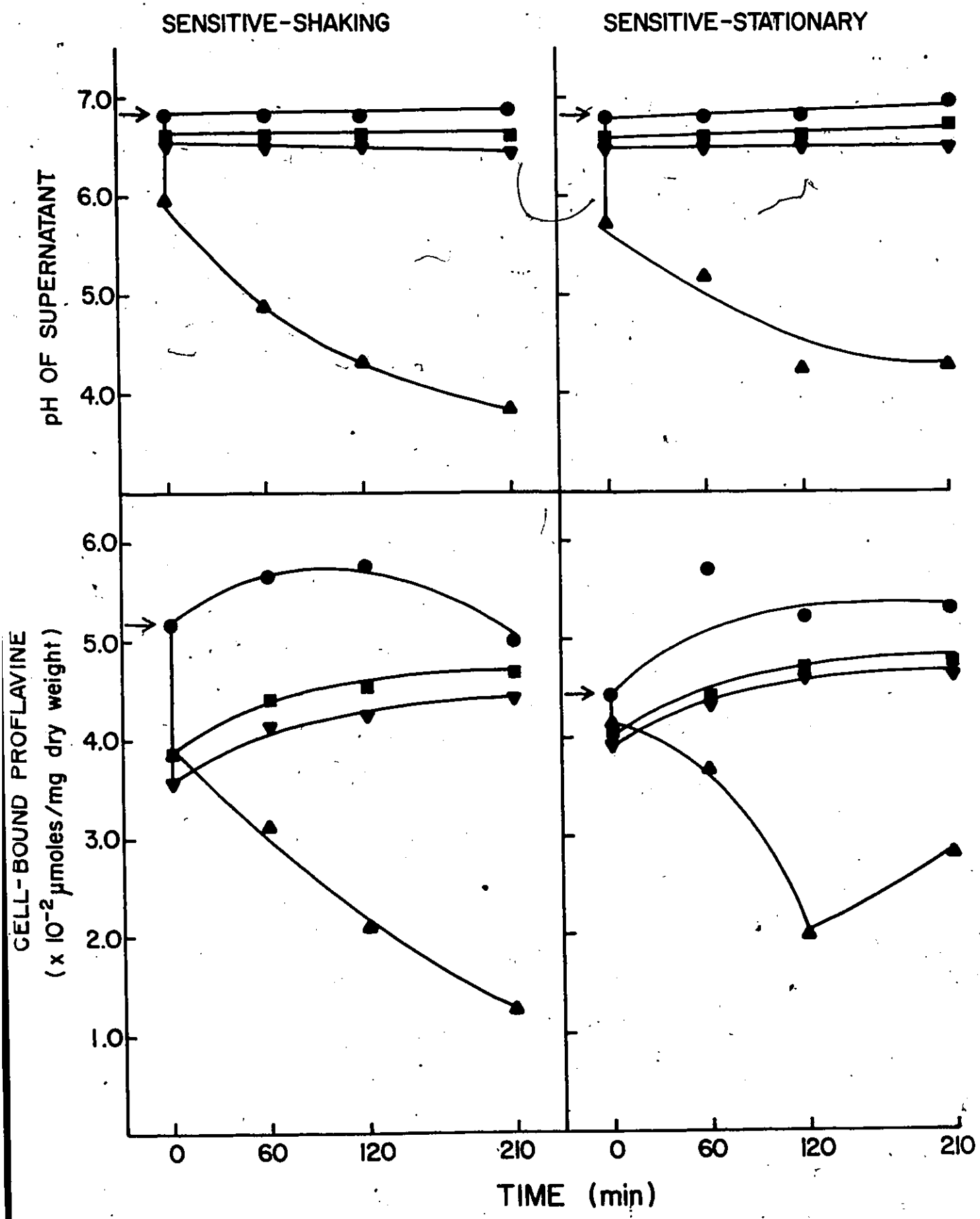

RESISTANT-SHAKING
RESISTANT-STATIONARY
pH OF SUPERNATANT
7.0
6.0
5.0
4.0
CELL-BOUND PROFLAVINE (x 10⁻² μmoles/mg dry weight)
6.0
5.0
4.0
3.0
2.0
1.0
0
60
120
210
TIME (min)

FIGURE 10c

pH CHANGES AND RELEASE OF BOUND PROFLAVINE FROM SENSITIVE CELLS TREATED WITH GLUCOSE, RIBOSE OR GLYCEROL IN POORLY BUFFERED SHAKING OR STATIONARY SUSPENSIONS

Conditions were as in Figure 10a. Cells treated with water (●), glucose (▲), ribose (▼) or glycerol (■) in the presence of proflavine. Arrows show levels of pH or bound proflavine before additions.


SENSITIVE-SHAKING
SENSITIVE-STATIONARY
pH OF SUPERNATANT
7.0
6.0
5.0
4.0
CELL-BOUND PROFLAVINE
(x 10⁻² µmoles/mg dry weight)
6.0
5.0
4.0
3.0
2.0
1.0
0
60
120
210
0
60
120
210
TIME (min)

The immediate decrease in pH was associated with an immediate release of bound proflavine from both sensitive and resistant cells (Figures 10b and c). In the aerated suspensions the release of bound dye was the same in both sensitive and resistant cells ($\sim$ 28%). Under stationary conditions more dye was released by the resistant cells. As the incubation progressed, shaken cells lost further amounts of proflavine (up to 75% with E. coli B). With sensitive, but not resistant cells, this further loss paralleled the gradual decrease in pH over the incubation period.

Under stationary conditions both cell types rebound substantial amounts of proflavine. The rebinding coincided with the stabilization of the pH. Thus, resistant cells had rebound considerable amounts of dye after 60 minutes of incubation. In contrast, sensitive cells lost proflavine during the first two hours of incubation, but, rebinding was evident by 210 minutes of incubation.

In the lower buffer concentration glycerol and ribose caused little or no immediate decrease in pH, although the overall decrease, especially in the aerated suspensions, was greater than that found in the usual buffer. Proflavine largely inhibited the lowering of pH following the addition of glycerol or ribose to sensitive cells under shaking and stationary conditions. The marked pH lowering produced by ribose in aerated suspensions of resistant cells was also reduced. These compounds were, nonetheless, as effective as glucose in causing an immediate release of cell-bound

proflavine from both sensitive and resistant cells. Glycerol- and ribose-treated resistant cells, aerated and stationary, lost further dye during the remainder of the incubation period. This loss was associated with a gradual decrease in the pH. In contrast, the glycerol- and ribose-treated sensitive cells rebound a small amount of proflavine.

In the above experiments using 2×10^{-5}M proflavine the glucose-induced pH lowering by sensitive cells was never completely inhibited. With higher concentrations (see section on glucose utilization, below) a much clearer distinction between metabolic susceptibilities of sensitive and resistant cells was obtained.

iv. Effect of Sodium Cyanide on pH Changes and Dye Release

In an experiment similar to those outlined above, the effect of 0.01M sodium cyanide (final concentration) on the release of bound proflavine was investigated (Figures 11a and b).

Cyanide inhibited the lowering of pH following glucose addition to vigorously aerated resistant or sensitive cells suspended in buffered proflavine. The inhibition was more pronounced with sensitive cells and correlated with a lesser and more gradual glucose-induced release of bound dye than was observed in the absence of cyanide. In resistant cells, cyanide inhibited the glycerol-induced release of bound proflavine but not that induced by glucose. This may reflect the fact that glucose can yield energy by both aerobic and anaerobic pathways, but glycerol only by an aerobic pathway.

Figure 11a

EFFECT OF SODIUM CYANIDE ON pH CHANGES AND PROFLAVINE RELEASE FROM RESISTANT CELLS TREATED WITH GLUCOSE OR GLYCEROL

Conditions were as in Figure 8a except that (i) cells were aerated during the incubation period, (ii) and at zero time sodium cyanide (0.01 M final concentration) was added immediately before the desired metabolite (0.5% final concentration). Cells treated with water (●), glucose (▲), or glycerol (■) in the presence of proflavine. Arrows show levels of pH or bound proflavine before additions.

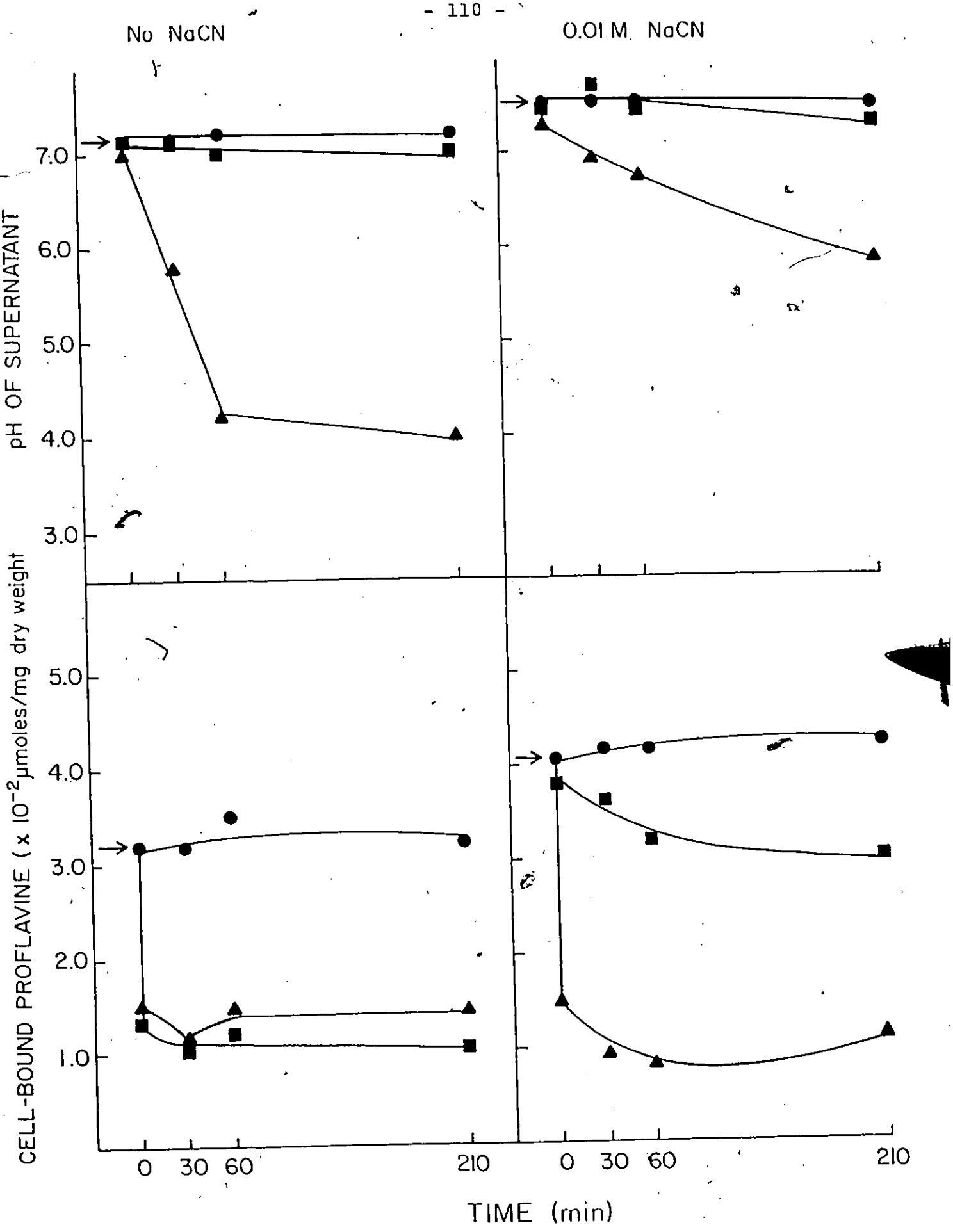
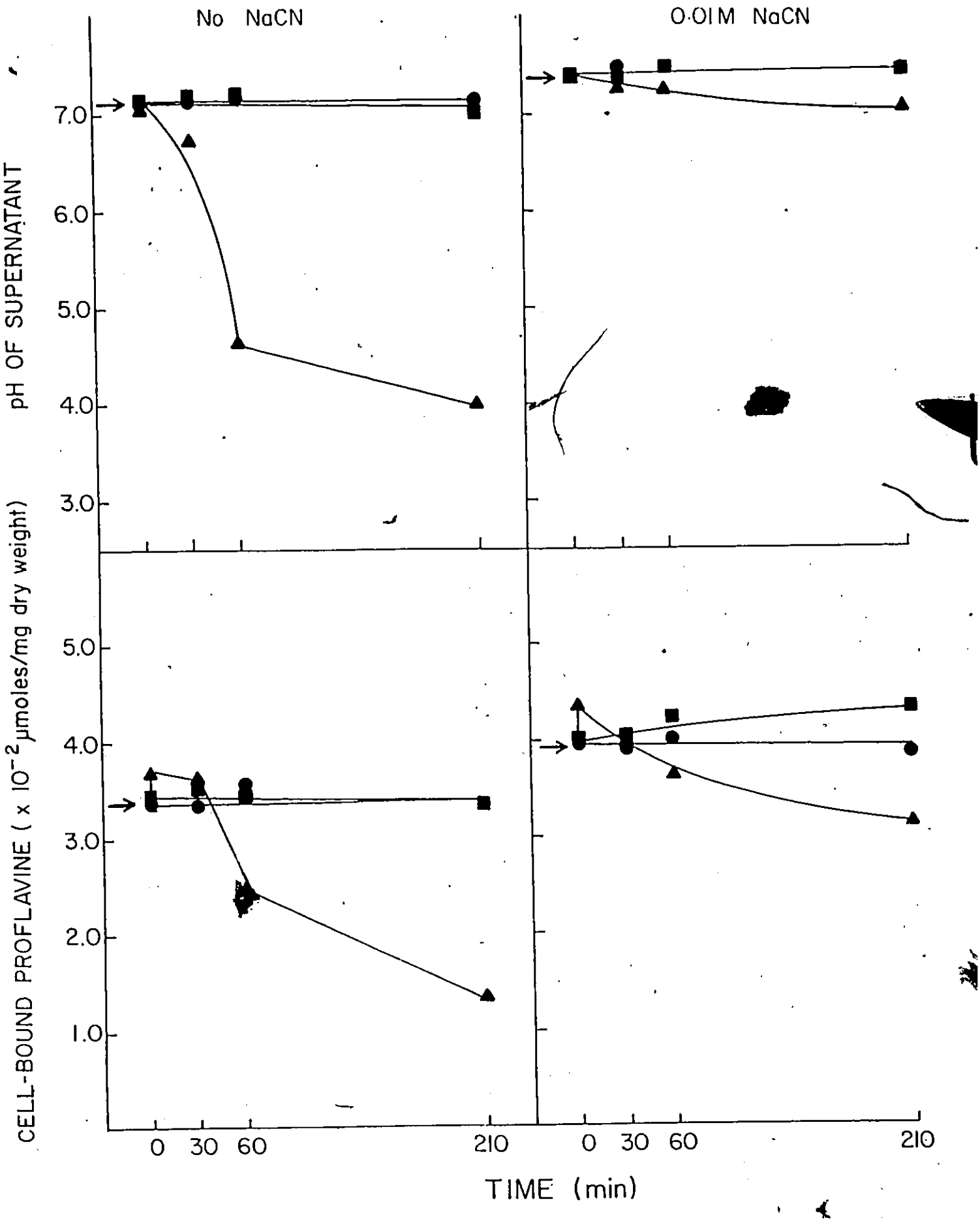

No NaCN
0.01 M. NaCN
pH OF SUPERNATANT
7.0
6.0
5.0
4.0
3.0
CELL-BOUND PROFLAVINE (x 10^{-2} µmoles/mg dry weight
5.0
4.0
3.0
2.0
1.0
0
30
60
210
0
30
60
210
TIME (min)

FIGURE 11b

EFFECT OF SODIUM CYANIDE ON pH CHANGES AND PROFLAVINE RELEASE FROM SENSITIVE CELLS TREATED WITH GLUCOSE OR GLYCEROL

Conditions were as in Figure 11a. Cells treated with water (●), glucose (▲), or glycerol (■) in the presence of proflavine. Arrows show levels of pH or bound proflavine before additions.


No NaCN
0·01M NaCN
pH OF SUPERNATANT
7.0
6.0
5.0
4.0
3.0
CELL-BOUND PROFLAVINE (x 10^{-2} µmoles/mg dry weight)
5.0
4.0
3.0
2.0
1.0
0 30 60 210
0 30 60 210
TIME (min)

EFFECT OF PROFLAVINE ON METABOLIC ACTIVITY

Studies on pH changes suggested that proflavine might be inhibiting glucose utilization in sensitive cells. Thus, the effect of proflavine on glucose utilization was determined by directly measuring the amount of glucose remaining when cells were incubated with glucose in the presence of the dye. As before, cells were preincubated with proflavine (the concentration x 10^{-5}M as indicated by the curves in Figures 12 and 13) under aerobic or anaerobic conditions. After measuring bound proflavine; glucose (0.01% final concentration) was added. A sample was centrifuged immediately (0 time) and at intervals for measuring proflavine bound to the cells and for determining glucose remaining in the supernatant fluid as described in 'Methods'.

In resistant cells, adding glucose led to the immediate release of proflavine from the cells, as well as the progressive disappearance of glucose itself from the external medium. After most of the glucose had been used, proflavine was again taken up by the cells (Figures 12a and b). The rate of glucose utilization was the same in the presence and absence of proflavine (up to 10 x 10^{-5}M), the highest concentration tested); glucose disappeared more quickly anaerobically, but less proflavine was released anaerobically than aerobically. After the glucose was used up, less rebinding of proflavine by the cells occurred anaerobically than aerobically.

In contrast, the glucose utilization of sensitive cells was inhibited by proflavine in concentrations of 2 x 10^{-5}M and higher. The degree of inhibition was greater under anaerobic than under

FIGURE 12a

GLUCOSE UTILIZATION AND RELEASE OF BOUND PROFLAVINE BY RESISTANT CELLS UNDER AEROBIC CONDITIONS

Cells (at 0.200 mg(dry weight)/ml) were incubated with shaking for 30 minutes at 37°C in the presence of different proflavine concentrations (the concentrations, x 10^{-5}M are indicated by the curves). After measuring bound proflavine (100% binding), glucose (0.01% final concentration) was added. A sample was centrifuged immediately ('0' time) and at intervals for measuring proflavine bound to the cells and glucose remaining in the supernatant fluid. The small decrease in glucose and large decrease in bound proflavine in the '0' time samples shows the changes occurring while the cells are being centrifuged. The values for 100% proflavine contents of the cells (x 10^{-2} u mol/mg(dry weight)) for added concentrations of 2, 5, and 10 x 10^{-5}M respectively, were 2.2, 4.9 and 14.1.

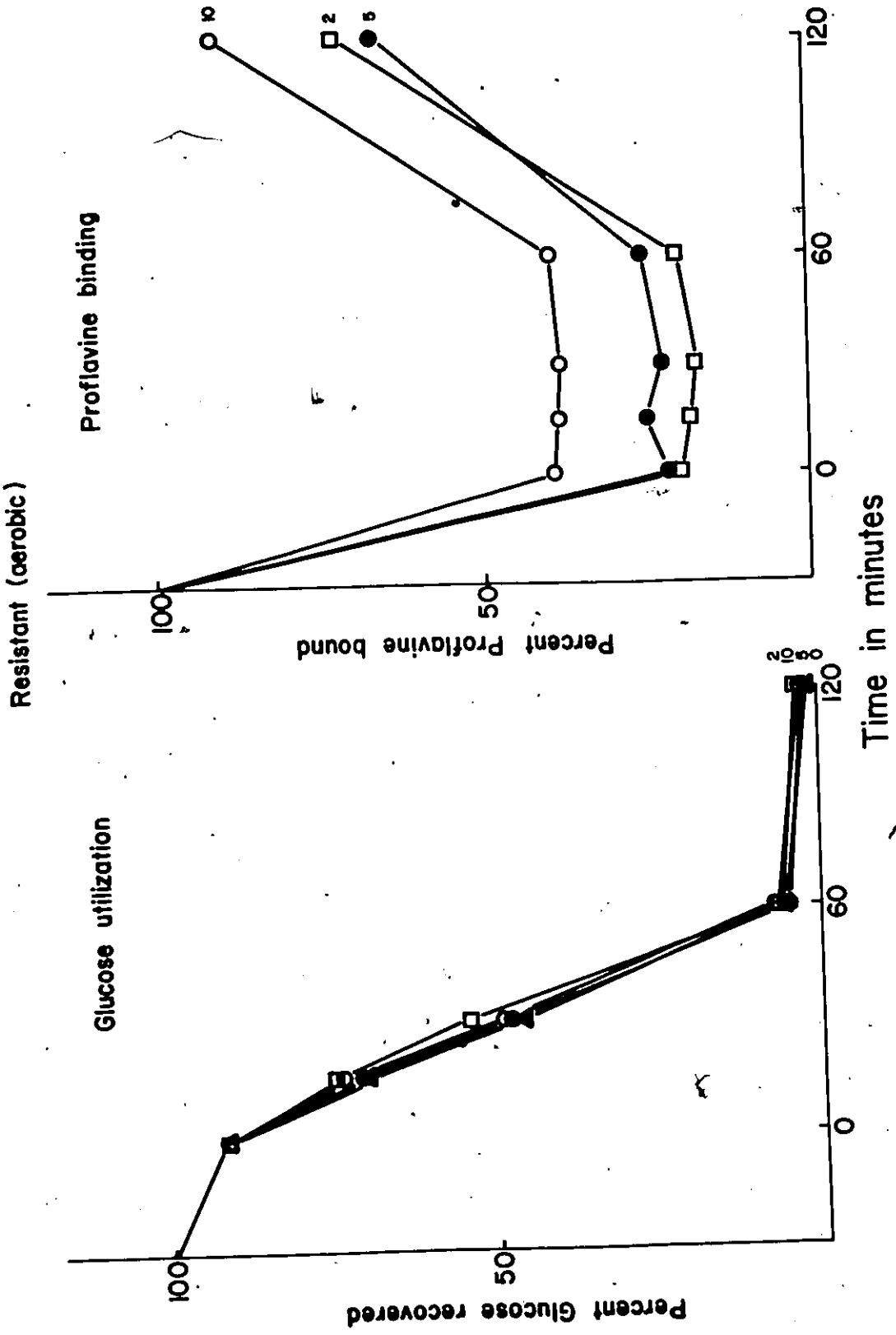
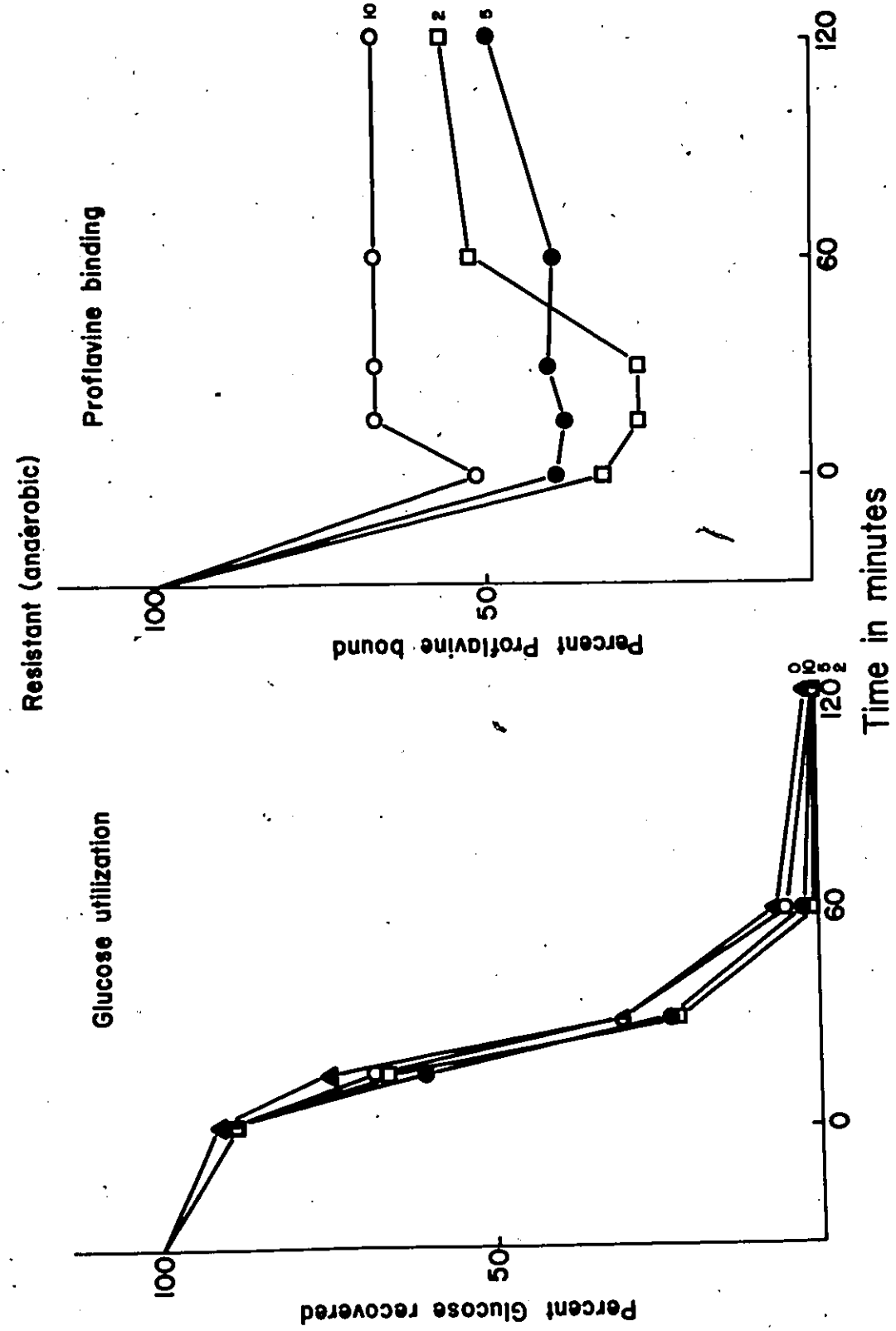

Resistant (aerobic)
Proflavine binding
Percent Proflavine bound
100
50
0
60
120
Time in minutes
10
2
5
Glucose utilization
Percent Glucose recovered
100
50
0
60
120
Time in minutes
20
60

FIGURE 12b

GLUCOSE UTILIZATION AND RELEASE OF BOUND PROFLAVINE BY RESISTANT CELLS UNDER ANAEROBIC CONDITIONS

Conditions were as in Figure 12a except that cells were incubated in standing flasks under nitrogen. The values for the 100% proflavine contents of the cells [x 10^{-2}u mol/mg(dry weight)] for added concentrations of 2,5 and 10 x 10^{-5}M respectively, were 2.5, 6.2 and 14.3.


Resistant (anaerobic)
Proflavine binding
Percent Proflavine bound
100
50
0
60
120
10
2
5
Glucose utilization
Percent Glucose recovered
Time in minutes

aerobic conditions (Figures 13a and b). Aerobically, even the highest proflavine concentration did not completely inhibit glucose utilization. These experiments also showed that if the amount of proflavine bound to the sensitive cells was too low to inhibit glucose utilization strongly, then adding glucose caused this bound proflavine to be released. This suggests that both sensitive and resistant cells are able to expel bound proflavine, but that in the sensitive cells proflavine itself inhibits the metabolic events responsible for its release.

In general, these experiments show that proflavine acts as a metabolic inhibitor affecting glucose metabolism more strongly in sensitive than resistant cells. In sensitive cells, glucose metabolism may be greatly or only slightly reduced, depending on the experimental conditions. The pH changes evoked by ribose and glycerol were also inhibited to a greater extent in sensitive than in resistant cells. Metabolism of any of these compounds is associated with the release of bound dye.

Both sensitive and resistant E. coli are able to release bound proflavine but the former may do so only if the amount bound is too low to inhibit glucose utilization. The extent and duration of the release is somewhat dependent upon the metabolite concentration and the incubation conditions. The fact that proflavine released during metabolism may be taken up again after the energy source has been depleted, suggests that a continuous supply of energy is needed to keep this dye out of the cells.

Figure 13a

GLUCOSE UTILIZATION AND RELEASE OF BOUND PROFLAVINE BY SENSITIVE CELLS UNDER AEROBIC CONDITIONS

Conditions were as in Figure 12a. Cells were at 0.185 mg(dry weight)/ml. The values for the 100% proflavine contents of the cells [x 10^{-2}u mol/mg (dry weight)] for added concentrations of 0.1, 2, 5 and 10 x 10^{-5}M, respectively, were 0.3, 2.8, 6.2 and 14.6

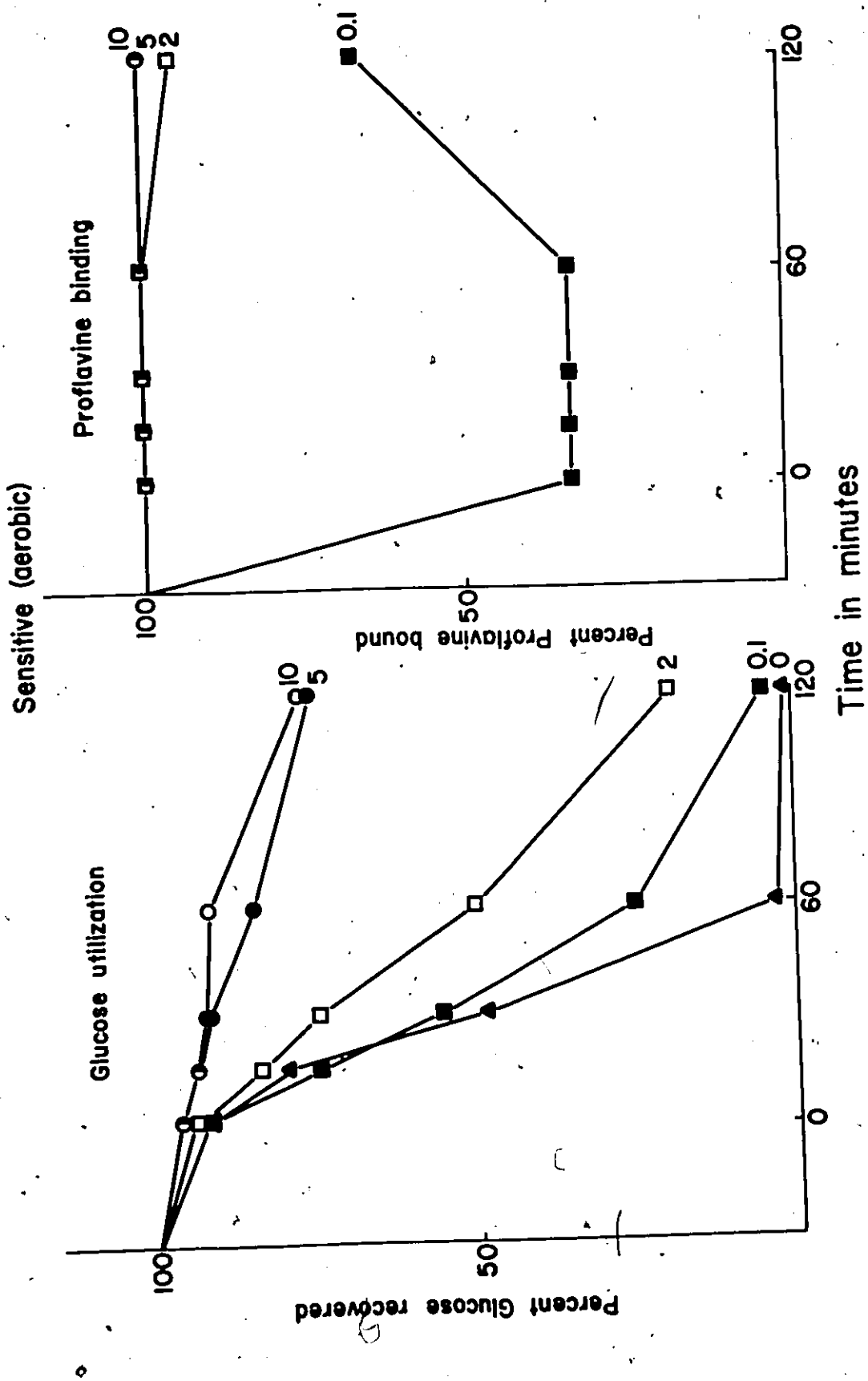
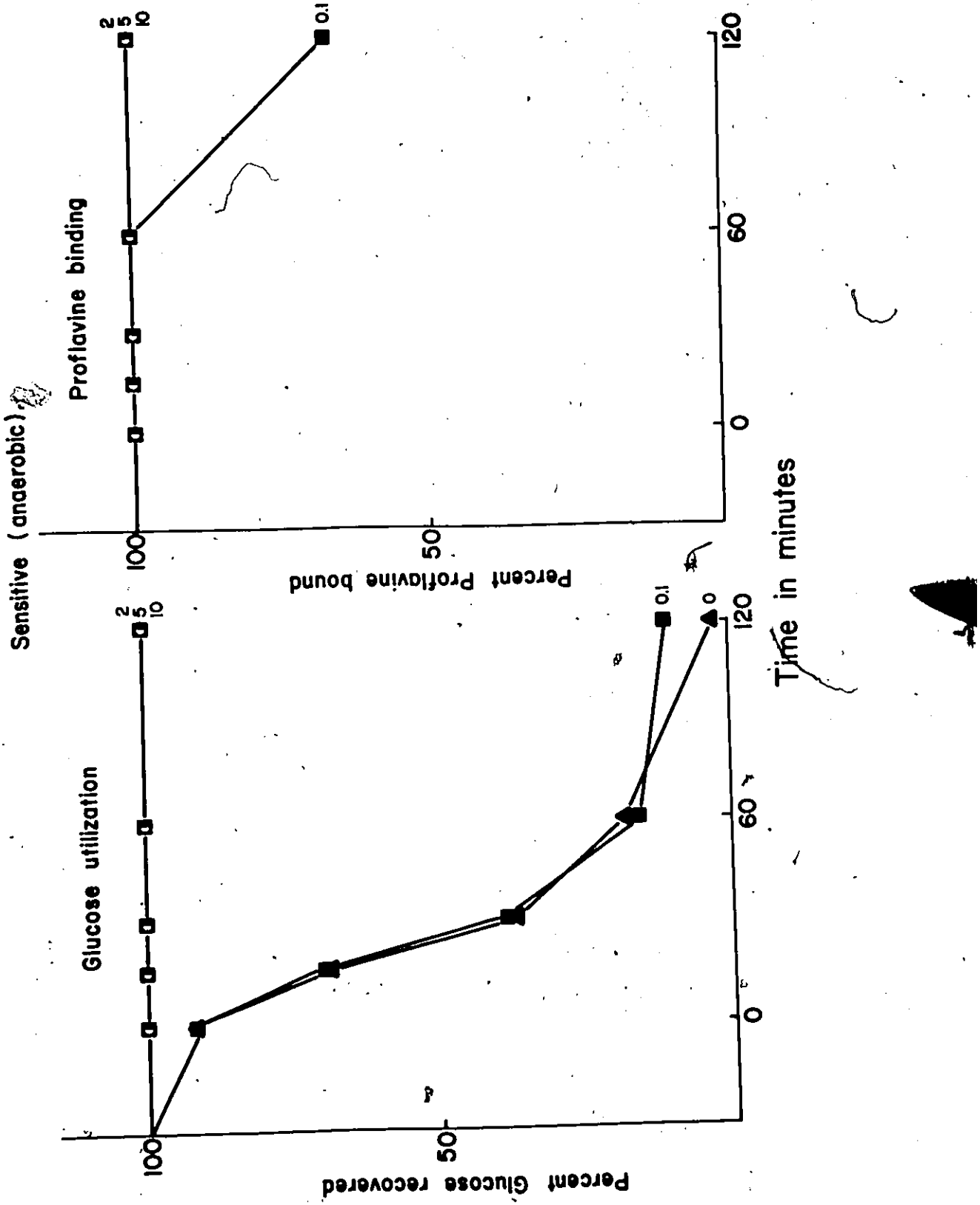

Sensitive (aerobic)
Proflavine binding
Glucose utilization
Percent Proflavine bound
Percent Glucose recovered
Time in minutes
100
50
0
60
120
10
5
2
0.1

FIGURE 13b

GLUCOSE UTILIZATION AND RELEASE OF BOUND PROFLAVINE BY SENSITIVE CELLS UNDER ANAEROBIC CONDITIONS

Conditions were as in Figure 12b. Cells were at 0.185 mg(dry weight)/ml. The values for 100% proflavine contents of the cells for added concentrations of 0.1, 2, 5, and 10 x 10^{-5} M, respectively, were 0.3, 2.4, 7.0, 13.3.


Sensitive (anaerobic)
Proflavine binding
Percent Proflavine bound
100
50
0
60
120
Time in minutes
2
5
10
0.1
Glucose utilization
Percent Glucose recovered
0

Hydrogen ions lower the binding of acridine dyes to cells and cell constituents (Peacocke and Skerrett, 1956; Nakamura, 1967; Silver, 1968; Woods et al., 1973). In the experiments reported above, the rapid, initial release of cell-bound proflavine from glucose-treated resistant cells preceded a detectable pH change. With sensitive cells under similar conditions the release of bound dye occurred gradually and paralleled the pH decrease. In dilute buffer the external pH and cell-bound dye decreased simultaneously in both sensitive and resistant cells. Both cell strains rebound large amounts of dye in acid pH. These findings suggest that the external pH has little influence on dye release in resistant cells. However, with the sensitive strain, dye displacement by hydrogen ions may possibly contribute to the release of bound proflavine. A decrease in the external pH (as a result of residual metabolism) may reduce cell-bound proflavine to a level which does not inhibit metabolism. Dye release could then proceed as with resistant cells.

In sensitive cells proflavine inhibits anaerobic glucose metabolism (or aerobic metabolism in CN-poisoned cells, Figure 11b) more strongly than aerobic metabolism. Since some dye was released by glucose in the sensitive cells, and eventually by glycerol in the resistant cells, it would appear that the concentration of cyanide used did not completely block aerobic metabolism.

DISCUSSION AND CONCLUSIONS

SITE OF PROFLAVINE ACTION

The main conclusions that emerge from the experiments reported here are that (i) proflavine binding is changed little by transforming E. coli cells to spheroplasts or by increasing spheroplast permeability; (ii) nucleic acids are not the only cellular binding sites for proflavine; and (iii) proflavine acts as a metabolic inhibitor, affecting metabolism more strongly in sensitive than in resistant cells.

Previous work from this laboratory showed that proflavine binding in E. coli and B. subtilis was a passive process. The experiments with spheroplasts have now shown that an osmotically supported membrane is not needed for proflavine binding by E. coli, nor does it present a barrier to such binding. However, an osmotically protected membrane does seem necessary for extrusion of proflavine by spheroplasts of resistant cells. Spheroplasts that had bound proflavine in 10% sucrose released some of the dye when glucose was added. In the absence of sucrose, they could bind proflavine but not release it. This supports the conclusion that such release is a metabolically active process. Possibly, osmotic lysis of spheroplasts causes loss or inactivation of enzymes responsible for the release of bound proflavine.

Resistant spheroplasts were not completely protected by 10% sucrose, but higher concentrations could not be used in studying proflavine release because these reduced proflavine binding in intact cells and inhibited proflavine release on glucose addition. According to Rose (1965), the internal

osmotic pressure of Gram-negative organisms is 8 atmospheres (or less), approximately equivalent to that exerted by a 10% sucrose solution. The presence of 20% sucrose reduced proflavine binding by about 50%. Possibly, the withdrawal of intracellular water resulting from this amount of sucrose concentrated intracellular salts and displaced bound proflavine.

A similar inhibition of acriflavine binding by high concentrations of sucrose-magnesium was recently reported by Woods et al. (1973) for acriflavine-sensitive and -resistant Serratia marcescens. They also found that the percentage survival after 18 hours of incubation in nutrient broth medium supplemented with 0.5M sucrose and 0.01M Mg++ was the same both in the presence and absence of acriflavine. This finding was taken to indicate that sensitive cells were resistant to acriflavine in such media because of their reduced dye uptake. However, since Woods et al. (1973) did not indicate whether or not any growth took place in the sucrose-magnesium supplemented media in the absence of acriflavine, percentage survival may not be a valid criterion of resistance.

Converting E. coli cells to spheroplasts by Tris - EDTA - lysozyme in the presence of hypertonic sucrose releases lipopolysaccharide, surface bound enzymes and elements of the acid soluble nucleotide pool (Russell, 1971), though we found that RNA, DNA and protein contents are not significantly affected (Table 3). In the absence of sucrose up to 40% of the nucleic acid and protein may be released (Table 3). When saturated with proflavine in 10% sucrose, sensitive spheroplasts bound as much dye as cells in sucrose. Unprotected spheroplasts of either

strain bound about as much dye as intact cells. Thus, despite the losses noted, proflavine binding was changed little by transforming cells to spheroplasts.

Histological, biochemical and genetic evidence indicates that nucleic acids are important sites of action of the acridine dyes. Elegant physicochemical studies have been made of the interactions between isolated DNA, RNA, ribosomes and nucleotides and acridines. The degradative studies presented here showed that almost all of the nucleic acids could be removed from mercury treated cells, from heated cells, and from spheroplasts without a corresponding loss in proflavine binding ability. Thus nucleic acids are not the only cellular binding sites for proflavine. Are they the most important ones?

In assessing the importance of nucleic acids as binding sites for acridine dyes it should be recalled that extensive, *in vivo* binding of acridines to nucleic acids occurs without any apparent deleterious effects on the cells (Hill *et al.*, 1960). Moreover, the complex between nucleic acids and acridines is reversible (Kann and Kohn, 1972). And as has already been noted, acridines bind to other cellular polyanions than nucleic acids, including proteins, mucopolysaccharides and phospholipids. Bradley and Wolf (1959) found that dye aggregation occurred more readily on some of these polymers than on DNA. Complexes with heme (Simpson, 1968) and interaction with membranes have also been reported (Azzi *et al.*, 1971; Nakamura and Suganuma, 1972). With binding sites of so many chemical natures available, it seems very unlikely that any selective degradation process could remove all such sites and leave any cell structure.

In vivo studies have also suggested a relationship between binding to non-nucleic acid sites and acridine action. When hemoflagellates are grown in the presence of low concentrations of acridines, a high percentage of organisms loose their kinetoplast * (Simpson, 1968). The loss of this organelle was thought to be caused by a specific inhibition of kinetoplast DNA synthesis, as a result of an apparently selective localization of acridines in the kinetoplast (Simpson, 1968; Luha, et al., 1971). Strauss (1972) studied the localization of the tritiated acridine acriflavine in L. tarentolae. She found that although the fluorescence of the dye was indeed localized in the kinetoplast, the label was widely distributed over the remainder of the mitochondrion, the cytoplasm, the nucleus and lipid droplets. Thus, acriflavine might have been acting at quite a different site than the kinetoplast.

In addition to the fact that acridines can bind to several cellular components, it also seems possible that the removal of one binding site may open up others. Miall and Walker (1967) observed that treatment with EDTA greatly increased the proflavine binding power of E. coli ribosomes. They thought this was caused by unfolding of the RNA, but it could also have been due to exposure of fresh protein binding sites to the dye. A similar mechanism may account for the enhanced dye binding capacity of heated cells observed here.

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* The kinetoplast of hemoflagellates is a specialized area of the mitochondrion containing mitochondrial DNA. In Giemsa stained preparations it is visible as a dense body at the base of the flagellum. Among hemoflagellates grown in the presence of acridines, a kinetoplast cannot be found in a high percentage of organisms stained with Giemsa; these forms are termed dyskinetoplastic (Strauss, 1972).

To add to these complications, *in vitro* studies have shown that acridines interact with polymers in a variety of ways. The particular binding mode which takes place is dependent upon the dye, the polymer and the environment. Lerman (1964b) found that intercalary binding was suppressed when external stacking was extensive. In turn, the degree of stacking may be affected by the free space available around the binding site. Such considerations explain why it does not seem possible to make a correlation between content of any one class of polymers and quantitative ability of intact cells to bind proflavine.

The penetration of ions through artificial membranes is strongly dependent on the double bond content and chain length of the constituent fatty acids (Haest *et al.*, 1969, cited by Johnson and Brown, 1972). It has been suggested that differences in permeability to acridines occur in sensitive and resistant organisms which arise from differences in their cell wall or cell membrane composition (Silver *et al.*, 1968; Johnson and Brown, 1972) Johnson and Brown (1972) studied the relation between acriflavine accumulation and fatty acid composition in yeasts which can ('petite positive') and cannot ('petite negative') give rise to respiratory deficient mutants. They found that yeasts of either strain absorbed similar, small amounts of acriflavine in buffer. The addition of a metabolizable sugar greatly enhanced dye uptake by 'petite positive' yeasts, but had no effect on dye uptake by 'petite negative' cells. Only 'petite negative' cells contained the polyunsaturated, linoleic and linolenic fatty acids. On the basis of these findings, Johnson and Brown suggested that uptake

and susceptibility to acriflavine were determined by membrane permeability, the latter being a function of its lipid composition. However, even in 'petite negative' yeasts acriflavine inhibits the synthesis of respiratory enzymes (De Deken, 1966). This effect implies a prior entry of the dye into the cell. Other studies have indicated that the above differences in fatty acid content reflect differences in the composition of mitochondrial membranes only (Kellerman et al., 1969; Linnane and Kellerman, 1971). Thus, although it seems unlikely that the two strains of yeast differ in the permeability of their cytoplasmic membranes to the dye, they may differ in this respect in their mitochondrial membranes. A difference in the latter could also influence the action of acriflavine.

A number of workers (Azzi et al., 1971; Dell'Antone, et al., 1972a) have reported that acridines were bound to de-energized submitochondrial membrane preparations; energization of the membrane lead to a marked increase in binding. Azzi et al. (1971) showed that anions were required for the energy-mediated uptake. Dell'Antone et al. (1972a) found that the lipophilic cation, nupercaine, competitively inhibited acridine uptake. Along with spectroscopic evidence (Dell'Antone et al., 1972b), this fact was taken to indicate that acridines were bound in the membrane itself. Dell'Antone et al (1972b) suggested that upon energization the membrane undergoes a conformational re-arrangement of the protein core. One consequence of this change was the unmasking of additional anionic groups which bind acridines.

Although these studies were performed with rat liver mitochondria, it seems possible that a similar phenomena could account for the increased acridine uptake by 'petite positive' yeasts in the presence of an energy source. The differences in fatty acid composition reported (Johnson and Brown, 1972) may be restricted to the outer layers of the mitochondrial membrane. In this position they may possibly influence the penetration of acridine to the protein core, or alter the type of structural rearrangement which takes place on energization and so limit the available dye binding sites.

Differences in fatty acid composition between acridine-sensitive and -resistant strains of E. coli have also been reported (Karkus et al., 1963, cited by Silver et al., 1968). Despite these differences, there is little evidence to suggest that sensitive and resistant strains of E. coli differ in overall permeability to acridines. In fact, both strains appear to be freely permeable to the dye. Kushner and Khan (1968) found that cells in buffer bound more proflavine than cells in growth media; moreover, in buffer, sensitive and resistant cells bound equal amounts of dye. They also showed that the reduced dye uptake of resistant cells in growth media was the result of their ability to actively expel the dye. Increased uptake of acridine by E. coli after bacteriophage infection or treatment with phenethyl alcohol has been considered evidence of increased permeability to these dyes (Silver, 1965, 1967a and b; Silver et al., 1968). However, in this work considerable amounts of dye were taken up by cells in growth media, even without the addition of agents

that are thought to increase permeability. The data presented in this thesis indicate that it may be very difficult to distinguish between increased membrane permeability and opening up of new cellular binding sites. It is suggested that the amount of dye bound by E. coli, and possibly other organisms, depends on the availability of binding sites, rather than differences or alterations in cytoplasmic membrane permeability.

A number of studies have suggested that acridines may inhibit growth by acting on sites other than nucleic acids. Acridines competitively inhibit certain proteases (Chen and Russo, 1971) and activate others (Hall et al., 1972; Skalski, 1973). Enzymes involved in carbohydrate breakdown from malarial parasites (reviewed by Albert, 1966) and from yeasts (Witt, 1968) are inhibited by acridines. Bovarnik (1946) showed that the inhibitory effects of mepacrine on Plasmodia were reversed by ATP. Karreman et al., (1957) found that the inhibition of muscle contraction produced by acridine orange could be reversed by ATP. Low (1959) reported that mepacrine stimulated (up to 0.75mM) or inhibited (at 3 mM) a mitochondrial ATPase. Mepacrine also uncouples oxidative phosphorylation in membrane preparations of mitochondria (Hunter, 1955) and Azotobacter vinelandii (Eilerman, 1970). Wolfe et al. (1971) have shown that Nitroakridine 3582 and mepacrine exerted bactericidal action that was independent of their effects on DNA synthesis.

Work reported here shows that proflavine acts as a metabolic inhibitor affecting glucose metabolism more strongly in sensitive than in resistant cells. Measurements of pH

change indicate that ribose and glycerol oxidation are also inhibited to a greater extent by proflavine in sensitive than in resistant cells.

MECHANISM OF PROFLAVINE RELEASE

Both sensitive and resistant E. coli are able to release bound proflavine after glucose is added, but the former can do so only if the amount bound is too low to inhibit glucose utilization. The fact that proflavine released during glucose metabolism may be taken up again after glucose disappears suggests that a continuous supply of energy is needed to keep this dye out of the cell.

The effects of certain metabolic inhibitors on glucose-induced proflavine release were studied. These agents included cyanide, 2,4 dinitrophenol, carbonyl cyanide m-chlorophenylhydrazone (CCCP), and sodium azide (Mehta et al., 1973). Chloramphenicol (100 ug/ml) did not inhibit proflavine release from resistant cells after the addition of glucose, ribose or glycerol; hence, proflavine release does not depend on protein synthesis. When proflavine prevented glucose utilization by sensitive cells, it also prevented its own release. The uncoupling agent, 2,4 dinitrophenol (5×10^{-3} M) prevented both glucose metabolism and dye release.

Sodium azide can inhibit cytochrome oxidase and adenosine triphosphatase and act as a proton conductor. CCCP and other uncouplers can prevent the active transport of cations into the cell (Harold, 1968). Sodium azide (10^{-1} M), and the uncoupling agent CCCP (10^{-4} M), prevented proflavine release

while permitting glucose utilization. The latter seems necessary, but not sufficient, for proflavine release.

The mechanism of proflavine release is still unknown. It was thought possible that metabolic energy stimulated transport of metallic cations into the cell thus displacing bound proflavine. Uncoupling agents could presumably block such transport. However, glucose released proflavine from resistant cells suspended in buffer which contained no metallic cation (Kushner and Khan, 1968; Mehta et al., 1973).

Kushner and Khan (1968) showed that resistant cells could grow while binding enough proflavine to inhibit the growth of sensitive cells. This suggests that the ability to expel bound proflavine is not the only mechanism of resistance.

A comparison of the figures reported here and those published by Kushner and Khan (1968) indicate that proflavine levels which cause little or no inhibition of glucose metabolism do not inhibit growth; concentrations inhibiting glucose utilization by 80% or more completely inhibit growth. These findings suggest that proflavine inhibits the growth of sensitive cells primarily through its action on metabolic processes. Thus, resistance at the metabolic level may determine whether or not growth is inhibited. By diminishing the amount of dye available for binding to sensitive sites, metabolically dependent expulsion of bound dye would act as a further mechanism of defense.

Further studies in this laboratory have been initiated to determine the nature of proflavine inhibition of glucose metabolism in E. coli and to elucidate possible mechanisms

of resistance (McKellar and Kushner, personal communication). These have shown that proflavine does not inhibit any of the enzymes in the glycolytic pathway and Krebs cycle. Moreover, glucose breakdown by cell free extracts was unaffected by proflavine. Work in progress suggests that a proflavine stimulated decrease in ATP content is responsible for the inhibition of glucose metabolism in sensitive cells (McKellar and Kushner, personal communication).

McKellar and Kushner (personal communication) found that in addition to affecting cellular ATP levels, proflavine stimulated the rate of NADH oxidation by sensitive cells and may act as an uncoupling agent. Implications of these findings are being investigated further.

To sum up briefly, the experiments with spheroplasts reported here have shown that an osmotically supported membrane is not needed for proflavine binding by E. coli, nor does it present a barrier to such binding. However, an osmotically supported membrane does seem necessary for proflavine extrusion by spheroplasts of resistant cells. These findings support the conclusions put forward by Kushner and Khan (1968), namely, that E. coli cells are freely permeable to proflavine and that proflavine binding by E. coli is a passive process, while its release is an active energy-dependent process.

Degradative studies have shown that nucleic acids are not the only cellular binding sites for proflavine. These experiments also suggested that treatments which remove one

class of binding site may open up others. For this reason, increased uptake of acridines after various treatments does not appear to be a reliable index of enhanced cell permeability.

The experiments on glucose utilization have shown that proflavine itself acts as a metabolic inhibitor, affecting glucose metabolism more strongly in sensitive than resistant cells; and that when proflavine inhibits metabolism, it also inhibits its own release. Thus, when the amount of dye bound by sensitive cells is too low to inhibit glucose metabolism, sensitive cells may also release bound proflavine.

The mechanism of the metabolite-induced release of bound proflavine (which is not the primary mechanism of resistance) is still unknown. Neither cations nor protein synthesis are required for such release to occur. The nature of the metabolic site responsible for sensitivity to proflavine is also unknown, and is currently being investigated.

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APPENDIX

Media Formulations:

1) Media of Davis and Mingioli (1950)

K_2HPO_4	0.7%
KH_2PO_4	0.3%
Na	0.05%
$MgSO_4.7H_2O$	0.01%
$(NH_4)_2SO_4$	0.1%
Glucose (autoclaved separately)	0.2%

pH: 7.0

2) Media of Beppu and Arima

KH_2PO_4	0.3%
$MgSO_4.7H_2O$	0.002%
$Na_2HPO_4.2H_2O$	0.88%
NH_4Cl	0.1%
Trace elements solution *	0.1%
Glucose (autoclaved separately)	0.4%

*Trace elements solution:

$ZnSO_4.7H_2O$	880mg
$FeCl_3.6H_2O$	970mg
$CuCl_2.2H_2O$	270mg
$MnCl_2.4H_2O$	72mg
$(NH_4)_6Mo_7O_{24}.4H_2O$	37mg
$Na_2B_4O_7.10H_2O$	88mg
H_2O	1000ml

pH: 7.0

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IN APPENDIX, NOT MICROFILMED.

The Elusive Permeability Barriers and Binding Sites for Proflavine in Escherichia coli, by M. Joan Gravelle, B.M. Mehta, and D.J. Kushner, Department of Biology, University of Ottawa, Ottawa, K1N 6N5, Ontario, Canada. Antimicrobial Agents and Chemotherapy, June 1972, Vol. 1, No. 6, pp. 470-475. American Society for Microbiology, 1972.

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