

**STUDIES ON THE BIOCHEMISTRY
OF DISINFECTION BY MONOCHLORAMINE**

**Thesis by
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ABSTRACT

The chemical characteristics of monochloramine and some of its effects on Escherichia coli were studied. Under certain conditions monochloramine-treated cells recovered in the presence of excess thiols or thiosulfate. Such recovery could not be attributed to activation of sulfur containing respiratory enzymes.

A mechanism of the reaction of monochloramine with amino acids was proposed. The sulfur containing groups were most reactive, followed by the guanidino, amino, imidazoyl and indole groups, and purines and pyrimidines. Sugars (lactose, glucose, ribose) were very unreactive. It was estimated that most of the cellular reactivity with monochloramine was instantaneous, resulting from reactions with sulfur groups.

Monochloramination of resting cells inhibited: (a) the oxidation of glucose, succinate, acetate and pyruvate; (b) the fermentation of glucose, fructose-1,6-diphosphate and pyruvate; and (c) the dehydrogenation of succinate, lactate, formate and fructose-1,6-diphosphate. Fermentation was the most sensitive, and formate and lactate dehydrogenases the least sensitive. In general, inhibition of enzyme activity by monochloramine was less than loss in viability. The induction of β -D-galactosidase was considerably less sensitive than any systems tested to monochloramine.

Results of these investigations implicate no one biochemical site of monochloramine action. Attempts to isolate monochloramine induced auxotrophic and monochloramine-resistant mutants were unsuccessful.

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I. INTRODUCTION

For over fifty years chlorine and its derivatives have been used in water treatment and waste treatment operations to kill pathogens and reduce bacterial numbers to a safe and esthetic level. In this period of time many investigations of the bactericidal efficacy of chlorine, and the many variables that affect it, have been initiated. While considerable field and laboratory data of an empirical nature are available, little light has been shed on the mechanisms of the bactericidal or bacteriostatic action of chlorine and its derivatives. Indeed, the mode of action of chlorine in relation to bacterial physiology has remained elusive.

The more stable but less effective disinfectant, monochloramine, is predominant in chlorinated water and waste streams high in ammonia and is, thereby, the critical disinfectant in many water treatment facilities. In addition, preformed monochloramine is used as the basic disinfectant in some water treatment facilities. Knowledge of the chemical, physical and bactericidal or bacteriostatic properties of monochloramine is sparse. Recent indirect information has suggested that monochloramine may be a strictly bacteriostatic reagent. Obviously, this question is of significance to public health. Serious questions can be raised concerning the rationale for using an agent, potentially bacteriostatic, in water treatment, especially when chlorine per se is more bactericidal. In any event, lack of information and an understanding of this compound are cause for some concern in such matters.

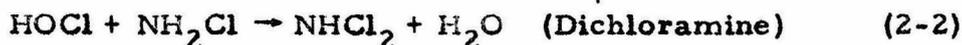
With these facts in mind, and in hopes of perhaps gaining insight into the mode of antibacterial action of monochloramine, a study was initiated to investigate in greater detail the chemical characteristics of monochloramine and some of its biological effects. The principal areas studied and reported herein dealt with: (a) bacterial recovery of monochloramine-treated cells; (b) chemical reactions between monochloramine and certain biologically important organic compounds; (c) the effects of monochloramine on a number of enzymes in resting cells of E. coli; and (d) mutagenic action of monochloramine.

Although chlorine and its derivatives are highly reactive with a great variety of biochemicals, so much so that, a priori, a specific mode of action seems a remote prospect, more definitive information than now exists is needed in each of the areas enumerated.

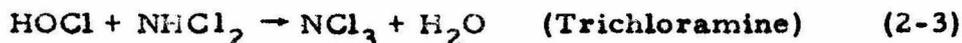
II. MONOCHLORAMINE - ITS PROPERTIES IN WATER AND SEWAGE

A. CHEMISTRY

Chlorine, or more specifically, its hydrolysis product, HOCl, in aqueous solution reacts with ammonia or similar nitrogenous compounds to form chloramines. The stepwise sequence of reactions with ammonia is as follows:



and in solutions of pH 4.4 or lower:



Morris and Weil (1) found that the formation of monochloramine (equation 2-1) is a second-order reaction, dependent upon the concentration of both ammonia and hypochlorous acid. The specific rate constant for the chlorination of ammonia is 3.7×10^8 liter mol⁻¹, min⁻¹ (1). Although the rate of formation of monochloramine is second-order at any fixed pH value, it is markedly dependent upon the hydrogen-ion concentration, being maximum at pH 8.3 at 25° C.

Monochloramine formation is greatly dependent upon temperature. Apparent activation energies, calculated from the Arrhenius expression, are 16.5 kilocalories at pH 4.58 and 11.0 kilocalories at pH 11.7. These energies correspond to a Q_{10} value of 2.5 at the low pH value and 1.9 at the higher value. The

rate of reaction is unaffected up to an ionic strength of 0.21 (1).

The basic ionization constant of monochloramine was found to be 1×10^{-15} (1). Thus at pH 1.0, 1% of the monochloramine would exist as the ion NH_3Cl^+ . The degree of ionization in water treatment, where pH ranges from 5 to 8, would be negligible.

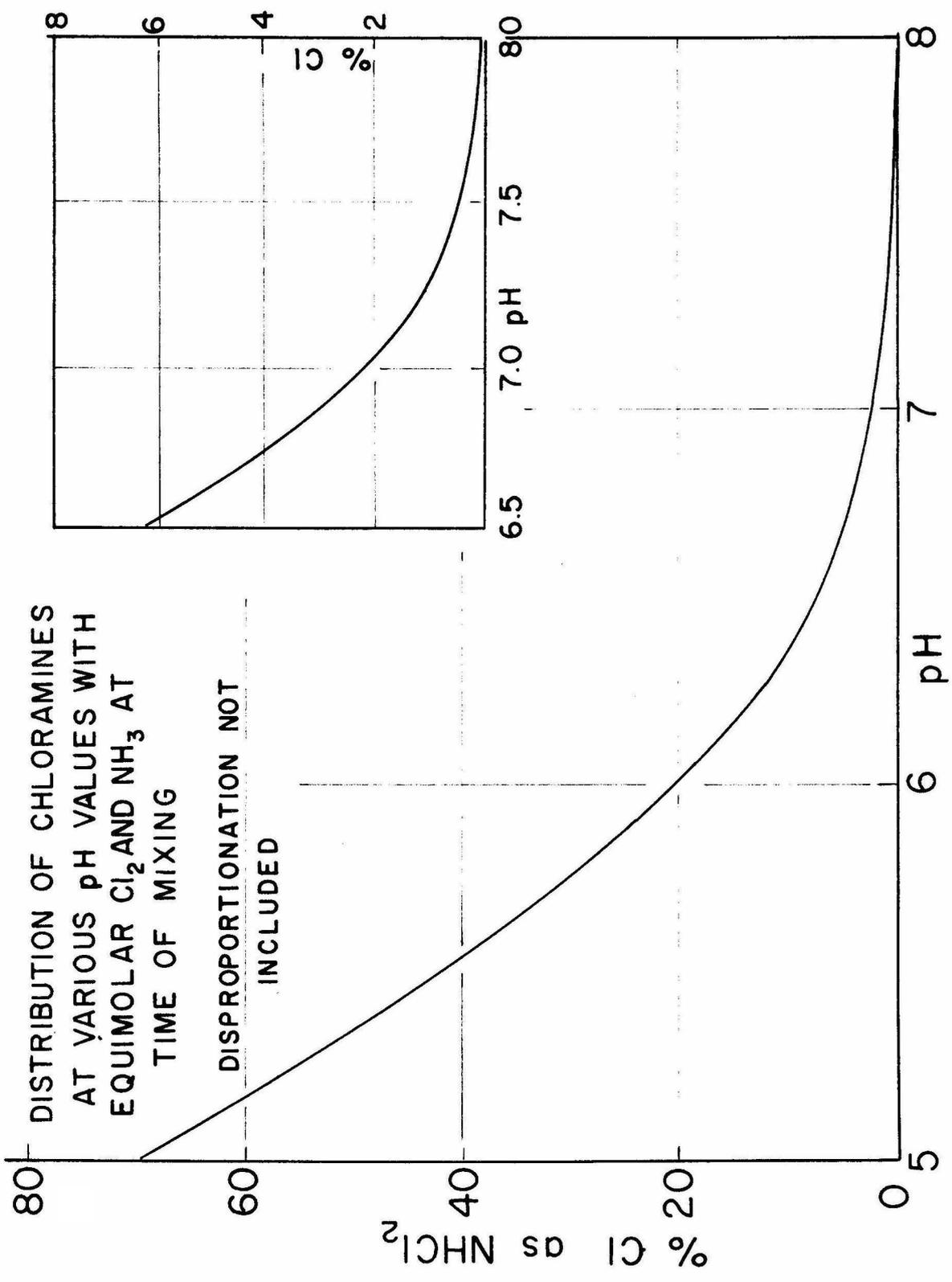
Nitrogen trichloride (trichloramine) appears to be formed principally below a pH of 4.0 and with high ratios of chlorine to ammonia. With respect to mono- and dichloramine, Chapin (2) found that below pH 5.0 only dichloramine is present, at pH 7.0 both compounds are present in about equal amounts, and above pH 9.0 only monochloramine exists. Morris and Weil (1) assumed that if the distribution Chapin found was a result of the equilibrium,



his results conformed to an equilibrium constant of 2.36×10^7 .

Palin (3) and Morris and Weil (1), working with concentrations found in water works practice, obtained a different distribution than that found at much higher concentrations by Chapin. They found that at low concentrations the relative proportions of mono- and dichloramine obtained depend on several factors. Upon mixing chlorine with ammonia, both mono- and dichloramine are formed as indicated above (equations 2-1 and 2-2). The relative rates of formation determine the initial relative concentrations, but those concentrations are not necessarily maintained (figure 2-1). Dichloramine is unstable and thus its decomposition

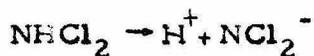
FIGURE 2-1
DISTRIBUTION OF CHLORAMINES
AT VARIOUS pH VALUES WITH
EQUIMOLAR Cl_2 AND NH_3 AT
TIME OF MIXING



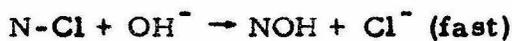
alters the relative amounts of mono- and dichloramine obtained from reacting chlorine with ammonia.

This decomposition of dichloramine causes the phenomenon known as breakpoint. Morris and Weil (1) proposed a mechanism for the decomposition of dichloramine as follows:

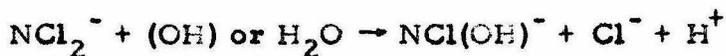
The dichloramine ionizes as a weak acid,



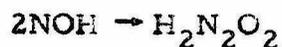
which then reacts with (OH^-) , either in accord with the reactions



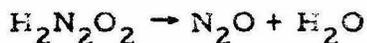
or by an alternate path,



Either pathway gives the same end product. It is likely that hyponitrous acid is formed,



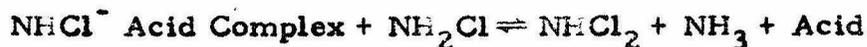
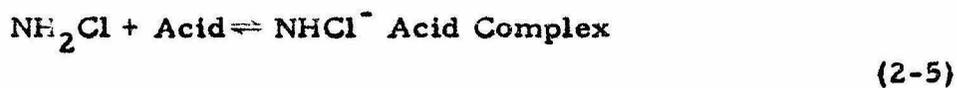
which decomposes slowly to give nitrous oxide,



An internal conversion of monochloramine takes place,



which shifts the relative concentrations of mono- and dichloramine. This internal conversion, known as a disproportionation, was studied by Granstrom (4) who found it to occur in two parallel and independent pathways, one a first-order reaction and the other a second-order reaction:



The first-order reaction (equation 2-4) is not pH dependent, whereas the second-order reaction is dependent on pH and buffer concentrations. The overall rate of disproportionation is second-order at low temperatures and low pH values. At high temperature (40 to 50° C) and relatively high pH values, the first-order reaction predominates.

B. FACTORS AFFECTING THE BACTERICIDAL ACTION OF CHLORINE AND ITS DERIVATIVES

1. Rates of Disinfection

Initially it was believed by many investigators that the rate of disinfection by chlorine and its derivatives followed Chick's Law (5):

$$\log \frac{N}{N_0} = -kt \quad (2-6)$$

where $\frac{N}{N_0}$ is the fraction of the original number of organisms at time t , and k is a proportionality constant. Such a relationship would yield a straight line if log survivors were plotted against time. However, survival curves obtained by several investigators (6-8) departed considerably from a linear relationship. This was true for spores and vegetative cells with both chlorine and chloramines. Careful studies by Rahn (9) and by Jordan and Jacobs (10) conclusively showed that disinfection rates with chlorine and its derivatives could not be expressed by first-order kinetics. They found that at high pH values or low concentrations of chlorine, the distribution of resistance was approximately normal. When plotting log survivors versus time they obtained a curve whose lower portion was approximately linear.

Fair et al. (11) have shown that linear relations are obtained if log survivors were plotted against the time squared. They proposed that a relationship of this nature could be explained: (a) as a combination of slow diffusion through the cell wall with a rate of kill proportional to the concentration of chlorine in a cell; or (b) on the assumption that there are several active centers in the organism and that the organism is not dead until all of these centers have been destroyed (9).

2. Effect of Temperature

If either the rate of diffusion through the cell membrane or the rate of reaction with an enzyme determines the rate of

disinfection, its variation with temperature is best expressed by the equation:

$$\log \frac{t_1}{t_2} = \frac{E(T_2 - T_1)}{4.575 T_1 T_2} \quad (2-7)$$

T_1 and T_2 represent the two temperatures in degrees Kelvin, t_1 and t_2 are the times required for equal percentages of kill at a fixed concentration of disinfectant and E is called the activation energy. Activation energies for chlorine and its derivatives increase with increasing pH and are greater for chloramines than for hypochlorous acid (11).

3. Effect of pH

The pH value plays an important role in the bactericidal action of chlorine compounds insofar as the ionization of hypochlorous acid is concerned. The concept that the undissociated hypochlorous acid molecule might be the active agent in solutions of chlorine probably originated with Holwerda (12). Fair et al. (13) compared values found experimentally for residual concentrations of chlorine required to kill cysts of Endamoeba histolytica in a given time and at various pH values with those computed from the equation:

$$R = A \frac{1 + \frac{K}{(H^+)}}{1 + \frac{BK}{(H^+)}} \quad (2-8)$$

where R is the required total chlorine, A is the concentration of HOCl alone required to produce the necessary kill, B is the ratio

of the efficiency of OCl^- ion to that of HOCl , K is the ionization constant for HOCl and (H^+) is the hydrogen ion activity (figure 2-2). Values for the ionization constant for HOCl obtained by critical analysis of all existing data are 2.0×10^{-8} at 0°C and 3.3×10^{-8} at 20°C (13).

Equation 2-8 describes a sigmoid curve when plotting the log of the chlorine concentration against pH (figure 2-2). Experimental data with E. histolytica, Bacillus meitens and Escherichia coli verified the hypothesis that hypochlorous acid (HOCl) is the main bactericidal agent in chlorine solutions. It has been shown that HOCl is approximately 80 times more bactericidal than OCl^- (13). One might attribute this phenomenon to the low isoelectric point of bacteria (generally less than 5), resulting in a high density of negative charges on the cell at higher pH values.

It is not so easy, however, to conceive of such a simple explanation for the effect of pH on monochloramine action in the presence of excess ammonia. The work of Butterfield and Wattie (7) indicates that the rate of disinfection of bacteria with monochloramine is affected by pH in a manner similar to that of free available chlorine (figure 2-3). It is important to note, however, that ammonia nitrogen was held constant in these experiments; thus the chlorine to nitrogen ratio increased with an increase in dosage. The studies of Granstrom (4) indicate that pH and excess ammonia are critical with respect to the hydrolysis of monochloramine:

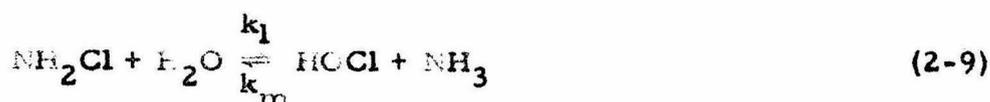


FIGURE 2-2
CONCENTRATIONS OF CHLORINE AS HOCl REQUIRED TO
KILL CYSTS OF E. histolytica AT 18° C

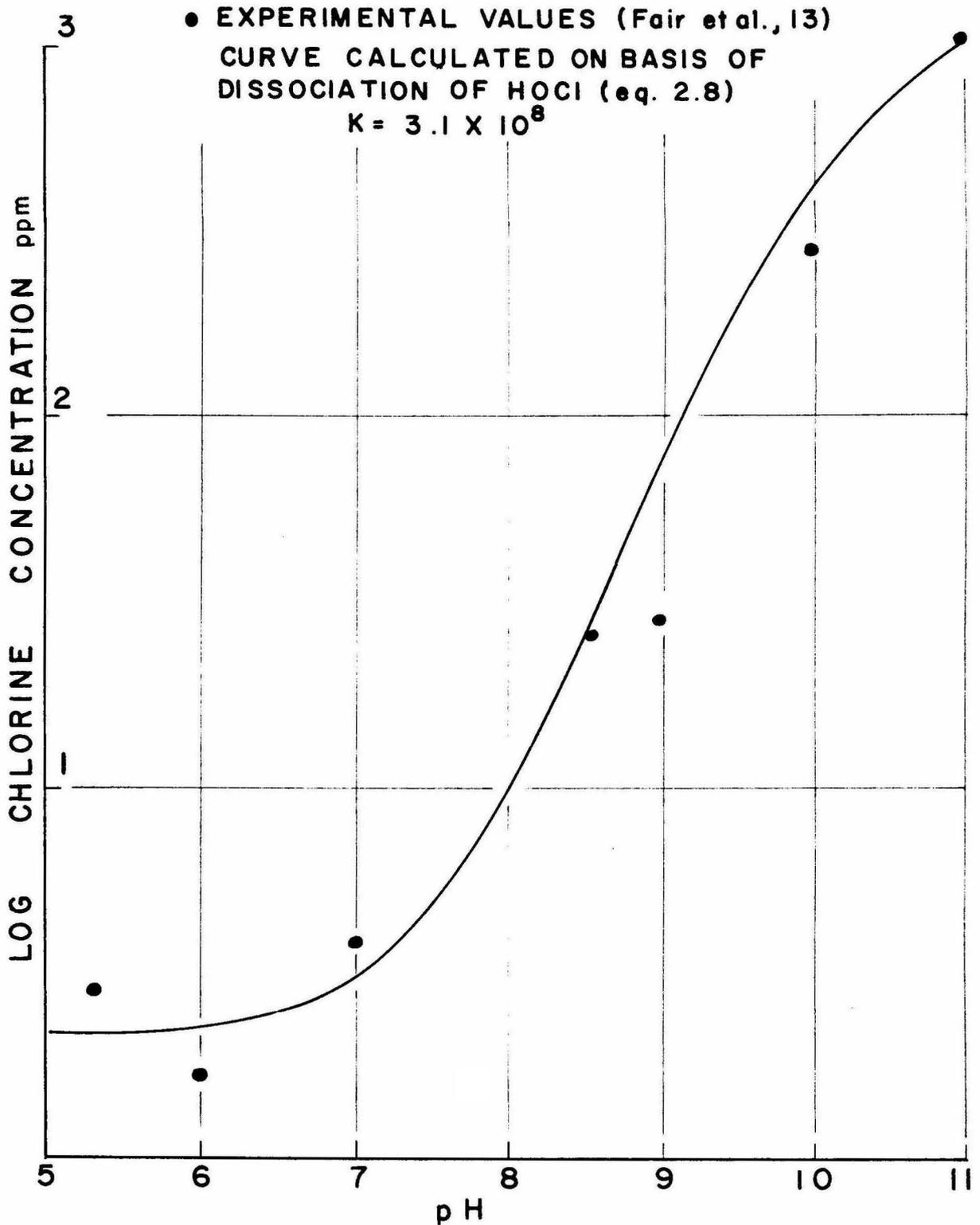
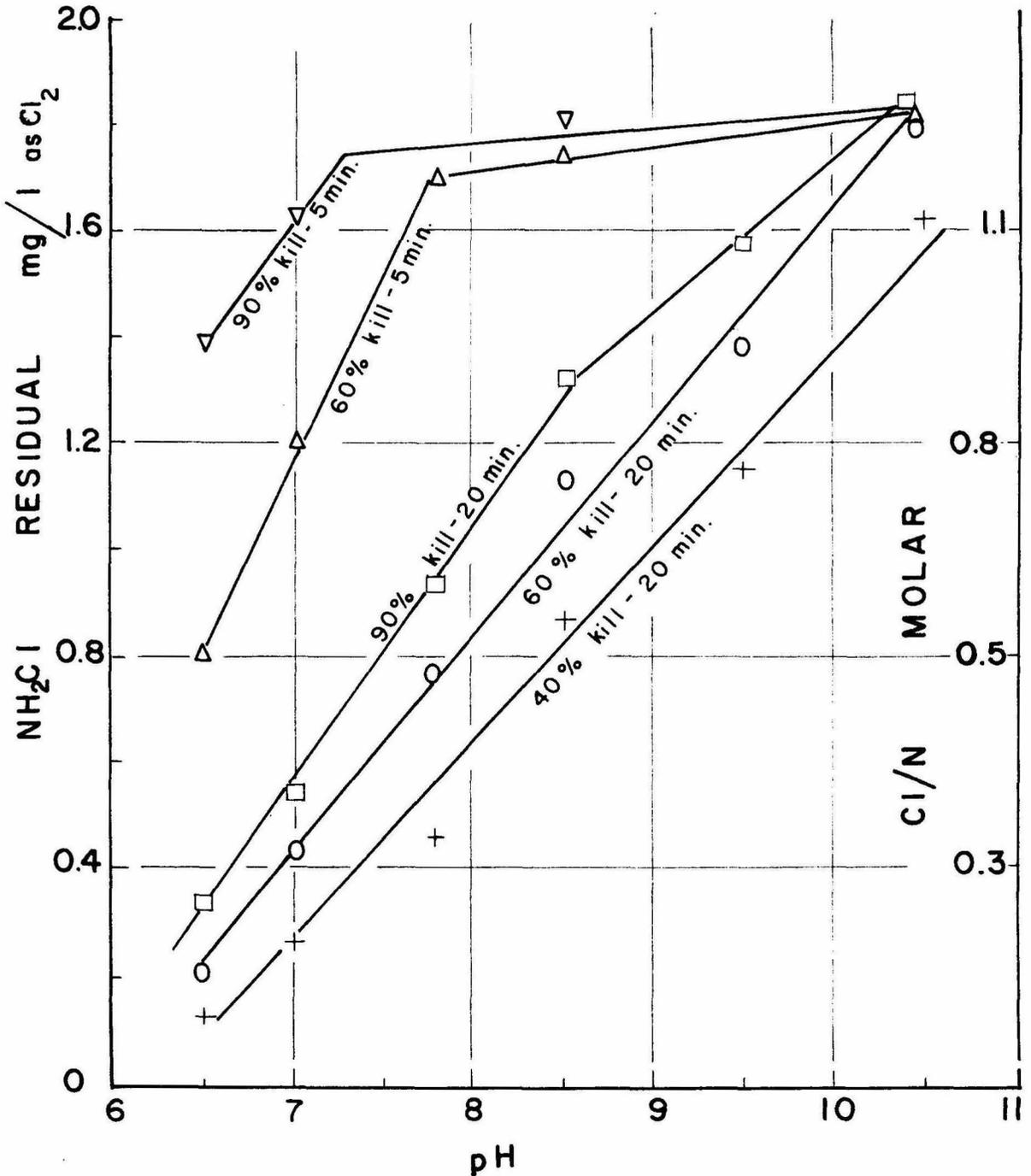


FIGURE 2-3
EFFECT OF pH ON BACTERICIDAL
EFFICIENCY OF MONOCHLORAMINE AT 25°C

E. COLI CELLS

NH₃-N constant = 0.3 mg/l



Using rate constants, k_m , derived by Morris and Weil (1) and k_1 derived by his experiments, Granstrom showed that $k_h = \frac{k_1}{k_m} = 9.0 \times 10^{-2} e^{-14,100/RT}$. He adjusted this constant in order to take into account the ionization equilibria of HOCl and NH_3 . This new equation gives the total free chlorine and ammonia-nitrogen in equilibrium with monochloramine:

$$k_h' = \frac{C \cdot N}{[NH_2Cl]} = k_h \left(1 + \frac{(H^+)}{k_a}\right) \left(1 + \frac{k_i}{(H^+)}\right) \quad (2-10)$$

where C is the concentration of total free available chlorine in moles/liter, N is the ammonia-nitrogen in moles/liter, k_i is the acid dissociation constant of HOCl, and $k_a = \frac{k_w}{k_b}$ where k_w is the ion product of water and k_b is the basic dissociation constant of ammonia. Values of k_i , k_a , k_h and k_h' for selected pH and temperatures are shown in table 2-1.

The concentration of HOCl present in monochloramine solutions at various pH values and ammonia excesses can be calculated using equation 2-10. Selecting the data points from figure 2-3 for the 40% kill in 20 minutes, the concentrations of HOCl are computed (table 2-2). The HOCl concentrations are negligible in this case throughout the entire pH range studied. It is of interest to note, however, that these values initially decrease with increased chlorine residual, after which they increase. This arises from: (a) the diminution of excess nitrogen which shifts equation 2-9 to the right and increases the HOCl concentration; and (b) an increase in the hydrolysis constant at about pH 9, further shifting the reaction to the right. (No computation was made in this analysis for the ionization of HOCl, but at the higher pH values the predominant form would be OCl^- .) This analysis indicates that the effects of pH on the efficacy of monochloramine disinfection are not due to hydrolysis to HOCl.

The second-order reaction of disproportionation (equation 2-5) might also be implicated in this analysis. However, close

TABLE 2-1

HYDROLYSIS CONSTANTS FOR NH_2Cl^*

Temp. °C	$K_h \times 10^{12}$ mol/l	$K_i \times 10^8$ mol/l	$K_a \times 10^{10}$ mol/l	5	6	7	8	9
0	0.47	1.5	0.83	57	5.8	0.65	0.141	0.096
10	1.2	2.0	1.9	65	6.6	0.77	0.20	0.163
20	2.8	2.6	4.0	70	7.2	0.88	0.26	0.27
25	4.2	2.9	5.7	74	7.6	0.96	0.30	0.35
30	6.1	3.2	8.1	76	7.8	1.01	0.34	0.45
40	13.	3.5	16.	83	8.6	1.14	0.44	0.78

$$K_h = \frac{[\text{HOCl}][\text{NH}_3]}{[\text{NH}_2\text{Cl}]} ; K'_h = K_h \left(1 + \frac{(\text{H}^+)}{K_a}\right) \left(1 + \frac{K_i}{(\text{H}^+)}\right) = \frac{C \cdot N}{[\text{NH}_2\text{Cl}]}$$

* From Granstrom (4).

TABLE 2-2

HYDROLYSIS OF NH₂Cl FOR VARIOUS NH₃ EXCESSES

T = 25°C
 NH₂Cl = Amt. for 40% Kill in 20 Min.
 NH₃ = 0.3 mg/L = 2.14 × 10⁻⁵ M
 N' = 2.14 × 10⁻⁵ - NH₂Cl

pH	NH ₂ Cl mg/L	NH ₂ Cl 10 ⁻⁶ mols/L	N'	k' _h	10 ⁻⁹ mols/L	C*	C*
			10 ⁻⁶ mol/L		10 ⁻⁹ mols/L	10 ⁻¹⁰ mols/L	10 ⁻⁵ mg/L
6.5	0.13	1.83	19.6	2.54	2.35	1.67	
7.0	0.27	3.66	17.8	0.96	1.95	1.38	
7.8	0.47	6.61	14.8	0.36	1.60	1.14	
8.5	0.88	12.68	8.7	0.28	4.00	2.84	
9.5	1.16	16.40	5.0	0.60	19.50	13.84	
10.5	1.63	22.95	-	4.04	3,045.0	2,162	

$$k'_h = \frac{C \cdot N}{[\text{NH}_2\text{Cl}]}$$

$$N = (C + N')$$

$$N' = (\text{Constant NH}_3 - \text{NH}_2\text{Cl})$$

$$\therefore C = \frac{-N' \pm \sqrt{(N')^2 + 4k'_h [\text{NH}_2\text{Cl}]}}{2}$$

* HOCl concentration instantaneously - does not take into account the ionization of HOCl.

inspection of the derivation of this rate constant indicates that it is important only at rather low pH values (< 5.0). In addition, any dichloramine present would probably go through the breakpoint reaction rather quickly. The ionization constant of NH_2Cl is slight and is probably not an important factor in the variation of its bactericidal activity with pH.

The preceding analysis is very important in visualizing the overall reaction of monochloramine with bacterial cells. In the past, the effect of pH on bactericidal efficiency was attributed to the hydrolysis of NH_2Cl to the principal disinfecting agent, HOCl . The detailed study of the disproportionation of NH_2Cl , however, has refuted this theory and once more opened up the question of pH effects. It could be argued that cells are "sensitized" to NH_2Cl by pH changes, perhaps in a manner similar to the phenomenon that renders cells more heat resistant at higher pH values than at lower ones (14). The matter of sensitization is purely conjecture at this time, although it might be suggested that the isoelectric points of many of the proteins and other complex organic molecules within cells may play an important role. It is also conceivable that pH affects the permeability of the cells, possibly imposing permeability barriers at the higher values.

It is of considerable interest to interject at this point the findings of Friberg (15), who used radioactive chlorine to study the absorption of this compound on bacterial cells. He found that greater quantities of free chlorine, in the reduced form, are tied up on or in bacterial cells at higher pH values than at lower ones.

This phenomenon was observed whether cells were viable or heat killed. Similar experiments using chloramines, however, revealed that no reduced chlorine was bound to the cells even after a 30-minute exposure at any pH.

4. Effect of Concentration of Disinfectant

Watson (16) has shown that the effect of concentration of a disinfectant on the death rate can be expressed empirically by $C^n \times K = T$, where C is the concentration, T is the time for a constant percentage killed, and n is a numerical value which depends upon the disinfectant, the organism and the temperature. The value of n characterizes the disinfectant with respect to dilution, i. e., high n values indicate that the disinfectant decreases rapidly in effectiveness with dilution. Chlorine and monochloramine have relatively low n values of about 0.5 to 1.5.

On the basis of experiments by Butterfield et al. (6-8) with various coliforms, one finds that about 1.4×10^{11} molecules of hypochlorous acid per organism are required for lethality at pH 7.0 at 20°C with a bacterial population of about 10^4 organisms per ml. Similarly, one finds the figure for monochloramine to be about 3×10^{12} .

5. Effect of Cell Concentration

Little is known about the effect of cell concentrations on germicidal efficiency of chlorine and its derivatives. Butterfield (6) claimed that with initial cell concentrations up to 50,000 per ml there was no increase in demand for chlorine for a given per cent kill. Knox et al. (17) reported that more chlorine was

needed for a constant per cent kill as cell densities were increased, but that the ratio of chlorine to cells remained constant for a given time of kill.

6. Effect of Growth Conditions of Treated Organisms

Milbauer and Grossowicz (18) found that E. coli cells grown in nutrient broth were more resistant than those grown in minimal media. Yeast extract in minimal media increased the cells' resistance to a level comparable to that of cells recovered from nutrient broth. Nitrogen starvation had no effect on the cells' resistance to chlorine. Ingols et al. (19) showed that the sensitivity to chlorine was enhanced by placing bacterial cells in distilled water two hours before chlorinating.

7. Effect of the Presence of Chemical Substances

The several investigations of the reaction of hypochlorous acid with organic materials have found that the high bactericidal reactivity of chlorine is easily reduced by foreign organic matter found in natural waters. Palin (3) studied the reaction of chlorine with amino acids and proteins and found that at pH 7.0 monochlor-amino acids and traces of dichloramino acids usually resulted. The stability of these derivatives varied with the amino acid. A plot of chlorine residual versus amino nitrogen resulted in curves similar to the breakpoint curves of chlorine in water systems. Friend (20) studied the kinetics of the chlorine-amino acid reactions at pH 7.0. He found that, in general, weak bases were most reactive to chlorine (30 times faster than ammonia), whereas

amides were quite slow to react. His figures showed that for a typical domestic waste, more than 50% of the chloramines formed in the waste were organic chloramines of little or no bactericidal effectiveness.

Of the non-nitrogenous organic compounds tested by Guiteras and Schmelkes (21), which included glucose, levulose, mannite, maltose, lactose, sucrose, raffinose, cholesterol and sodium oleate, only levulose consumed an appreciable amount of chlorine. Allen and Brooks (22) found the chlorine demand of 0.005% sodium palmitate, 4% sodium acetate, 0.1% glycerol, 0.1% starch, 0.1% acetaldehyde, 0.1% furfural, 2% methyl alcohol, 2% ethyl alcohol, 2% lactose and 2% glucose to be less than 1 mg per liter. Substances with appreciable demands were acetoin, ascorbic acid, and catechol. Chlorine is also quite reactive with phenol, indigo and aniline (22).

There has been very little information reported in the literature regarding the interaction of monochloramine with organic materials. Langheld (23) and Dakin et al. (24) found that hypochlorites and chloramine-T react with amino acids to form mono- and dichloro substitution products according to the concentration of chlorine and the number of hydrogen groups directly attached to the nitrogen. Dakin et al. suggested that there was no difference in the reactions, other than rates, of hypochlorite or chloramines with amino acids. However, he reported that chloramines did not react with aniline, indigo and phenol.

Although no supporting data were furnished, Ingols et al. (19) reported that monochloramine reacts with: (a) alanine to yield a rather stable monochloroalanine; (b) cysteine to form cystine; (c) peptides to form terminal monochloro-derivatives; and (d) hemin to form an irreversible addition or oxidation product.

C. RELATIVE BACTERICIDAL EFFICIENCY OF CHLORINE AND CHLORAMINES

From all experimental results reported, it is evident that the biologically destructive effect of chloramine is very much less than that of hypochlorite. The relative merits of each in water-works practice have been the subject of controversy for many years. Rideal (25) reported in 1905 that in polluted water, the addition of ammonia to hypochlorite greatly increased the disinfectant action of chlorine. He found that the phenol coefficient of 2.18 for a solution of 1% available chlorine was increased to 6.36 by addition of ammonia. Race (26) also found these results in an application of ammonia or hypochlorite to a water supply system. He noted that with the addition of ammonia, bacterial aftergrowths decreased and taste and odors were completely eliminated. Since hypochlorites are much more reactive with organic matter than chloramines, it follows that the disinfecting power of the more potent disinfectant is greatly reduced in the presence of certain types of organic matter. The resultant organic chlorinated compounds are to a large extent not bactericidal and exert little or no effect on the reduction of organisms. Under such conditions

chloramine may be a much more effective germicide than chlorine alone. In addition, chloramines are much more stable than the highly reactive chlorine and, thereby, would persist for longer periods of time.

D. MECHANISMS OF BACTERIOSTATIC OR BACTERICIDAL ATTACK BY CHLORINE AND ITS DERIVATIVES

At one time the disinfectant action of chlorine was believed to result from the liberation of nascent oxygen from hypochlorous acid, which then oxidized cell protoplasm (27). This theory appeared to be contradicted when it was discovered that small concentrations of chlorine were much more lethal than corresponding concentrations of other strongly oxidizing substances (26).

Green and Stumpf (28) and Knox et al. (17) have stated that the powerful bactericidal character of chlorine suggests operation of an "enzyme-trace substance theory." This theory states that whenever a substance influences biological reactions in trace amounts it must be part of an enzyme or influence or modify an enzymic reaction. On the basis of this theory and certain experimental results, these workers have attributed the bactericidal effect of chlorine to the irreversible oxidation of a sulfhydryl enzyme essential in glycolysis. Analysis of respiration of glucose by cells treated with chlorine showed a degree of parallelism between the inhibition of glycolysis and death of the cells. They further observed that the minimal concentration of chlorine required to give 100 per cent inhibition of glucose oxidation by a bacterial cell suspension always sterilized the

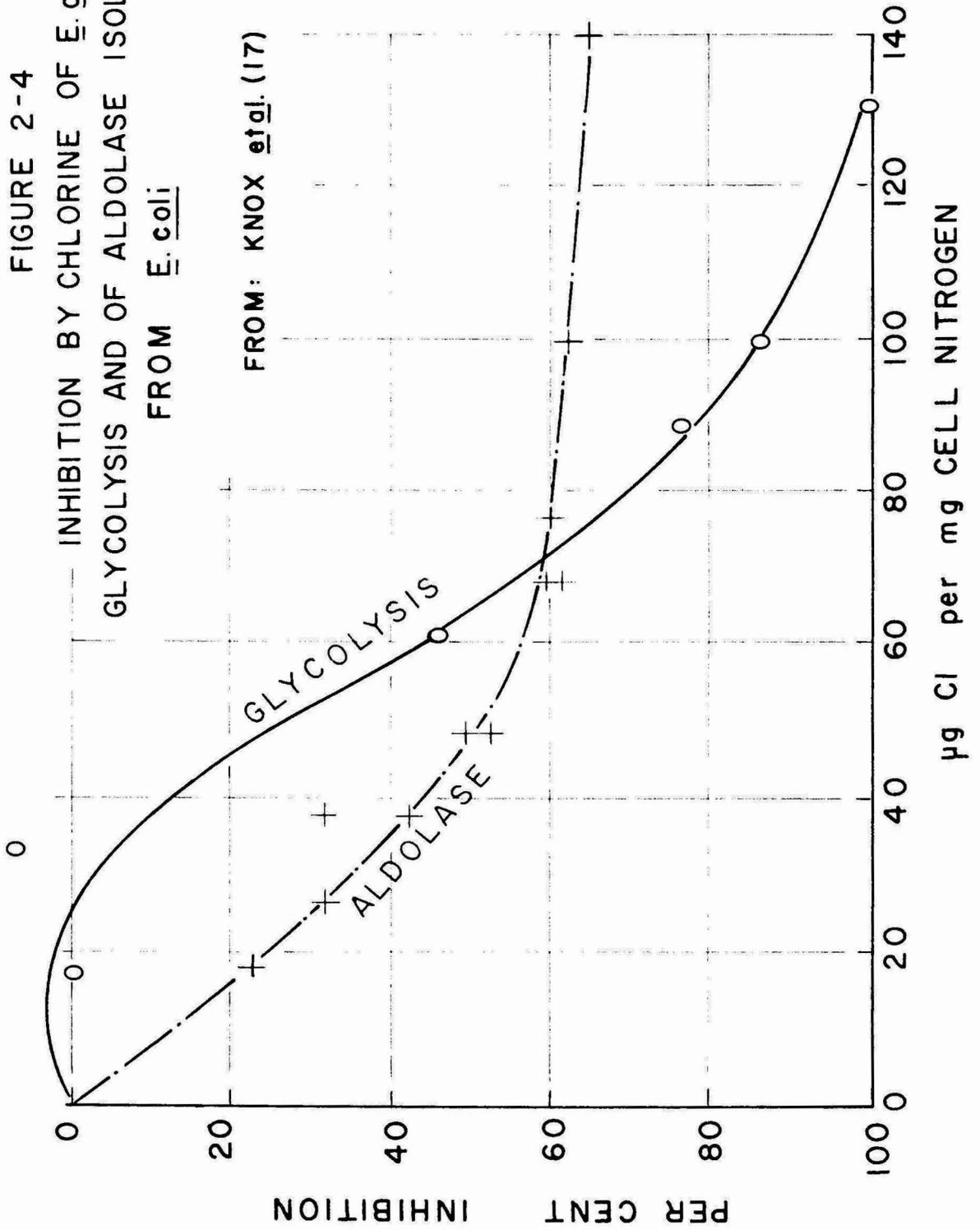
suspension. The time required for the glucose oxidation enzymes to be inhibited was also identical with the time required for complete killing. All attempts to reverse this reaction by the addition of cysteine or glutathione were unsuccessful.

Knox et al. (17) studied the reaction of chlorine with several known sulfhydryl enzymes, including the aldolase-triose phosphate dehydrogenase system from rabbit muscle, D-amino acid oxidase, transaminase and succinic oxidase; catalase, a non-sulfhydryl enzyme, was tested also. They found these systems, with the exception of catalase, to be sensitive to low concentrations of chlorine and, thereby, they categorized chlorine as a sulfhydryl enzyme inhibitor.

Of the sulfhydryl enzyme systems occurring in E. coli, Knox et al. reasoned that the aldolase-triose phosphate dehydrogenase system in the glycolysis chain was most critical to cell integrity and function. They found that only one-fifth as much HOCl was required completely to inactivate the aldolase-triose phosphate dehydrogenase system isolated from rabbit skeletal muscle as was required to inhibit glucose oxidation of E. coli. This system appeared to be much more sensitive to chlorine than other isolated animal sulfhydryl enzymes.

The aldolase enzyme was isolated from Escherichia coli and partially purified by these investigators. In figure 2-4, the chlorine inhibition of this enzyme is presented along with the inhibition of glycolysis in resting cells of E. coli. Aldolase activity was analyzed by measuring inorganic phosphorous from

FIGURE 2-4
INHIBITION BY CHLORINE OF *E. coli*
GLYCOLYSIS AND OF ALDOLASE ISOLATED
FROM *E. coli*



triose phosphate cyanhydrin exposed to "N soda" and $Mn SO_4$ for 20 minutes (28). It was claimed that both systems had about the same degree of sensitivity; deviations were explained on the basis of the properties of the in vitro system in which the enzyme activity was measured.

Extending their theory to bacterial spores, Knox proposed that spores are inhibited by mechanisms other than those of vegetative cells, as spore survival does not depend upon the ability to oxidize glucose. Concentrations of chlorine that inhibited glucose oxidation in vegetative cells also inhibited glucose oxidation in spores, but these spores were not killed. This suggests that the spore is able to regenerate the affected enzyme, or that it does not rely upon this oxidative pathway for growth. While recognizing that chlorine is reactive at many sites within the bacterial cell, these researchers felt that the inactivation of aldolase is the primary cause of death from chlorine.

Since he found that cysteine would not reactivate monochloraminated cells, Ingols et al. (19) suggested that, at least for monochloramine, the sulfhydryl group is not the locus of any critical reaction. He argued that hemin may be the critical site, as monochloramine reacts with it irreversibly. He has also theorized that catalase may be destroyed by monochloramine.

Marks, Wyss, and Strandkov (30) studied the action of the monochloro-derivatives of several nitrogenous compounds by varying the concentration of the nitrogen compound while holding the concentration of chlorine constant. They believed that, at

equimolar concentrations, death resulted from the sum of the action of the hydrolyzed hypochlorous acid and the intact monochloro molecule. At higher concentrations of the nitrogen compound the hydrolysis equilibrium was shifted to the left, thereby decreasing the amount of hypochlorous acid and the disinfecting action of the system. A point was reached where further additions of the nitrogen compound had no effect upon the disinfecting action. The killing action at that point was ascribed to the unhydrolyzed N-chloro compound. The disinfecting action of the various N-chloro derivatives varied widely with the compound tested and the pH of the medium.

In addition to enzyme inactivation, Fair et al. (13) have raised the question of the ability of chlorine to penetrate the cell membrane. Other oxidative agents, such as potassium permanganate, will react in a similar manner with enzyme systems but do not exhibit as marked an effect as does chlorine on the cell. Laboratory studies (13) indicate that it is this phenomenon, one of penetration, which largely determines the rate of disinfection and relative efficiency of the disinfectant. Thus, Fair et al. believed hypochlorous acid to be a superior disinfectant because of its small size and neutrality which promote cell penetration.

Hess et al. (30) attempted to derive from Chick's Law a theoretical explanation of the deviation of death curves with chlorine. Their equations are based on the diffusion of toxic

materials into bacterial cells where the susceptible sites are presumed to be randomly distributed. Although the theoretical curves fit certain laboratory and field data, the validity of such a theory has not been established.

E. AFTERGROWTHS, BACTERIAL REACTIVATION, AND CHLORAMINES

Occasionally a great increase in bacterial population is observed after sewage has been treated and discharged into nearby streams (32, 33, 34, 35, 36). These increases are generally preceded by a lag of many hours after discharge. It is not clear at present whether such increases result from the proliferation of surviving organisms, the emergence of previously protected organisms, or to the resuscitation of partially blocked cells.

Heukelekian (32) observed that when chlorinated sewage is diluted with stream water, a significant increase in coliform organisms takes place, generally within 24 hours following a lag period. He found greater coliform increases to accompany higher chlorine residuals provided that all chlorine residual was lost within 24 hours. Similar increases were not found, however, when chlorinated samples were diluted with sea water.

In work with peptone water and sewage, Allen and Brooks (22, 36) found that additions of ammonium sulfate (250 mg per liter) prior to chlorination yielded survivor curves which were reversible after several hours, i. e., a marked recovery in cell

count occurred. In work with E. coli in raw sewage Allen and Brooks (36) found that chlorination below the amount needed to satisfy the chlorine demand resulted in definite recovery of organisms in less than 12 hours. The rate of recovery was markedly decreased as chlorine doses approached the chlorine demand of the waste, but the final population eventually approached the initial density. Allen and Brooks (36) also found that with low doses of chlorine in sewage, recovered populations attained only modest levels of growth, whereas at higher doses the levels were much higher after a considerable lag. These phenomena were explained on the basis of the presence of protozoa which were completely killed at these higher doses. Pure cultures of Proteus sp. inoculated in sterile sewage and chlorinated to the ten minute demand showed recovery in eighteen hours to three days and regained their original density level.

Milbauer and Grossowicz (37) have shown that resting cells of E. coli B/r⁴ recovered more readily when chlorinated in the presence of ammonium sulfate. The extent of recovery could be enhanced if cells were initially grown in rich liquid media. Recovery was estimated on the basis of the ratio of nutrient agar plate counts to minimal agar plate counts. They hypothesized that high concentrations of cells released factors which induced the recovery of injured cells.

Upon comparing dilution counts with membrane filter counts in chlorinated wastes, McKee et al. (38) found the former to give higher counts of coliform bacteria than the latter method. They

reasoned that this discrepancy might reflect the bacteriostatic action of monochloramine, the effect being more pronounced in one medium than in the other.

Heinmetz et al. (39) reported that after chlorine or heat treatment a high percentage of E. coli could be revived by incubation in mixtures of Krebs-cycle metabolites. This action was interpreted to be an indication that chlorine exerts a bacteriostatic action through reactions with numerous enzymes.

In a series of well designed experiments, Garvie (40), Hurwitz et al. (41), and Chambers et al. (42, 43, 44, 45) independently tested the validity of Heinmetz's interpretation. They found that any increases of organisms could be explained as the proliferation of survivors, and that reactivation could not be implicated as a factor in increased cell density.

The occurrence of aftergrowths seems to be the result of a temporary upset in the natural balance in sewage and water. There is not, at present, sufficient evidence in the literature to suggest reactivation as the chief cause of these increases in coliforms. Other causes include: (a) the destruction of predatory forms of life; (b) the breaking up of protecting material; and (c) temporary increases in the amount of nutrients.

III. METHODS AND MATERIALS

This section covers a discussion of all basic materials and experimental procedures used throughout the entire study. Owing to the variety of methods used in this multifaceted study, the section is divided into a description of general procedures and a detailed presentation of methods and materials used in each phase of the study.

A. PREPARATION OF MONOCHLORAMINE

For most experimental work with monochloramine it was essential to use water with little or no chlorine demand. To achieve this, enough chlorine was added to distilled-deionized water in large carboys to yield a concentration of about 2 mg/liter. The chlorinated water was stored about one week to insure the loss of chlorine-demand materials. Sodium thiosulfate was then added until only a slight chlorine residual remained, and the entire carboy was autoclaved for 1 hour at 121° C.

Aqueous chlorine solutions were prepared by bubbling chlorine gas into chlorine-demand-free water until it showed a pale yellow-green color. This solution was immediately titrated and used in the preparation of monochloramine solutions.

Monochloramine solutions of about 2×10^{-3} M were prepared by adding, with rapid stirring, appropriate volumes of freshly prepared chlorine solution to 10% molar excesses of aqueous ammonium sulfate. Prior to mixing, the chlorine solution was adjusted to pH 11.0 with 1 N sodium hydroxide, and the final mixture was again readjusted, if necessary, to a pH of 10.5.

Preparation in this manner prevented the formation of dichloramine. Moreover, any dichloramine that might have formed at the relatively high pH would immediately decompose, thus insuring reasonably pure monochloramine solutions (4). The monochloramine in the stock solution, stored in amber flasks at room temperature, was found to decompose at a rate of about 10% per week. No solution was used for a period longer than two weeks.

B. MONOCHLORAMINE ASSAY

Monochloramine was measured by the acid thiosulfate titration method using starch as an indicator (46). Sodium thiosulfate of approximately 0.01 N concentration was standardized daily with 0.015 N potassium iodate.

On occasion, when small quantities of monochloramine were analyzed or when substances that might interfere with color changes were present, amperometric techniques were used. The Fisher Electropode equipped with a rotating platinum electrode was used as an end point indicator for the titration, with sodium thiosulfate, of the iodine liberated by monochloramine from potassium iodide. For the monochloramine-potassium iodide solution the potential employed was -0.25 volts versus the standard calomel electrode. By holding this potential constant across the cell, the end point of the titration was determined by measuring the current change against additions of sodium thiosulfate. The titration curve (current versus volume of sodium thiosulfate) usually resolved itself into two straight lines which intersected at the end

point of the titration. This method was satisfactory for determining small quantities of combined chlorine, but care was necessary to keep the platinum electrode clean.

Monochloramine exhibits maximal absorption in the ultraviolet range at 244 m μ (figure 3-1). On occasion monochloramine solutions were checked for purity by obtaining an ultraviolet spectrum of the solution in a Beckman DU Spectrophotometer. Using the basic Beer's Law equation for spectrophotometric analysis ($A = abc$, where A is the absorbance, a is the molar absorptivity, b is the internal cell width, and c is the molar concentration of absorbing material), monochloramine exhibited a molar absorptivity of 458 liter mole⁻¹ cm⁻¹ at 244 m μ . Hypochlorous acid, dichloramine and nitrogen trichloride exhibit maximal absorptivities considerably remote from this value (table 3-1).

C. BACTERIA STUDIED

Because of its relatively simple nutritional requirements, its use as an index of fecal pollution, and because it has been the bacterium of choice in a vast majority of chlorine disinfection studies, Escherichia coli (strains ATCC 26 and B) was selected as the biological system for most of the work to be reported. Streptococcus faecalis R, a more fastidious bacterium, was also tested on a few occasions.

Escherichia coli was maintained by bimonthly transfer on nutrient agar slants. Freshly inoculated agar slants were incubated overnight at 37° C and stored in the refrigerator at 4° C. Streptococcus faecalis R was preserved in 2 ml of trypticase soy broth

FIGURE 3-1

UV SPECTRUM OF MONOCHLORAMINE

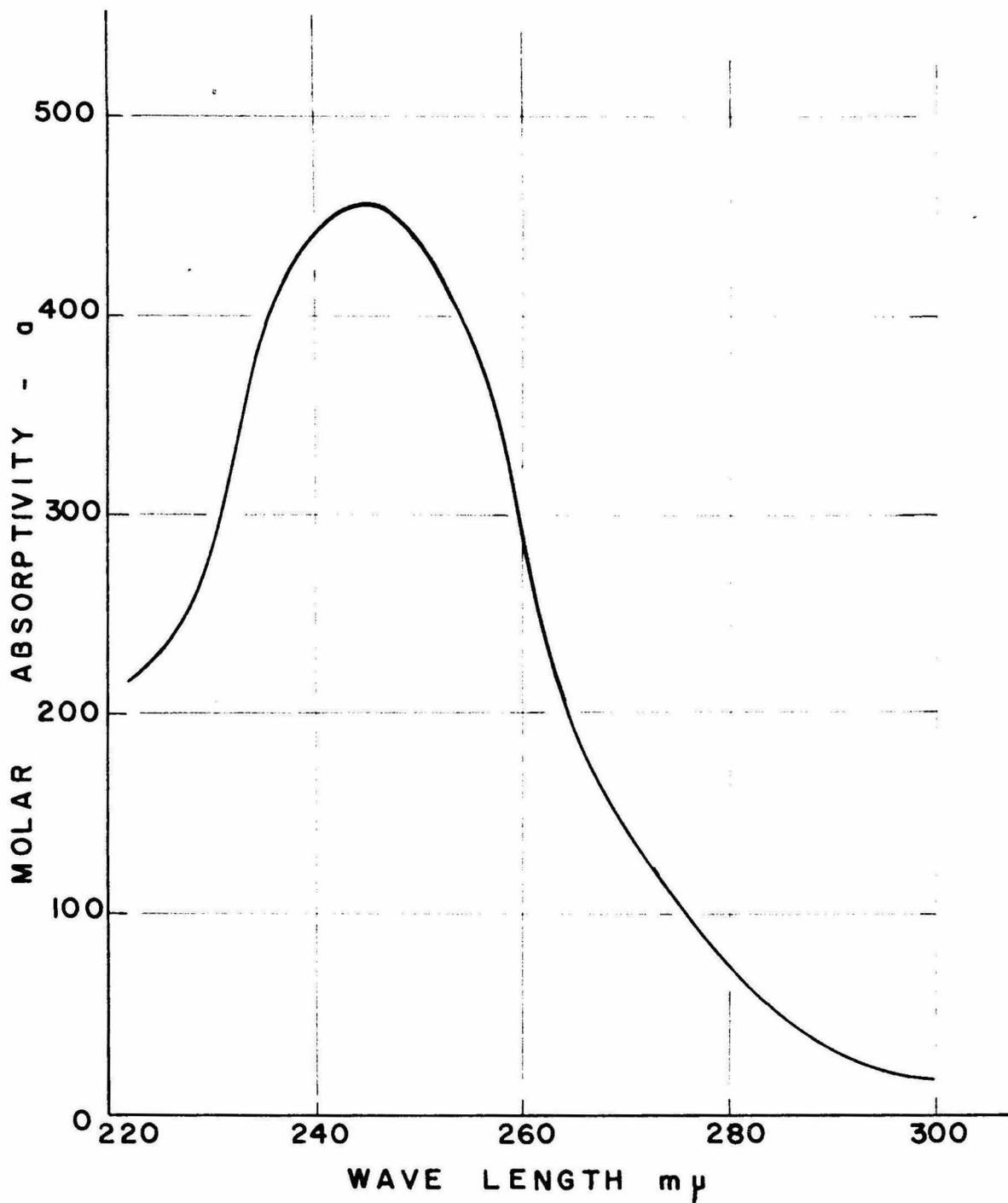


TABLE 3-1

MAXIMUM ABSORPTIVITIES OF CHLORINE AND DERIVATIVES

Compound	Wave Length m μ	Maximum Molar Absorptivity	
		(1)	(2)
HOCl	233		95
	225	110	
OCl ⁻	291.5		325
	295		340
NCl ₃	220		8120
	340		260
NH ₂ Cl	244	458	455
NHCl ₂	206		2150
	295		295

(1) This Investigation

(2) Samples (47)

(Baltimore Biological Laboratories) which had been inoculated with one drop of an 18-hour, 37°C trypticase soy broth culture and promptly frozen at -60°C. In this state, the organism remained viable for many months. Upon placing the frozen, inoculated medium in a 37°C incubator, excellent growth occurred overnight, and the culture was ready for further use.

D. GROWTH MEDIA

The medium of Davis and Mingioli (48), employed in most of the investigations of E. coli, has the following composition:

K_2HPO_4	7.0 g
KH_2PO_4	3.0 g
$MgSO_4 \cdot 7H_2O$	0.1 g
$(NH_4)_2SO_4$	1.0 g
Sodium citrate	0.4 g
Energy source	5.0 - 10.0 g
Water	to 1000 ml
(Final pH)	6.9 to 7.0)

The presence of the chelating agent, sodium citrate, greatly enhanced the growth of E. coli, especially when sodium acetate was used as the energy source. The choice of energy source for this medium was dependent upon the analysis to be made. Three energy sources were employed throughout this investigation, viz., glucose, sodium succinate and sodium acetate. Sodium succinate and sodium acetate were added directly to the basal medium prior to autoclaving (121° C, 15 to 20 minutes), whereas glucose was

autoclaved separately as an aqueous 25% solution and the appropriate volume added just before inoculation. If sterilized with the medium, glucose imparted a yellowish discoloration to the medium, presumably a result of caramelization.

Large quantities of S. faecalis R were obtained by cultivating this organism in a medium of the following composition:

Beef extract	4.5 g
Tryptose	15.0 g
Glucose	7.5 g
Sodium chloride	7.5 g
Water	to 1000 ml

On most occasions plate counts were made using a standard Difco preparation containing:

Tryptone	5 g
Yeast extract	2.5 g
Glucose	1 g
Agar	15 g
Water	to 1000 ml

This agar medium permitted prolific growth of both E. coli and S. faecalis.

For the detection of auxotrophic mutants, a minimal agar medium containing the following constituents was used:

NaCl	5.0 g
MgSO ₄ ·7H ₂ O	0.2 g
NH ₄ H ₂ PO ₄	1.0 g

K_2HPO_4	1.0 g
Glucose	10.0 g
Washed Agar	20.0 g
Water	to 1000 ml

It was essential that the agar be washed free of impurities since Adelberg (49) found such materials in unwashed agar to decrease survival of auxotrophs by 75 per cent when employing the penicillin technique. Fifty-gram quantities of Difco's Bacto agar were washed six times with two-liter volumes of distilled water, followed by two washes with 200-ml volumes of 100% ethanol. The glucose, agar and mineral salts were autoclaved separately and mixed after cooling.

For the penicillin technique the appropriate weight of sodium penicillin G (Nutritional Biochemicals Corp.) to give a final concentration of 4000 Oxford units/ml was added to 10 ml sterile distilled water and aseptically filtered through a 0.45 μ HA millipore filter. One ml of this reagent was added to 100 ml minimal agar just prior to pouring plates. The remaining penicillin solution was immediately frozen at -60° C for future use; if not used within 7 days the frozen solutions were discarded. A sterile, powdered preparation of penicillinase (Neutrapen, Riker Laboratories, Inc.) with an equivalent neutralizing capacity of 800,000 Oxford units at pH 7.0 was reconstituted with 2 ml of sterile distilled water, and 1 ml was added to 100 ml minimal agar immediately before use.

E. CULTIVATION OF BACTERIA AND PREPARATION OF CELL SUSPENSIONS

The manner in which bacterial cells were grown and prepared were important parameters in this study. Since bacterial cultures or cell suspensions are variable from one experiment to the next and are sometimes difficult to characterize, superimposition of a highly reactive material like monochloramine only compounds the difficulty. The following procedures were designed to eliminate as many variables as possible, and were vigorously adhered to during this study.

Fifteen ml of the defined medium were inoculated with E. coli and incubated in a rotary shaker-incubator for 8 hours at 30° C. From this culture 2-ml aliquots were aseptically transferred to 200-ml portions of fresh defined medium contained in 300-ml Erlenmeyer flasks to obtain secondary cultures by overnight incubation at 30° C (13 to 16 hours) in the rotary incubator-shaker. Cells were centrifuged with buffered deionized water in a Sorvall SP/X anglehead centrifuge at 2500 rpm for 15 minutes. After three washings the organisms were resuspended to the required density with buffered water and agitated for 20 to 30 minutes in a mechanical shaker in order to break up clumps and obtain a homogeneous suspension. Cell densities varied with the analysis but generally ranged between 4 and 12 mg dry weight/ml.

Similar procedures were employed in preparing suspensions of S. faecalis which had been harvested from the crude medium after overnight incubation at 37° C without agitation.

In some cases it was desirable to examine the effects of monochloramine on cell-free systems. Fifty ml of a concentrated (14 - 20 mg dry weight/ml) suspension of E. coli in 4.5×10^{-4} M phosphate buffer (pH 7.2) was subjected for 2 minutes to sonic vibrations in a 10 Kc - 250 W Raytheon magnetostriction oscillator. The temperature of the suspension was maintained at 4° C by means of a circulating pump arrangement on a Forma refrigerated water bath. The crude sonicated suspension was used immediately for tests of enzymic activity (to be described later).

All glassware, previously thoroughly cleaned and rinsed in deionized water, was rinsed two times with a strong solution of monochloramine followed by several rinsings of chlorine demand-free water. Flasks to be sterilized by autoclaving were treated in a similar manner.

All cultures were suspended in buffer solutions since pH has a marked effect on enzymic function and on chemical reactions of chloramines and related compounds. The buffer used in most experiments was made from a stock solution of 1.5×10^{-2} M KH_2PO_4 and 3.0×10^{-2} M K_2HPO_4 diluted 1:100 to give a pH of 7.2. A Beckman Model G pH meter was used to measure pH. A sodium bicarbonate buffer (0.0104 M bicarbonate, final concentration) was used in some experiments where cells were tested for rate of fermentation.

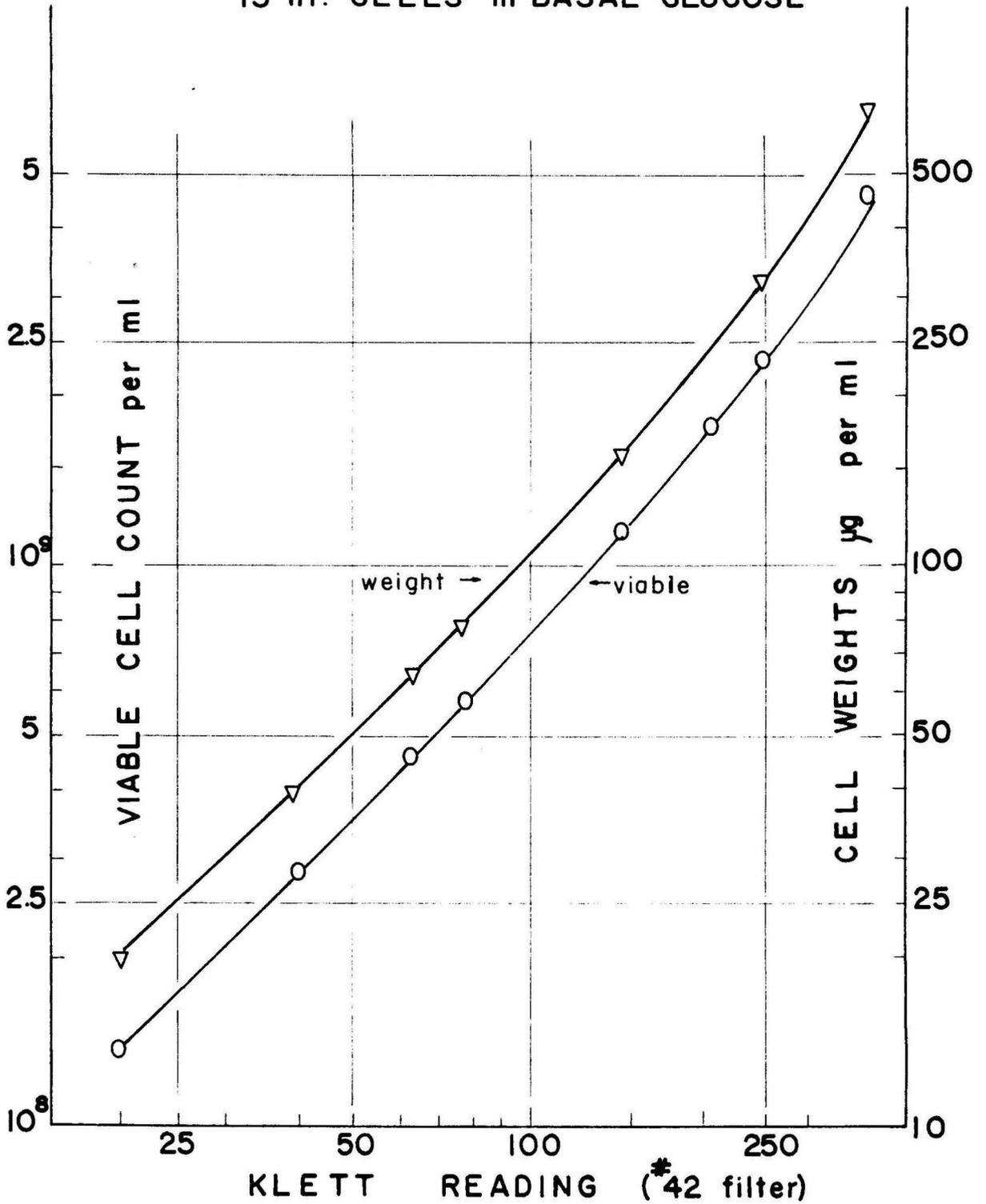
F. ENUMERATION OF BACTERIA

The two methods of enumerating bacteria were dry weight measurements and viable counts.

For dry weight determinations, HA millipore filters of 0.45 μ pore size, which had been stored in a vacuum desiccator for several days, were carefully tared. A thick cell suspension was prepared in deionized water and serially diluted in 6 separate flasks. Samples were withdrawn from these flasks, and density readings were obtained on a Klett-Summerson photoelectric colorimeter using a number 42 filter (blue). An appropriate volume of the cell suspensions was filtered under 0.5 atmosphere vacuum and rinsed with water several times. Several controls (water only) were included. The filters on which cells had been deposited were placed in a desiccator and weighed several days later. The \log_{10} of cell weights plotted against the \log_{10} of the density readings resulted in a curve which was used in subsequent tests to convert colorimeter readings of cell suspensions to dry weight (figure 3-2).

Viable counts were made from serial decimal dilutions of the same 6 samples as outlined above. Sterile Petri dishes were inoculated with 1-ml portions of the appropriate dilutions (in triplicate). Plate count agar (Difco), cooled to 45° C, was added immediately afterward, and the plates were inverted and incubated at 37° C for 24 hours. The colonies on each plate of one of the dilutions were counted on a Quebec colony counter. The average colony count multiplied by the reciprocal of the dilution resulted in a close approximation of the number of viable cells per ml of undiluted suspension. The \log_{10} of these counts were plotted against the \log_{10} of the colorimetric readings as

FIGURE 3-2
STANDARD DRY WEIGHT AND VIABLE
COUNT CURVE FOR E. coli B
- 13 hr. CELLS in BASAL GLUCOSE



described above (figure 3-2). In later experiments it was found that pouring a shallow protecting layer of plate count agar in the bottom of the Petri dishes prior to inoculation prevented the development of spreading colonies on the bottom of the dish, thereby enhancing reliability of these plate counts. The standard plate count and dry weight curves were checked every few months.

G. EXPERIMENTAL PROCEDURES IN MPN-MF AND BACTERIAL RECOVERY STUDIES

Bacteria were enumerated in this phase of the study by the membrane filter and the dilution to extinction methods. The latter is henceforth referred to as the most probable number (MPN) technique.

The MPN analyses for coliform bacteria were performed according to "Standard Methods" (46) using serial decimal dilutions and five tubes per dilution in lactose broth. In general, eosin-methylene-blue (EMB) agar was used to confirm the presence of coliforms in tubes from the highest dilution showing gas after 48 hours of incubation. Occasionally brilliant green bile (BGB) confirmatory tests were run. No confirmatory tests were made on pure cultures of E. coli. Counts were estimated from the most probable number tables of Thomas (50).

For membrane filter counts, the Lawrence Experiment Station (LES) technique recently developed by McCarthy (51) was adopted. Volumes of sewage or cell suspensions estimated to yield between 10 and 60 colonies were filtered through HA membrane filters with 0.45 μ pore size at 0.5 atmosphere vacuum

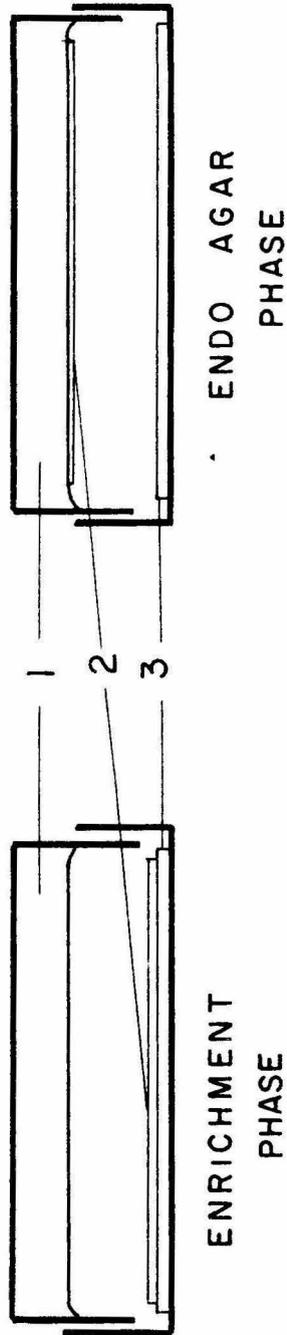
and washed with 50 ml of sterile buffer. The filter was placed on sterile absorbent pads soaked with lauryl tryptose broth (Difco) and incubated in Petri dishes at 37° C for 1.5 ± 0.5 hours (figure 3-3). The filter was then transferred to m-Endo agar (Difco), which had been poured into the bottom of the same dish, and was incubated for 22 hours at 37° C. Only sheen-type colonies were counted. (Presumably only the sheen colonies are of the E. coli species.) Each serial dilution was run in triplicate, and counts were averaged from the triplicate dilution giving between 10 and 60 colonies.

The samples tested included settled sewage, phosphate buffer suspensions of E. coli (strain B or ATCC 26) and sterile sewage inoculated with these bacteria.

Sewage samples were prepared by siphoning off the supernatant from raw sewage after a settling period of 20 minutes. The 15-minute chlorine demand of this supernatant ranged from 2.8 to 3.1 mg/liter as measured by the starch-iodide titration. These supernatants, contained in 100-ml Erlenmeyer flasks, were treated with monochloramine (10 mg/liter) at room temperature with gentle agitation for 10 minutes. The residual monochloramine was neutralized with excess sodium thiosulfate (1 ml of 0.01 N solution), and the coliform population was estimated by the membrane filter and/or the MPN technique.

Sterile sewage tests were made from settled supernatant which had been autoclaved at 121° C for 30 minutes. Appropriate dilutions of buffered cell suspensions were added to the sewage,

FIGURE 3-3
INCUBATION POSITIONS OF MEMBRANE
FILTER IN THE TWO-PHASE LES TECHNIQUE



1. ENDO AGAR
2. MEMBRANE FILTER
3. ABSORBENT PAD, SATURATED WITH LAURYL TRYPTOSE BROTH

and the preparation was thoroughly mixed. These samples were treated with 10 mg monochloramine per liter for 10 minutes at room temperature, and coliform populations were estimated as above.

Pure culture samples were prepared by washing E. coli harvested from lauryl tryptose broth after 12 to 15 hours of incubation at 37° C. These cells were diluted in phosphate buffer to give a final concentration of about 2×10^9 per ml. The samples, while being gently agitated, were treated with monochloramine (1 mg per liter) for 10 minutes at 25° C, at which time the excess monochloramine was neutralized with 0.1 ml of 0.01 N sodium thiosulfate. Coliforms in these preparations were enumerated as with the sewage samples.

H. EXPERIMENTAL PROCEDURES FOR STUDIES OF CHEMICAL REACTIVITIES BETWEEN MONOCHLORAMINE AND ORGANIC COMPOUNDS

The basic feature of this series of tests was the measurement of the monochloramine demand of the various compounds studied. In order to eliminate as much error as possible, all glassware and equipment used was rendered demand-free by a wash in monochloramine followed by several rinsings in demand-free water. Compounds to be tested were made up to the appropriate concentrations (usually 1×10^{-2} M) in demand-free water.

Into one Erlenmeyer flask was placed the desired amount of monochloramine; into a second flask was placed the compound to be tested, diluted to the appropriate concentration with phosphate buffer sufficient to raise the pH of the entire mixture to

7.2. (The stock phosphate buffer was the same as that used in preparing pure cultures for chlorination.) The final concentrations of monochloramine varied between 2×10^{-4} and 2×10^{-3} M depending upon the compound being tested (molar ratios of monochloramine to the test compound ranged from 1:1 to 1:10). A blank containing all the constituents of the sample flasks except the compound tested was also prepared.

Contents of the two flasks were then poured simultaneously into a larger flask, and this mixture was agitated on a mechanical mixer. Samples were withdrawn at regular intervals for starch-iodide thiosulfate titration of monochloramine. No temperature control was provided, but the temperature in the flasks usually remained between 24° and 26° C over the testing period.

It was desirable to study in more detail the chemical reaction of monochloramine and amino acids. Several amino acids, L-alanine, L-cysteine and L-cystine, were selected for this purpose. The methods that follow were used to gain a more complete understanding of the nature of this reaction.

Total nitrogen was determined by the Kjeldahl method (46) using a semi-micro apparatus with steam distillation. Appropriate volumes of sample were digested for one hour in concentrated H_2SO_4 with a copper catalyst. Afterwards, the sample was diluted with ammonia-free water, neutralized to a phenolphthalein end point with concentrated NaOH, and distilled into 5 ml of 0.645 M borate buffer. The sample was added to a

Nessler tube, and 2 ml Nessler's reagent (46) was added. The transmittance of the yellow color was read on a Lumitron colorimeter at 420 m μ , and the concentration of organic nitrogen as ammonia nitrogen in the digest was determined by comparison with a standard curve.

Ammonia nitrogen was determined by the direct Nesslerization method (46). Color was determined on a Lumitron colorimeter at 420 m μ and compared with a standard curve. Nitrate nitrogen was analyzed according to the Brucine method as described in "Standard Methods" (46). Excess monochloramine in samples was partially neutralized with 0.01 N sodium thiosulfate to leave approximately 4 mg available chlorine per liter. Sodium arsenite was then added to neutralize completely the remaining monochloramine. A slight excess of sodium arsenite does not interfere with this test. The transmittance of the yellow color was read on the Lumitron colorimeter at 420 m μ , and nitrate nitrogen concentration was determined from a standard curve. Nitrite nitrogen was obtained according to the sulfonic acid-naphthylamine hydrochloride colorimetric procedure as outlined in "Standard Methods" (46). The pink color was read at 515 m μ on a Lumitron colorimeter and compared with a standard curve.

A relatively simple qualitative measurement for acetaldehyde was obtained by placing the sample in the receiving tube of an aeration tube system. One milliliter of concentrated H₂SO₄ and 1 milligram of *p*-hydroxydiphenyl were placed in the reaction

tube. A vacuum was drawn on the system and was continued for about 60 minutes. In the presence of acetaldehyde a violet color appears in the reaction tube (52).

A simple qualitative test was used for the determination of α -keto acids. To a 1-ml sample, 0.5-ml of 2,4-dinitrophenylhydrazine solution (0.1% in 2 N HCl) was added. After standing for 10 minutes, 1 ml of 2 N NaOH was added. An intense red color indicates the presence of an α -keto acid (53).

The analyses of both sulfhydryl groups and disulfide bonds were carried out in several of the studies with L-cystine and L-cysteine. Sulfhydryl groups were determined by the colorimetric procedure as described by Barron (54). A 3.0-ml sample aliquot was placed in a test tube, and 0.5 ml 0.5 M sodium phosphate buffer (pH 7.0) and 1.0 ml 0.1 M ferricyanide solution were added. After standing at room temperature for 10 minutes, 0.1 ml of 5 N HCl, 0.3 ml ferric sulfate solution and 1.5 ml H_2O were added to the tubes, and they were placed in the dark for 30 minutes. The absorbance of the Prussian blue color was measured at 710 m μ on the DU spectrophotometer with a tungsten lamp. These readings were compared with a standard curve prepared from L-cysteine and the results expressed as μM -Sh.

The ferric sulfate solution was prepared by suspending 20 grams of gum ghatti overnight in 1 liter of deionized water and filtering this solution through filter paper. This extract was added to a solution containing 5 g anhydrous ferric sulfate, 75 ml

of 85% phosphoric acid plus 100 ml deionized water. Fifteen ml of 1% potassium permanganate was added further to oxidize impurities in the gum ghatti. This test combines a high degree of sensitivity (10 μ moles SH) with simplicity of analysis.

Disulfide was measured by the procedure described above after a 5-ml aliquot of sample was treated with 1 to 2 ml of fresh 5% sodium cyanide solution for 10 minutes. The colorimetric readings were compared with the L-cysteine standard curve and the results expressed as μ M-SH.

I. RESPIRATION STUDIES

Resting cell respiration was measured manometrically by means of the Warburg apparatus. The respirometer is based on the principle that at a constant temperature and constant gas volume any changes in the amount of gas can be measured by changes in its pressure. The oxidative utilization of carbohydrates by E. coli involves the uptake of O_2 and the release of CO_2 and H_2 . Carbon dioxide is absorbed by an alkaline solution added to the center well of the Warburg flask. Hydrogen is released through the enzymatic split of formic acid ($HCOOH \rightarrow H_2 + CO_2$). It has been shown by Billen (55) that this enzyme, formic hydrogenlyase, is not produced by E. coli grown in a mineral salts - glucose medium. There is also good evidence (56) that formic hydrogenlyase is not present in aerobically grown E. coli suspensions. The release of hydrogen was no problem in these experiments, for the organism was grown in a mineral salts-glucose medium

under vigorous aeration.

In these studies an Aminco rotary Warburg apparatus with provision for 18 manometers was used. The calibrated single-stem manometers were filled with Brodie's solution (density = 1.033). The 15-ml Warburg flasks had two sidearms, one vented, and a center well. Temperature of the water bath was maintained at 29.4° C by means of a mercury thermostat. Flask constants were computed according to methods outlined by Umbreit (57).

Two milliliters of a washed cell suspension in buffer were added to the main compartment of the Warburg flasks followed by an amount of distilled, chlorine demand-free water to bring the total volume of liquid in the flask, including contents of the center well and sidearms, to 3.2 ml. An appropriate volume of substrate and monochloramine were added to each arm of the flask. In order to absorb carbon dioxide, 0.2-ml of a 10% KOH solution was placed in the center well. Whatmann No. 1 filter paper was cut into 2-cm squares, folded accordian style and placed in the center well. The paper increased the absorptive surface of the alkali.

A thermobarometer was prepared for each run by placing 3.2-ml of distilled water in one of the Warburg flasks. As its name implies, this flask measures fluctuations in barometric pressure and temperature during the experimental runs so that appropriate corrections can be made for each test flask.

All flasks were fitted on Vaseline-coated ground glass joints of the sidearms on the manometers, and the units were placed in the temperature controlled bath and agitated at 112 oscillations per minute. A vacuum was pulled on the open end of the manometers so that the readings after the manometer was closed were between 28 and 30 cm (when the closed end was set on 15 cm). This reduced the need to reset the manometers during subsequent tests. After the endogenous oxygen uptake in all flasks appeared to be reasonably constant, the substrate was tipped into the flask. Twenty to thirty minutes later the monochloramine solution was tipped into the flask. Readings were made at 10 to 15 minute intervals for as long as 3 hours. The $\mu\text{l O}_2$ taken up per mg dry weight of cells per hour was calculated from the average rate of two flasks after subtracting the endogenous activity. Triplicate plate counts from thiosulfate-neutralized serial dilutions were made from the Warburg vessels at various intervals of time by sacrificing selected vessels during the experimental test.

The method above was modified in some cases wherein cell suspensions were treated with monochloramine prior to the respiration study. Treated cells were then diluted, centrifuged and resuspended to original density in buffer prior to inoculation into flasks. In some cases cells were directly neutralized with thiosulfate and assayed. Triplicate plate counts from serial dilutions were made from the suspensions before these inoculations.

The substrates used in these experiments were glucose, acetate, pyruvate and succinate. The stock concentrations and quantities of these substrates used were as follows:

	Stock Solution	Volume to Warburg	Final Concentration
Glucose	15% (0.83 M)	0.20 ml	1.0% (0.055 M)
Sodium succinate	10% (0.72 M)	0.25 ml	0.8% (0.056 M)
Sodium acetate	10% (1.22 M)	0.25 ml	0.8% (0.098 M)
Sodium pyruvate	5% (0.45 M)	0.30 ml	0.5% (0.045 M)

During the course of these experiments it was noted that better rates could be obtained for succinate and acetate if cells were grown in a defined medium with sodium acetate as the energy source.

The concentration of monochloramine used in experiments of this nature was extremely important but very deceptive. The basic reason for this is the monochloramine side reactions which occur in the presence of cellular material. Thus, if a given amount of monochloramine is found to kill a certain proportion of cells in suspension, it is impossible to ascertain how much of the monochloramine activity lost exerted a lethal effect and how much reacted at non-lethal sites.

Concentrations of monochloramine were generally confined to levels which were not 100% lethal so as to detect threshold sensitivity of enzymic mechanisms to this disinfectant. Concentrations used ranged from 8×10^{-4} to 1.25×10^{-2} mg NH_2Cl per ml for cell densities of from 5 to 12 mg dry weight per ml. Stock

solutions of monochloramine were diluted immediately before use so that between 0.1 and 0.4 ml could be added to the sidearm of the vessel. The buffer capacity of the cell suspension was sufficient to hold the pH at 7.2 after monochloramine was tipped.

J. FERMENTATION STUDIES

The anaerobic dissimilation of carbohydrates and other organic carbonaceous compounds was measured by manometric techniques with the Aminco rotary Warburg apparatus. Anaerobic conditions were created by gassing flasks with a nitrogen-carbon dioxide mixture (5% carbon dioxide). In the absence of oxygen, with bicarbonate buffer in the flasks and an atmosphere containing some carbon dioxide, CO_2 released from a substrate by the cells will escape to the atmosphere to be measured manometrically. In addition, any acids formed in the fermentation dissociate to yield H^+ ions which combine with HCO_3^- and release CO_2 via H_2CO_3 . The former CO_2 released is known as metabolic CO_2 ; the latter, acid CO_2 (57).

The flasks were prepared in a manner similar to that described for respiration studies with the exception that a bicarbonate buffer was used. The concentration of buffer required to give an initial pH of 7.2 under an atmosphere of 5% CO_2 at a temperature of 29.4° C was calculated to be 0.0104 M, according to the Henderson-Hasselbach equation (57). A 0.0445 M stock solution of bicarbonate buffer was prepared and 0.7 ml of the stock was added to each flask along with 2 ml cell suspension.

Flasks and manometers were assembled and placed on the Warburg drum. A hose from the nitrogen-carbon dioxide tank was connected to the gassing assembly, and the individual hoses were attached to the closed ends of the manometers. The flask vents were opened and flasks were gassed for 30 minutes. Each manometer was set by pulling a slight vacuum on the closed end with rubber tubing and shutting the stopcock so that the fluid rested between 0 and 3 cm on the open side when equilibrated. Substrates were tipped following equilibration and readings were taken every 10 to 20 minutes for periods as long as 90 minutes.

To differentiate metabolic CO_2 from acid CO_2 , 0.3 ml 3 N H_2SO_4 was added to the sidearms of each of two flasks containing cells, buffer substrate and bicarbonate. After gassing and equilibrating, acid and substrate were tipped into one flask, and the pressure caused by the CO_2 evolved was recorded. At the end of the run, acid was tipped into the other flask and the CO_2 remaining was read. The difference in these two readings represented the acid CO_2 ; any other CO_2 released was that produced by the cells as metabolic CO_2 (57).

The substrates used in these analyses included glucose, fructose-1,6-diphosphate and pyruvate. All substrates were prepared as aqueous 5% solutions; 0.3 ml of substrate solution in a total volume of 3.0 ml in the Warburg flasks resulted in a final concentration of 0.5%.

All cell suspensions were pretreated with monochloramine-- some diluted, centrifuged and resuspended to original densities

and others neutralized with 0.1 ml .01 N sodium thiosulfate and used directly. Triplicate plate counts from serial dilutions of cell suspensions were usually made preceding Warburg runs.

K. DEHYDROGENASE STUDIES

The study of the catalytic transfer of hydrogen by cells was carried out by the use of methylene blue which acted as a hydrogen acceptor. Methylene blue is particularly suitable for the assay of dehydrogenase activity because it is colorless in its reduced form (leuco-methylene blue) and blue in its oxidized form. The well-known Thunberg method of estimation of dehydrogenase activity depends upon the reduction of methylene blue by hydrogen (and electrons) transferred from an enzymatically activated substrate.

Thunberg tubes were provided with a sidearm cap arranged to hold materials to be added to the main tube after evacuation. The main tube was provided with an outlet fitting, and the sidearm cap was turned so that a hole drilled in its standard taper coincided with the outlet fitting. The cap was rotated after evacuation, sealing off the tube. The tubes were of a size (19.5 mm diameter × 17.5 cm long) which fit directly into the receptacle of a Klett-Summerson photoelectric colorimeter.

The procedures employed were a modification of those described by Umbreit (57). One ml of 1:10,000 methylene blue, 2 ml 0.066 M phosphate buffer ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ adjusted to pH

7.0) and 2 ml 0.02 M substrate were placed in the Thunberg tube. One ml of cell suspension was added to the sidearm, and two lines of Vaseline were applied to the tapered joint. In order to insure anaerobic conditions, nitrogen was bubbled through the open tube for 2 to 5 minutes prior to pressing the cap in place. The tubes were evacuated for 5 or 6 minutes with a water aspirator. Each tube was tapped intermittently to rid the system of dissolved gases and gas bubbles. When evacuation was complete the cap was rotated 180°, and the tubes were placed in a water bath at 37° C for 10 minutes. Cells were tipped in at the end of the equilibrium period, and colorimeter readings were made at appropriate intervals. A No. 66 (red) filter was used.

Prior to analysis of dehydrogenase activity, cells were treated with sublethal levels of monochloramine and neutralized with excess sodium thiosulfate. Triplicate plate counts of serial dilutions were made before cells were dispensed into the sidearms of the Thunberg tubes. Controls consisting of dilutions of untreated cells were also assayed for dehydrogenase activity so that activity could be related to the viable population, a relationship which was not always linear.

L. β -D-GALACTOSIDASE STUDIES

Escherichia coli B was grown in the basal defined medium containing 0.1 M sodium succinate. Succinate was used as the sole source of energy and carbon because it does not specifically affect the induced synthesis of β -D-galactosidase (58).

Beta-D-galactosidase activity was determined by the method of Brock and Brock (59), with modifications, using toluene-treated cells (60). The β -galactosidase system is composed of two factors: (a) a specific transport system (permease) for bringing galactosides into the cell; and (b) β -galactosidase itself (58). Toluene treatment reduces permeability problems to permit full expression of β -galactosidase activity. Non-toluenized cells gave a measure of the induced activity of the entire system. Five ml of a cell suspension (0.7 mg dry weight/ml), 0.1 ml toluene and 0.1 ml 0.05% sodium deoxycholate were mixed together for 15 minutes in a screw cap test tube by tilting the tube slowly (20 times per minute).

The chromogenic galactoside, o-nitrophenyl β -D-galactoside (ONPG), which has a negligible optical density at visible wave lengths, was employed as the substrate for assaying β -D-galactosidase. Free o-nitrophenyl (ONP) is capable, however, of a tautomeric change that gives it a yellow color in alkaline solution, with an absorption peak at 420 m μ .

One ml of appropriately prepared cell suspension was added to a test tube along with 4 ml M/600 ONPG (Calbiochem) in a 0.1 M Na_2HPO_4 buffer at pH 7.0. This mixture was incubated in a water bath at 37° C for 45 minutes. The reaction was stopped with the addition of 5 ml 1 M sodium carbonate and the tubes were quickly cooled, but not frozen, at -60° C. The suspension was centrifuged at 2400 rpm for 10 minutes, and the

supernatant was placed in 2-cm Beckman quartz cells for analysis on a Beckman DU Spectrophotometer at 420 m μ . Galactosidase activity was expressed in terms of an arbitrary unit: one activity unit is defined as the liberation of 10^{-9} moles of o-nitrophenyl (ONP) per minute per mg dry cells. The molar absorptivity of ONP (Calbiochem) in 0.01 M Na₂CO₃ (pH 10.8) was 4.4×10^3 liter mole⁻¹cm⁻¹ at a wave length of 420 m μ .

Cells were treated with monochloramine before or after toluenization of the cell suspensions and neutralized with excess sodium thiosulfate. Triplicate plate counts were made from serial dilutions prior to assay. In the case of treatment of toluenized cells with monochloramine, parallel controls without toluenization were made to give an estimate of survival of the organisms tested. (Toluenization reduced viability to less than 0.001% of original population, although β -galactosidase activity was considerably enhanced.)

M. DETECTION OF AUXOTROPHIC MUTANTS

Two methods were used to isolate auxotrophic mutants, viz., the replica plate technique and the penicillin technique.

In Lederberg's (61) replica plate technique, a velveteen cloth is employed to transfer colonies from one plate to another in the same pattern. This facilitated routine tests involving repetitive inoculations of many isolated colonies on different media. In these tests, 12-cm squares of velveteen cloth were cut, placed in Petri dishes and autoclaved. When the master agar plates

containing colonies were ready, a square of cloth was placed, nap up, on a cylindrical aluminum disk 9 cm in diameter and fastened securely with a rubber band. The agar plate was inverted onto the fabric with slight digital pressure to transfer the growth. The imprinted fabric then acted as a pattern for transferring replica-inocula to subsequent plates. In these investigations, initial plates were usually made by spreading, with an L-shaped glass rod, 0.1 ml of an appropriate serial dilution of cells on the partially dried surface of plate count agar. Plates were inverted and incubated for 20 to 24 hours prior to transfer. Transfers were usually made to minimal agar or minimal agar fortified with 1% yeast extract or 0.1% casein hydrolysate (Nutritional Biochemicals, vitamin free-enzymatic).

The penicillin technique is based on early developments by Lederberg (62) and Davis (63), with modifications as prescribed by Adelberg (49). The basis for this technique is that penicillin kills only growing bacterial cells; thus, incubation of a mixture of wild type and auxotrophic cells in minimal medium containing penicillin greatly increases the proportion of auxotrophs in the surviving population. Adelberg suggested that by using a solid medium each mutation would give rise to only one colony at the end of the procedure, thereby facilitating computations of mutation rates. In addition, he found that by applying penicillin to micro colonies instead of single cells, the yield of auxotrophs would be raised to a high and reproducible level.

To each of a series of Petri dishes 7-ml of minimal agar was aseptically added and cooled. Treated and control cell suspensions were diluted to result in a viable population of between 10^4 and 10^5 /ml. One ml of the properly diluted suspension was pipetted into each dish, an additional 5 ml of minimal media was poured, and the dishes were carefully agitated in a circular motion. Upon cooling, a third 7-ml layer of minimal agar was pipetted into each dish and the plates were incubated for 7 hours at 37° C. All tests were carried out in triplicate. It is during this period that auxotrophy, if present, will be expressed and, therefore, it is a critical period in the efficacious use of this technique.

After the intermediate cultivation period, a 7-ml layer of minimal agar with sodium penicillin G (1000 Oxford units/ml) was pipetted into each plate. Plates were immediately refrigerated overnight (15 hours) at 4° C to allow penicillin time to diffuse and reach all cells.

Plates were then moved to a 37° C incubator for 24 hours. (This was later changed to 48 hours for reasons to be discussed later in the text.) At the end of the secondary cultivation period, 7-ml of penicillinase, sufficient to neutralize 1000 units of penicillin, was pipetted to each plate. These plates were reincubated at 37° C for 48 hours, and colonies that arose were marked. Seven ml of nutrient agar was added to the plates at this time, and plates were again reincubated for 48 hours. Colonies arising during this final incubation period were considered to be auxotrophic mutants.

IV. RECOVERY OF BACTERIA TREATED WITH MONOCHLORAMINE

In considering methods for studying the mode of action of monochloramine, the existing inconclusive evidence of recovery of bacteria subjected to its action merits review (38). Certainly, if one could conclusively demonstrate reversibility of the anti-bacterial action of monochloramine by specific reagents, some leads to the biochemical lesions might ensue. This subject has far-reaching implications in the field of water disinfection, for if the action of monochloramine is truly reversible, the foundation of basic water treatment practices would be badly shaken.

This phase of the study was divided into two general areas. The first, a study of discrepancies in coliform populations of sewage and cultures as measured by the dilution technique and the membrane filter method, was an indirect approach. The second, a study of the recovery of treated coliforms in neutralized sewage or buffer, was a more direct study of this phenomenon and the variables which affect it.

A. RESULTS - PHASE I

The coliform population of 15 samples of raw, settled Pasadena sewage was assayed by the membrane filter and MPN techniques. Then, 18 samples were treated with 10 mg/l of monochloramine at 25° C, held for 10 minutes, and then dechlorinated prior to assay by MPN and MF procedures. The results of these tests are recorded in table 4-1. A probability plot of the MPN-MF ratios, figure 4-1, shows the geometric mean to be 16.0,

TABLE 4-1

MPN-MF ANALYSIS OF MONOCHLORAMINE TREATED SEWAGE

Test No.	Controls			Samples**			
	MPN (/100 ml)	MF (/100 ml)	$\frac{MPN}{MF}$	Test No.	MPN (/100 ml)	MF (/100 ml)	$\frac{MPN}{MF}$
1A	13×10^6	19×10^6	.70	1A	35×10^2	1×10^2	35.0
2A	18×10^6	15×10^6	1.20	2A	24×10^2	0	24.0
3A	16×10^6	12×10^6	1.35	3A	13×10^2	2×10^2	6.5
4A	17×10^6	11×10^6	1.55	4A	220×10	6×10	36.6
5A	25×10^6	51×10^6	0.49	5A	22×10^2	2×10^2	11
				6A	35×10^2	9×10^2	3.9
1B	28×10^6	15×10^6	1.86	1B	130×10^3	$0 \times 10^{3*}$	-
2B	17×10^6	32×10^6	0.54	2B	33×10^3	6×10^3	5.5
3B	50×10^6	28×10^6	1.80	3B	300×10^3	11×10^3	27.3
4B	11×10^6	41×10^6	0.27	4B	790×10^2	$0 \times 10^{2*}$	-
5B	55×10^6	15×10^6	3.65	5B	172×10^3	6×10^3	28.6
				6B	542×10^3	53×10^3	10.1

TABLE 4-1 (Cont'd)

Test No.	MPN (/100 ml)	MF (/100 ml)	$\frac{MPN}{MF}$	Test No.	MPN (/100 ml)	MF (/100 ml)	$\frac{MPN}{MF}$
1C	46×10^6	70×10^6	0.66	1C	1700	31	55.0
2C	70×10^6	68×10^6	1.02	2C	110×10^2	6×10^2	18.4
3C	58×10^6	66×10^6	0.88	3C	120×10^2	17×10^2	7.1
4C	23×10^6	66×10^6	0.35	4C	35×10^2	1×10^2	35.0
5C	63×10^6	22×10^6	2.85	5C	190×10^2	27×10^2	7.1
				6C	75×10^2	7×10^2	10.7

$$\bar{M} \frac{MPN}{MF} = 1.02$$

Fig. 4-2

$$\sigma_g = 2.02$$

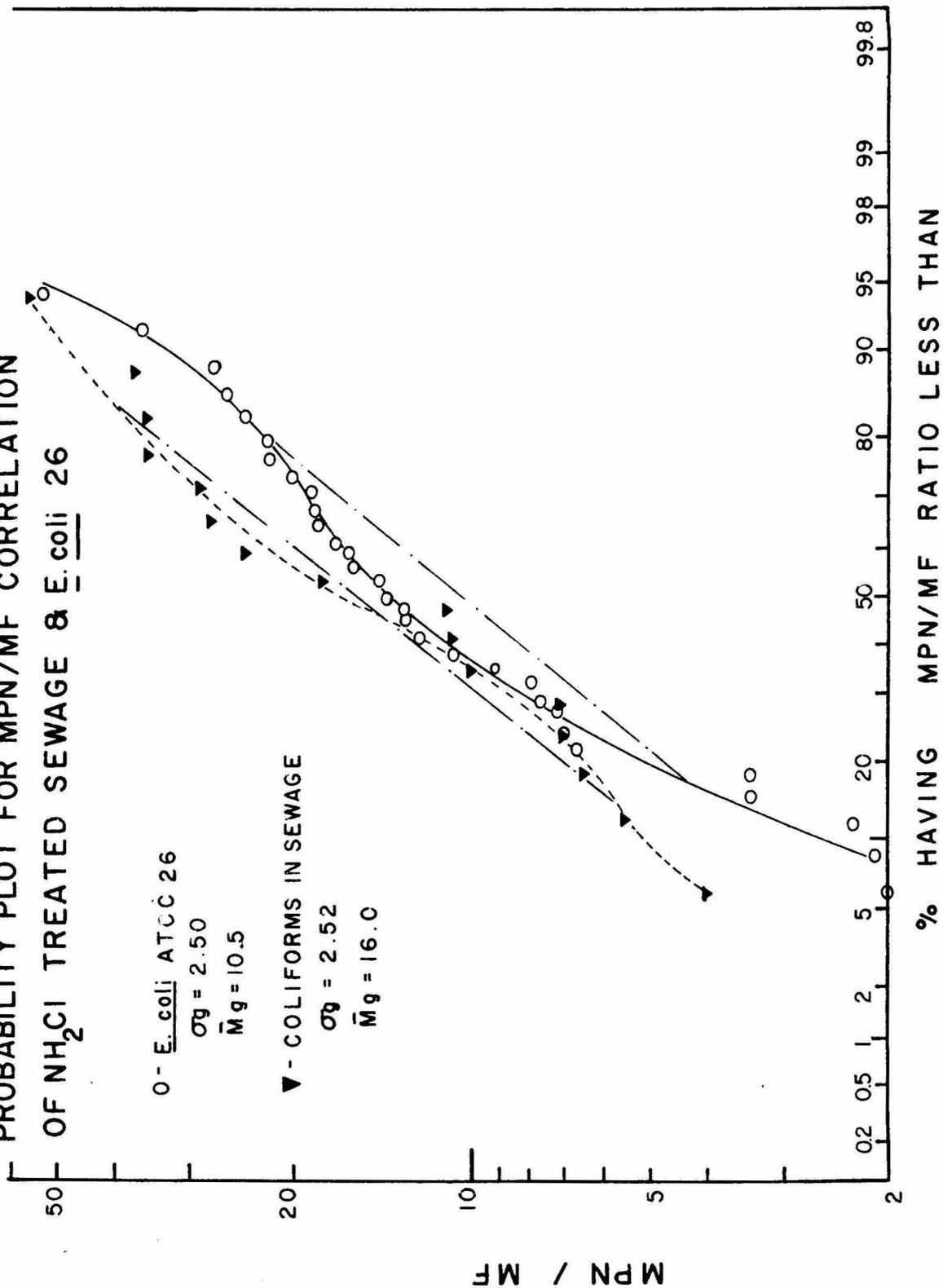
$$\bar{M} \frac{MPN}{MF} = 16.0$$

Fig. 4-1

$$\sigma_g = 2.52$$

** All samples treated with 10 mg/L Na_2Cl for 10 min. at 25° C.
 * These figures not used in MPN/MF computation.

FIGURE 4-1
PROBABILITY PLOT FOR MPN/MF CORRELATION
OF NH_2C TREATED SEWAGE & E. coli 26



with a geometric standard deviation of 2.52. The data are too limited to determine whether they are normally distributed. The validity of such an analysis will be discussed in greater detail later in this chapter. Note that the control samples yielded a geometric mean very close to 1.0, with a geometric standard deviation of 2.0 (figure 4-2).

Since sewage-borne coliforms are a heterogeneous group of species and strains of lactose fermenters, it was desirable to examine the effects of monochloramine on the MPN and MF counts in buffered suspensions of pure cultures of E. coli. Initial tests were performed with E. coli ATCC 26 using 1 mg monochloramine per liter for 10 minutes at 25° C. Results from 33 tests on 9 different suspensions are compiled in table 4-2. A probability plot of the data from this group of experiments, figure 4-1, indicates that the geometric mean of the MPN-MF ratio of treated E. coli suspensions was slightly lower (10.5) than ratios for sewage (16.0), but that the geometric standard deviations were virtually the same (2.50 vs. 2.52). The MPN-MF ratios of the controls for strain 26 are plotted on probability paper together with sewage controls (figure 4-2). Note that they do not appear normally distributed. A plot of MPN versus MF (figure 4-3) shows that all points for the treated suspensions fall outside Woodward's (64) 95% confidence limits.

Further tests were made to learn what effect holding treated cells in phosphate buffer would have on recoverable E. coli and on the MPN to MF ratios. Typical findings in table 4-3

TABLE 4-2
 MPN-MF ANALYSIS OF MONOCHLORAMINE TREATED E. COLI ATCC 26

Controls				Samples*			
Batch No.	MPN (/100 ml)	MF (/100 ml)	MPN / MF	Batch No.	MPN (/100 ml)	MF (/100 ml)	MPN / MF
1	3.3×10^5	11×10^6	0.29	1	210×10^3	8×10^3	26.2
	22×10^6	15×10^6	1.46		330×10^3	15×10^3	22.0
	17×10^3	6×10^3	2.83		490×10^3	20×10^3	24.5
					490×10^3	5×10^3	98.1
2	22×10^5	45×10^5	0.49	2	11×10^4	3.2×10^4	3.4
	78×10^5	40×10^5	1.95		2×10^4	1×10^4	2.0
	78×10^3	74×10^3	1.05		33×10^4	4.7×10^4	7.1
					110×10^4	5×10^4	22.0
3	64×10^6	47×10^6	1.35	3	540×10^3	50×10^3	10.8
	16×10^6	49×10^6	0.33		130×10^3	9×10^3	14.4
	11×10^4	9×10^4	1.10		54×10^3	7×10^3	7.7
					160×10^4	3×10^4	53.4

TABLE 4-2 (Cont'd)

Controls				Samples*			
Batch No.	MPN (/100 ml)	MF (/100 ml)	$\frac{MPN}{MF}$	Batch No.	MPN (/100 ml)	MF (/100 ml)	$\frac{MPN}{MF}$
4	79×10^5	70×10^5	1.10	4	54×10^5	2×10^5	27.0
	13×10^6	16×10^6	0.81		54×10^5	3×10^5	18.0
	11×10^3	14×10^3	0.74		160×10^4	8×10^4	20.0
					92×10^4	5×10^4	18.4
5	92×10^7	90×10^7	1.02	5	130×10^2	18×10^2	7.2
	38×10^4	40×10^4	0.95		170×10^2	14×10^2	12.1
					130×10^3	8×10^3	16.2
6	35×10^6	28×10^6	1.25	6	13×10^3	1×10^3	13.1
	23×10^6	28×10^6	0.82		490×10^3	13×10^2	37.6
	84×10^4	83×10^4	1.40		33×10^4	21×10^4	1.6
					17×10^4	8×10^4	2.1
7	13×10^6	12×10^6	1.08	7	49×10^3	6×10^3	8.7
	17×10^6	12×10^6	1.43		70×10^4	5×10^4	14.0
					28×10^4	1.5×10^4	18.6

TABLE 4-2 (Cont'd)

Controls			Samples*		
Batch No.	MPN (/100 ml)	MF (/100 ml)	MPN (/100 ml)	MF (/100 ml)	MPN/MF
8	79×10^5	12×10^6	45×10^3	20×10^3	2.3
	68×10^6	70×10^6	68×10^3	20×10^3	3.4
			79×10^4	5×10^4	15.8
			70×10^4	4×10^4	17.5
9	35×10^8	34×10^8	78×10^3	6×10^3	13.0
	41×10^5	38×10^6	54×10^4	8×10^4	6.7
			92×10^4	10×10^4	9.2

$$\frac{MPN}{MF} = 0.96$$

Fig. 4-2

$$\sigma_g = 1.55$$

$$\bar{M} \frac{MPN}{MF} = 10.5$$

Fig. 4-1

$$\sigma_g = 2.50$$

* Treated with 1 mg/l of NH_2Cl for 10 min. at 25° C.

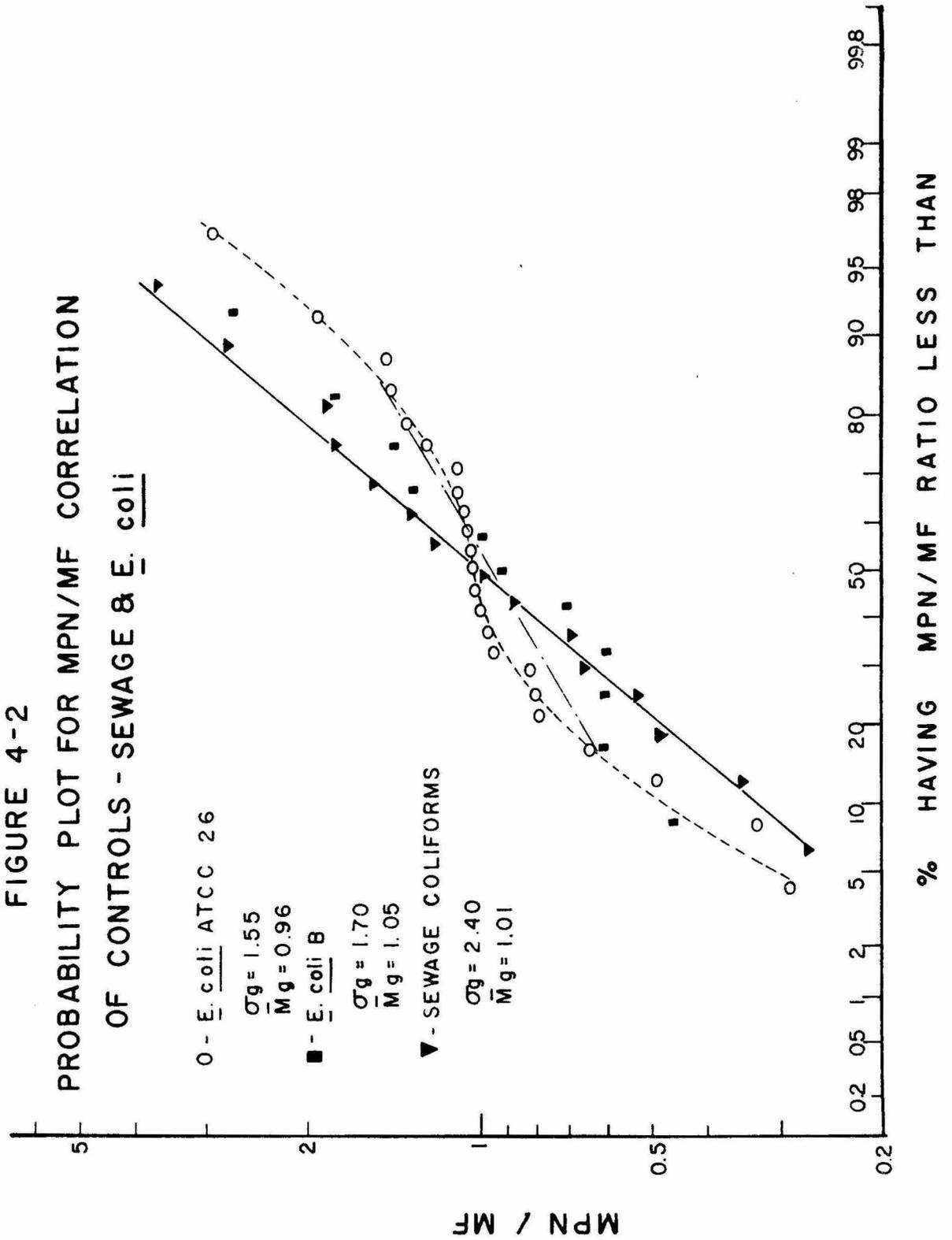


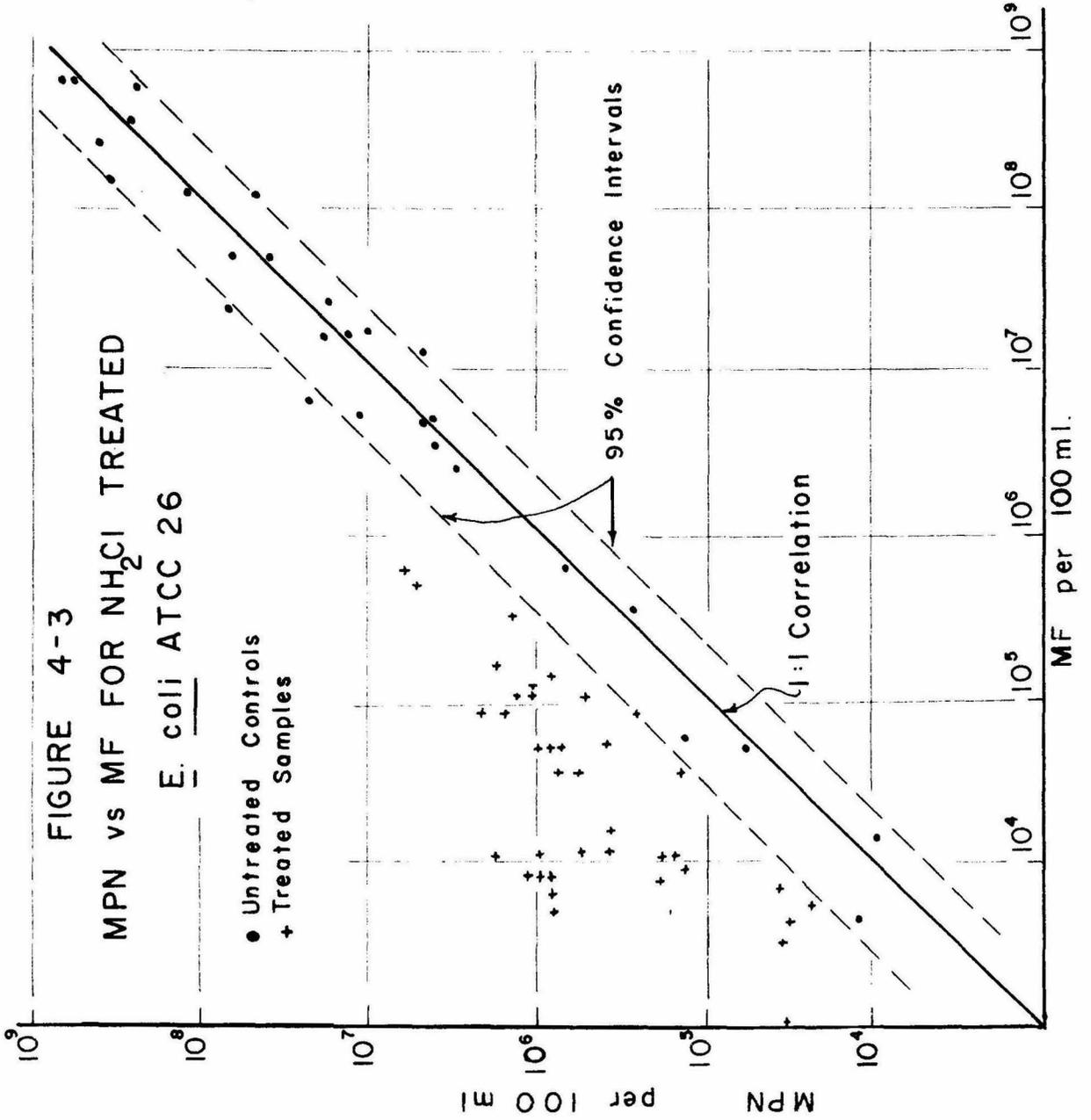
TABLE 4-3

EFFECT OF EXPOSURE TIME TO NH_2Cl AND
HOLDING TIME IN BUFFER ON MPN/MF OF E. COLI ATCC 26

Trial	Exp. * Time Min.	Initial		t** Hrs.	Count t		Initial MPN MF	Final MPN MF
		MPN	MF		MPN	MF		
1	5	49×10^3	32×10^2	2	14×10^3	7×10^3	15.3	2.0
	10	33×10^2	7×10^2	2	49×10^3	15×10^2	4.7	3.2
	15	330	4	2	80	6	82	13.2
2	5	92×10^4	18×10^4	3	92×10^4	23×10^4	5.1	4.0
	10	35×10^4	41×10^3	3	35×10^4	90×10^3	8.5	3.9
	15	92×10^3	20×10^2	3	35×10^3	63×10^2	46	5.5
3	5	8×10^4	27×10^3	11	33×10^4	25×10^4	3.0	1.3
	10	8×10^3	100	11	46×10^2	26×10^2	80	1.8
4	5	22×10^5	38×10^4	22	13×10^6	3.5×10^6	5.75	3.7
	10	13×10^3	44×10^2	22	17×10^4	23×10^4	3.0	0.7
	15	49×10^2	5×10^2	22	23×10^3	1×10^3	9.8	2.3

* All samples exposed to 1 mg/liter NH_2Cl at 25° C

** Neutralized with excess sodium thiosulfate and held in PO_4 buffer at 25° C



show that both MPN and MF counts increased over the incubation periods, but that MF counts increased at a higher rate, thereby lowering the overall MPN to MF ratio.

Another group of experiments were made to investigate the effects of holding the prepared membrane filters on a preservative medium for 24 hours. One such holding medium described in Standard Methods (46) was prepared by adding 3.2-ml of a 12% solution of sodium benzoate to 100-ml of m-Endo broth. Sterile nutrient pads were soaked with this reagent, and filters were placed on the pads and incubated for 24 hours at room temperature. Results of a typical set of experiments appear in table 4-4. This preservative medium held cell concentrations constant. The results imply that increases of E. coli held in buffer resulted from growth of survivors and not from resuscitation of injured cells.

E. coli B, grown under the same conditions as the ATCC 26 strain and treated in the same manner, yielded results considerably different from those obtained with strain 26 (table 4-5). A series of tests was also designed to compare the effects of monochloramine on fresh versus refrigerated (held at 4° C for several days) suspensions. A probability plot, figure 4-4, indicates that these data are insufficient to determine geometric means accurately. Note that the MPN ratios are considerably lower for strain B (1.7) than for ATCC 26 (10.5). It was difficult to determine the effects of refrigerated storage of cells with respect to

TABLE 4-4

MPN-MF COUNTS OF MONOCHLORAMINATED E. COLI
(ATCC 26) HELD IN BUFFER VERSUS BENZOATE-ENDO BROTH

Sample	Immediate Count		24 Hours* Count		24 Hour** Holding	Initial	24 Hr.
	MPN	MF	MPN	MF	MF	$\frac{MPN}{MF}$	$\frac{MPN}{MF}$
Control	54×10^4	28×10^4	92×10^6	70×10^6	28×10^4	1.9	1.3
A	92×10^3	8×10^3	54×10^3	14×10^3	10×10^3	11.5	4.1
B	35×10^3	14×10^3	35×10^3	13×10^3	10×10^3	2.5	2.7
C	13×10^4	5×10^3	79×10^4	10×10^4	5.2×10^3	26.0	7.9
D	35×10^4	20×10^3	35×10^4	12×10^4	10×10^3	17.5	2.8
E	54×10^4	56×10^3	17×10^4	9×10^4	42×10^3	9.6	1.9

* Held 24 hours at 25° C in phosphate buffer after excess thiosulfate neutralization

** Holding media - sodium benzoate and m-Endo broth

Note: Samples treated with 1 mg NH_2Cl /liter for 15 min.

TABLE 4-5

MPN-MF ANALYSIS OF MONOCHLORAMINE TREATED *E. COLI* D

Batch No.	Controls			Samples*		
	MPN (/100 ml)	MF (/100 ml)	MPN/MF	MPN (/100 ml)	MF (/100 ml)	MPN/MF
1	11×10^6	6×10^6	1.80	2.4×10^9	9.0×10^8	2.67
	9.2×10^6	13×10^6	0.71	7.9×10^7	6.0×10^7	1.32
	7.9×10^6	13×10^6	0.61	1.3×10^6	5.7×10^6	0.23
	9.2×10^6	15×10^6	0.61	1.1×10^5	4.0×10^5	0.28
2				4.5×10^5	4.0×10^5	1.12
				3.5×10^5	1.6×10^5	2.18
				9.2×10^4	4.3×10^4	2.14
	1.7×10^9	1.2×10^9	1.42	7.9×10^7	2.2×10^7	3.58
	7.9×10^7	13×10^7	0.61	11×10^5	8×10^5	1.38
	11×10^8	11×10^8	1.00	14×10^4	4.5×10^4	3.12
				13×10^5	12×10^5	1.08
				7.9×10^4	8×10^4	0.99
				5.4×10^4	2.0×10^4	2.70
				7.0×10^7	18×10^7	0.39
			13×10^6	5.2×10^6	2.50	
			22×10^5	7.9×10^5	2.82	

TABLE 4-5 (Cont'd)

Batch No.	Controls			Samples*		
	MPN (/100 ml)	MF (/100 ml)	MPN/MF	MPN (/100 ml)	MF (/100 ml)	MPN/MF
3	11×10^8	12×10^8	0.92	9.2×10^5	7.7×10^5	1.18
	7.9×10^6	17×10^7	0.46	3.5×10^4	2.8×10^4	1.25
	7.9×10^6	5.9×10^6	1.34	2.4×10^6	1.7×10^6	1.41
	4.9×10^7	1.8×10^7	2.70	9.2×10^4	2.3×10^4	4.00
				2.0×10^4	2.1×10^4	0.95
				9.2×10^2	3.5×10^2	2.63

$$\frac{MPN}{MF} = 1.05$$

Fig. 4-2

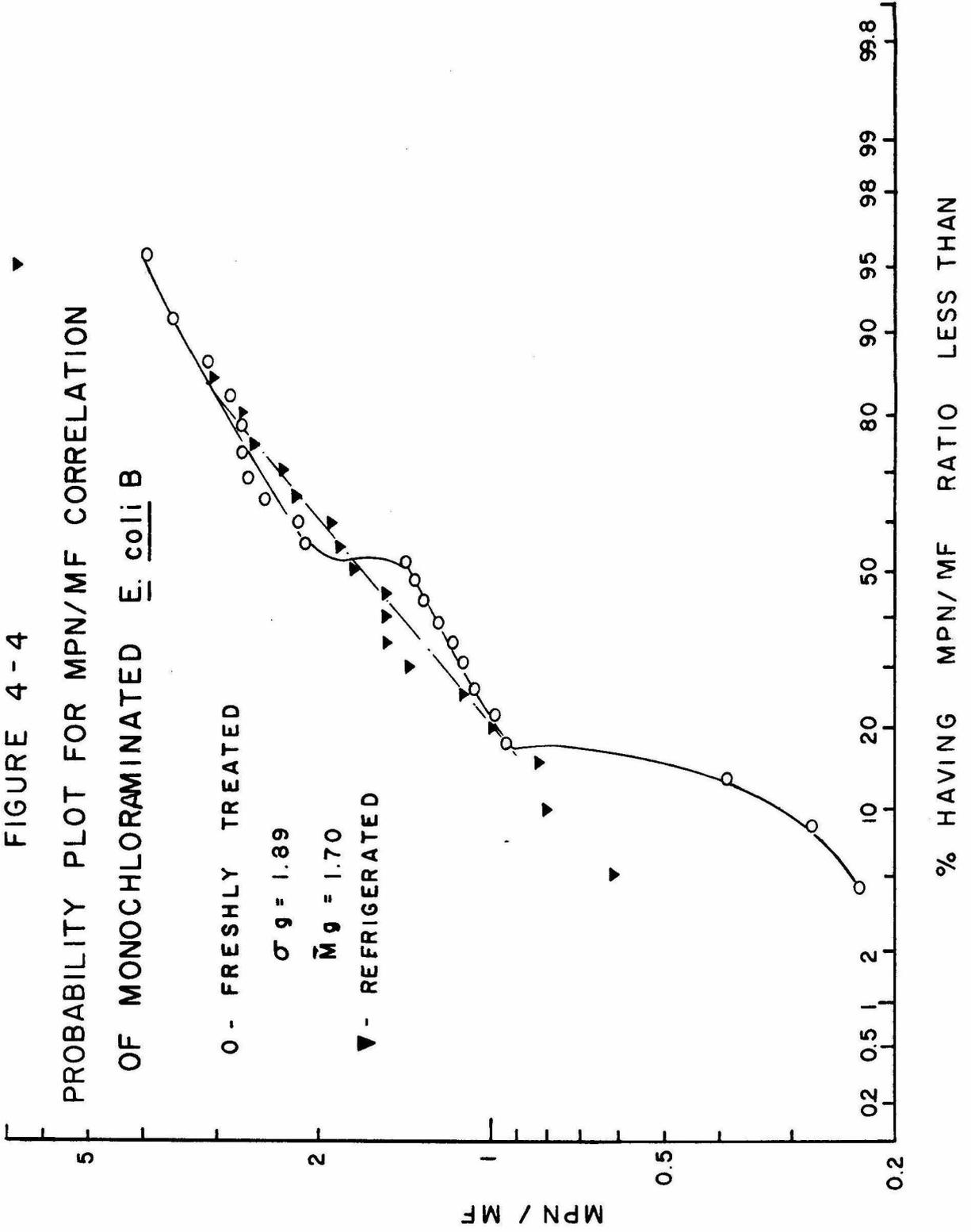
$$\sigma_g = 1.70$$

$$\frac{MPN}{MF} = 1.70$$

Fig. 4-4

$$\sigma_g = 1.89$$

* Treated with 1 mg/1 of SO_2Cl for 10 min. at 25° C



MPN-MF discrepancies. The controls were plotted on probability paper (figure 4-2), but the number of samples were not great enough to determine the geometric mean or standard deviation.

Both strains of E. coli were tested in autoclaved sewage, the results of which are compiled in table 4-6. It is interesting to note that MPN-MF discrepancies are approximately the same in sewage as in buffered water, and, that again, strain 26 gave markedly higher ratios of MPN to MF counts than did strain B.

D. RESULTS - PHASE II

During the study of MPN-MF enumerations, it was noted on several occasions that neutralization with heavy excesses of sodium thiosulfate yielded appreciable increases in numbers of viable cells recovered from the buffer held for a 48-hour period. This phenomenon was studied in greater detail using a carefully designed set of controls.

One hundred ml of washed E. coli ATCC 26 cells in phosphate buffer were added to each of seven 200-ml flasks. Five flasks, designated as controls which received no monochloramine, were prepared as follows:

Control 1 - E. coli in buffer.

Control 2 - E. coli cells diluted to approximate viable population of treated sample. At the appropriate time this flask was inoculated with monochloramine-killed cells plus excess sodium thiosulfate.

Control 3 - E. coli cells adjusted to approximate population of treated cells and exposed to excess sodium thiosulfate.

TABLE 4-6

MPN-MF COUNTS FOR MONOCHLORAMINE
TREATED E. COLI ATCC 26 AND B IN STERILE SEWAGE

Sample	<u>E. coli</u> ATCC 26		<u>E. coli</u> B	
	MPN	MF	MPN	MF
Controls	24×10^6	18×10^6	7.9×10^6	13×10^6
	35×10^6	42×10^6	16×10^6	14×10^6
	17×10^6	21×10^6	19×10^6	15×10^6
A	49×10^2	13×10	7.9×10^2	6×10^2
	78×10^2	6×10^2	24×10^2	13×10^2
	70×10^2	5×10^2	35×10^2	21×10^2
B	49×10^3	5×10^3	5.4×10^3	2×10^3
	17×10^3	2×10^3	7×10^3	5×10^3
	24×10^3	2×10^3	2.4×10^3	1.7×10^3
Avg $\frac{\text{MPN}}{\text{MF}}$ of A and B	15.8		1.8	

Note: Samples treated with 10 mg/1 NH_2Cl - 15 min. at T = 25° C.
pH = 7.1

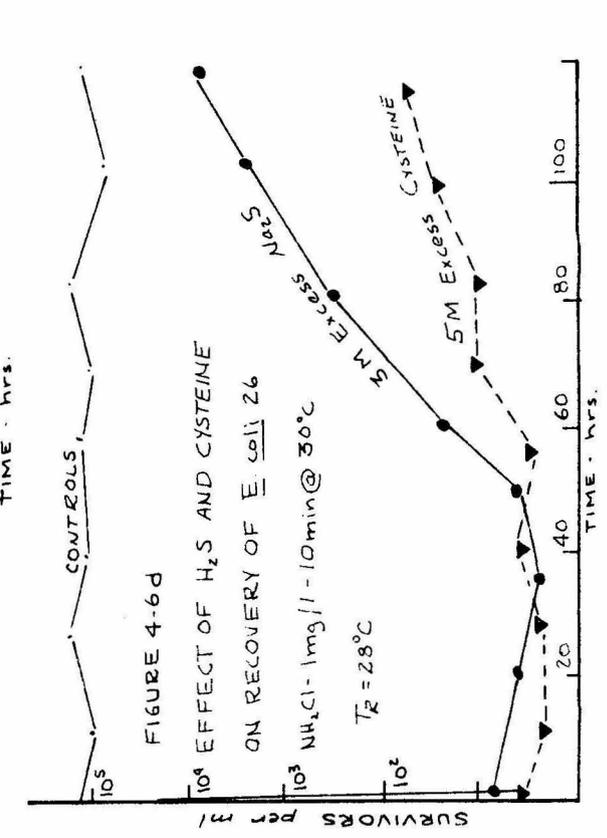
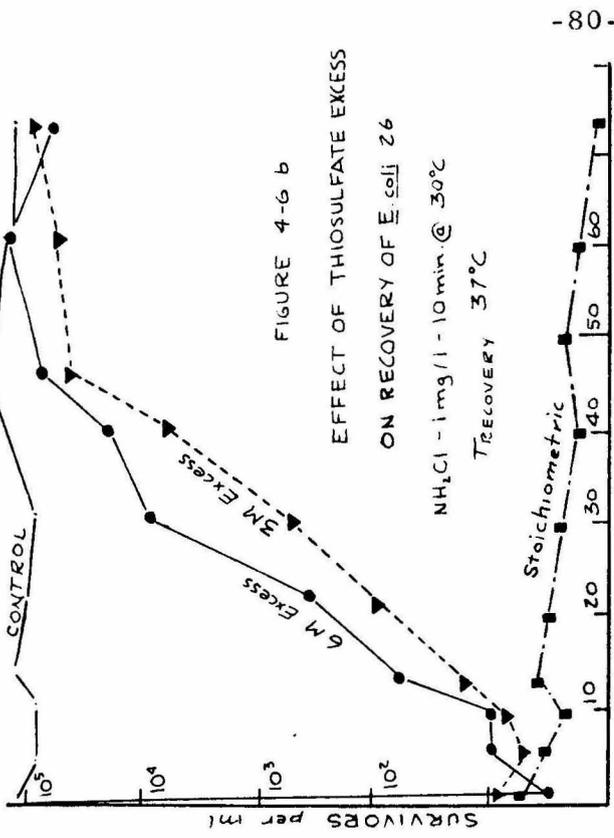
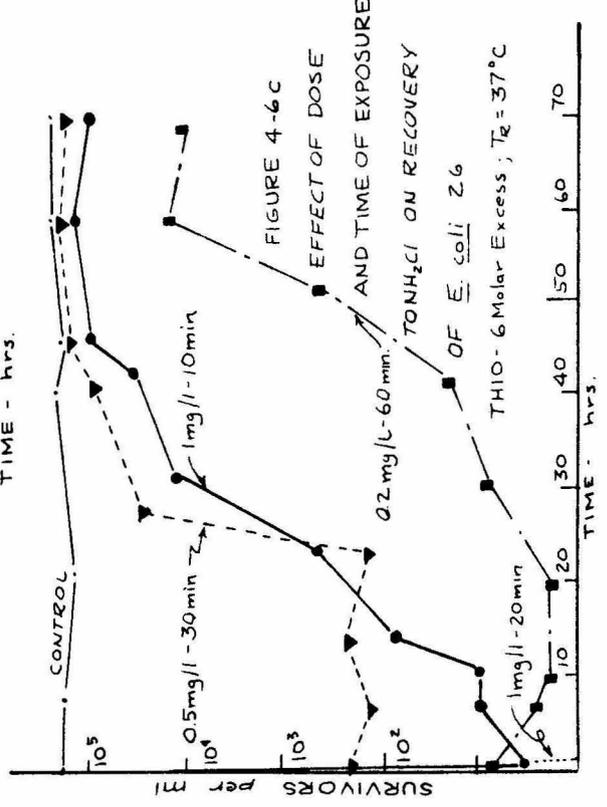
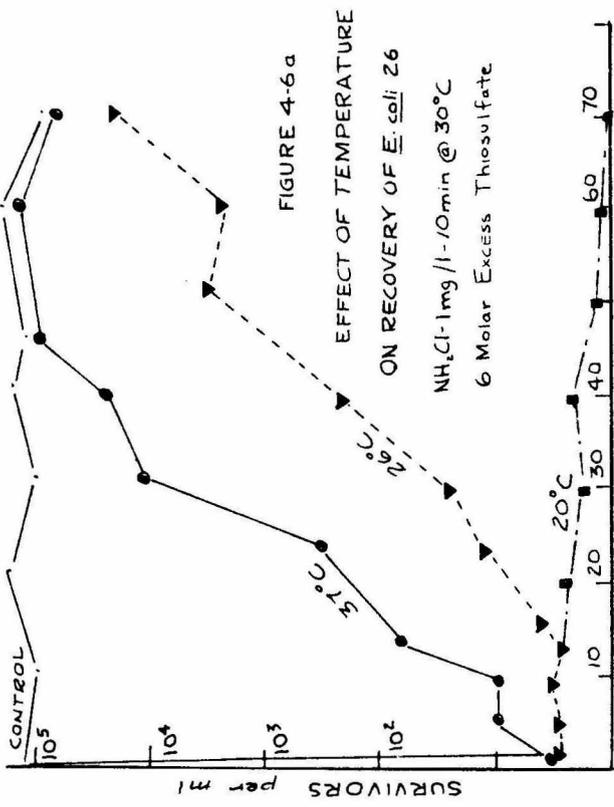
Control 4 - E. coli cells killed with heat to an extent comparable to that of monochloramine-treated cells and exposed to sodium thiosulfate.

Control 5 - E. coli cells diluted to approximate population of treated cells. At appropriate times this flask was inoculated with heat killed cells plus excess sodium thiosulfate.

Duplicate samples were treated with 1 mg monochloramine/liter for 10 minutes, at which time an excess of sodium thiosulfate was added (3 times the stoichiometric amount according to the equation: $\text{NH}_2\text{Cl} + 2 \text{H}^+ + 2 \text{S}_2\text{O}_3^{--} \rightarrow \text{NH}_4^+ + \text{Cl}^- + 2 \text{S}_4\text{O}_6$). Samples were withdrawn at intervals, diluted and plated in triplicate. Results of a typical test are shown in figure 4-5. As can be seen, the controls maintained a steady level, whereas monochloramine treated suspensions neutralized with excess sodium thiosulfate showed considerable recovery starting within 10 hours, apparently complete in approximately 50 hours.

In a series of further experiments (figure 4-6) the dose of monochloramine, time of exposure, holding temperature and the amount of thiosulfate excess used in neutralization were varied. The results from these tests are summarized as follows:

(a) In all cases the addition of stoichiometric amounts of sodium thiosulfate to monochloramine treated suspensions of E. coli never permitted cell recovery. In fact, the surviving cells (after neutralization) usually disappeared completely within 24 hours.



(b) Additions of from 3- to 6-fold stoichiometric excesses of sodium thiosulfate resulted in enhanced recovery of the cells. At 37° C. there was a definite increase in population over a 60-hour period, recovery beginning within 6 to 8 hours after treatment and neutralization. This recovery was also characterized by a closer correlation in the MPN and MF counts. Recovery was also detected at 26° C. with these excesses of sodium thiosulfate, being initiated some 6 hours later than at 37° C. At 20° C. excess sodium thiosulfate did not affect recovery of E. coli for periods as long as 72 hours.

(c) High concentrations of chlorine (2 mg/liter) and long periods of exposure rendered cells incapable of recovery regardless of the quantity of thiosulfate used. In general, exposure of organisms to 1 mg monochloramine/liter for over 20 minutes prohibited recovery of cells, whereas even after 1 hour at 0.2 mg/liter, recovery was noted.

(d) In most recovery studies the cell densities approached those of the original sample.

To study further the notion that the disinfecting activity of monochloramine is related to the oxidation of certain critical sulfhydryl groups (17), several good reducing agents were tested in a manner similar to that of sodium thiosulfate. A 1×10^{-3} M solution of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ in phosphate buffer was sterilized by filtration through a 0.22 μ HA millipore filter and used immediately. Two to three molar excesses yielded recovery phenomena similar to those found with sodium thiosulfate (figure 4-6). Usually

recovery at 25° C. was initiated within 20-40 hours after neutralization.

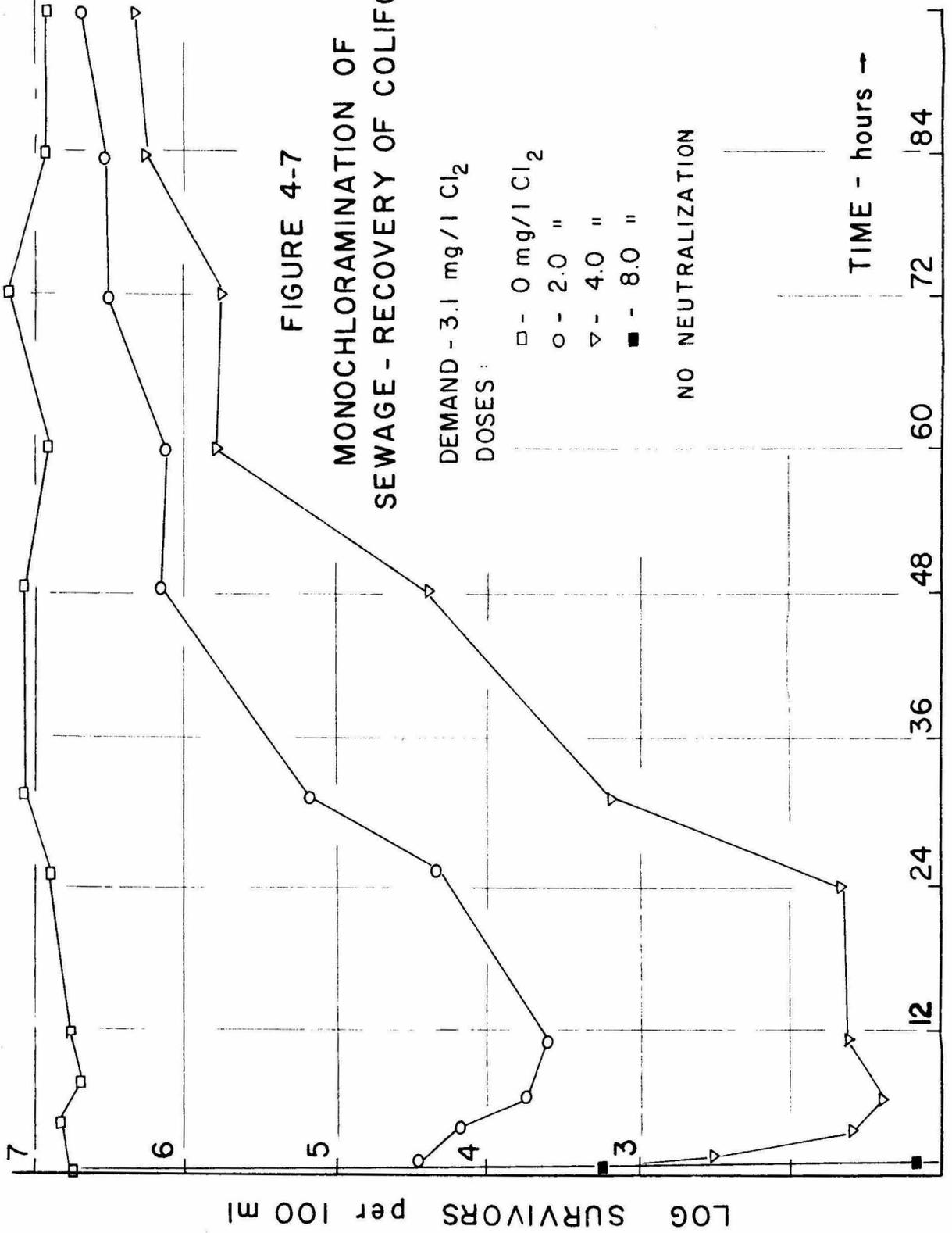
Similar experiments were also performed with cysteine and cystine. Great excesses of cysteine (5-10 molar excesses) did not yield significant recovery, although on occasion some recovery was detected. Thus, cystine proved to be a poor reagent for recovery of monochloraminated coliforms.

A series of samples of settled sewage were treated with monochloramine without thiosulfate neutralization. It was found that if monochloramine residuals reached zero in less than 2 hours, recovery occurred (figure 4-7). Dilution of chloraminated sewage, however, in phosphate buffer did not result in noticeable recovery of coliforms, although other organisms began to increase in population.

C. DISCUSSION

The foregoing studies were designed to investigate the suspected bacteriostatic nature of monochloramine through indirect counting techniques and through studies of variables affecting the recovery of chloraminated bacteria. It is important to note that the statistical analysis of MPN-MF ratios is not completely valid. It has been shown that the reproducibility of the 5-tube serial dilution technique is theoretically inferior to the plate count (and MF) procedure, assuming that the coliform species grow equally well under the environmental conditions presented by both procedures (65). Replicates in plate count

FIGURE 4-7
MONOCHLORAMINATION OF
SEWAGE - RECOVERY OF COLIFORMS



techniques follow a normal (Poisson) distribution, whereas the dispersion of MPNs from multitube tests accords with an entirely different probability model. Cochran (66) has shown that the logarithm of the MPNs of replicate tests have an approximately normal distribution. Thus, if one compares 10 replicate plate counts against ten 5-tube MPN serial dilutions for a true bacterial population of 50 per ml, the range for plate counts would be 39 to 61 per ml, whereas the range for the MPNs would be 21 to 120 per ml. Thomas (50) has further shown that the MPN is inherently biased on the positive side resulting in a skewed distribution about the true value. Woodward (64) has prepared tables of confidence limits for the 5-tube MPN tube technique taking these factors into account. He shows that for a 5-tube serial dilution technique the 95% confidence intervals are on the average from 31% to 289% of the estimated MPN. However, the limits range even wider for highly improbable positive combinations (0, 2, 5, for example) or for those which nearly missed the range (1, 0, 0 or 5, 5, 4). In the experiments performed in this investigation, triplicate MF counts were analyzed along with single 5-5-5 MPN counts. It is difficult to conceive that the ratio of these numbers (or any comparison of these values) could be used in a valid statistical study. In addition, the data are, in many cases, insufficient to derive a reasonable value of the geometric mean and geometric standard deviation. The chief purpose of the MPN-MF analysis was to determine trends in these techniques on chlorinated wastes, and no attempt will be made to

utilize the statistical data for purposes other than to describe trends. Experiments revealing marked variation in ratios of MPN:MF coliform counts emphasize the difficulty in interpretation of such kinetic studies. Obviously there are many variables, several of which were noted; namely, the conditions under which the test organisms were grown and treated and the strain of bacterium studied. Since two entirely different media are used in the two counting procedures, it is not surprising that the methods do not agree; nor from such results can one even speculate on the affected mechanisms in the bacterial cell. The results with sewage indicate that a significant portion of coliform bacteria treated with monochloramine which do not survive on membrane filters will thrive in lactose broth. This difference is not nearly as striking with treated pure cultures of E. coli in buffer and sterilized sewage as with sewage coliforms. These facts suggest that the two techniques detect a different spectrum of strains and species of coliforms. Thus, the enumeration of only the sheen colonies appearing on m-Endo medium will not include all of the gas-producing lactose fermenters present, i. e., Aerobacter aerogenes does not produce sheen colonies on Endo agar, but does produce gas from lactose (67). Although control samples show these differences to be slight, treatment with monochloramine might have exerted a selective action on the coliforms, favoring survival of those organisms which ferment lactose but do not produce a sheen on Endo agar.

This argument does not answer the question of why the MPN and MF methods did not agree when pure cultures of E. coli were tested. There are, however, several valid arguments for these discrepancies. The typical metallic sheen produced on Endo agar by colonies of E. coli results from entrapment of the metabolite, acetaldehyde, by sulfite to result in the release of fuchsin dyes (67). Treatment of cells with monochloramine (a) may render them incapable of producing acetaldehyde when fermenting lactose or (b) may enhance the sensitivity of cells against sulfite or basic fuchsin so that metabolic processes cease to function. It has been shown that the sulfite-fuchsin environment is often toxic to normal coliforms (51), although the concentration of these compounds in the LES m-Endo agar preparation is lower than is customarily found in differential media for coliforms. It is also conceivable that monochloramine might affect cell permeability, thus disrupting diffusion of materials in and out of the cells, especially for those on solid media. The cells, it must be remembered, are impinged upon the membrane leaving only a small area in contact with essential nutrients. Moreover, cells weakened by monochloramine -- and even "healthy" cells -- may be damaged when they strike the membrane, rendering them incapable of normal function (68).

The MPN-MF discrepancies, however, pointedly suggest that treated cells recover more readily in lactose broth than on membrane filters. Under this pretext it is possible that the

differential in counts results from the ability of the cell in lactose broth to reverse those functions inhibited by monochloramine through such means as: (a) diffusion of monochloramine out of the cell (b) biochemical reduction of affected chemical groups, or (c) substitution of another compound in an affected metabolic pathway. Thus, lactose broth would seem to provide such mechanisms more easily than m-Endo agar.

The data obtained in recovery tests with treated sewage substantiate to a great degree the findings of Heukelekian (32), Allen and Brooks (36), Howard (33), and Rudolfs and Gehm (34). Allen and Brooks and also Rudolfs and Gehm attributed growth of coliforms in streams receiving chlorinated sewage to the death of predatory organisms in sewage. Hanging drop techniques in this investigation showed that in all cases where after-growths of coliforms occurred in chloraminated sewage, such predators as protozoa and certain of the ciliates were absent or inactive. Sewage is also very rich in its variety of nutrients, some of which might stimulate recovery of injured cells and/or growth of survivors. The theories of reactivation of treated cells in sewage are difficult to prove because of the many complexities of the medium.

The implications of the results from chloramination of pure cultures are far reaching, but the mechanisms of the observed phenomena are still not clear. It was hoped to determine whether recovery of chloraminated coliforms was the result of resuscitation (revival of injured cells) or growth of chloramine-

resistant survivors. On the basis of the reactions of the controls, it appears that cells were resuscitated in the presence of excess thiosulfate. Yet one might argue that recovery resulted from slow growth of surviving cells (0.5 to 1 cell division per hour). It has been shown by Garvie (40) that E. coli can multiply in buffered water and that the presence of dead bacteria improved this growth considerably. If one also assumes that monochloramine treatment may result in the leakage of certain constituents of cells, this argument would be strengthened further. Unfortunately no data exist to support this notion. Controls 2 and 4 (figure 4-5) were included to determine whether dead cells (or their constituents) provide nutrients which can be utilized by survivors to permit growth. Since these controls exhibited no increase in cell population over the test period, this possibility seems unlikely, regardless of whether the cells added as nutrients were killed by heat or by monochloramine.

It is interesting to note that the recovery phenomenon is related to temperature. Cells recovered more rapidly at higher temperature than at lower ones, and, in fact, no recovery was detected below 20° C. A chemical reversibility would be temperature linked, undoubtedly, but one would expect that chemical reaction rates would not cease at 20° C. On the other hand, bacterial growth may well be static at a temperature of 20° C. for long periods of time.

Another argument in favor of recovery of partially blocked cells relates to the ultimate populations attained after recovery

was initiated. In most cases, viable counts approached original cell densities; never once did cell recovery exceed the population of the original population, although it would be surprising if such a weak "culture medium" were able to sustain a large amount of growth. Such results, while not incontrovertible, suggest a true recuperation effect.

The role played by sodium thiosulfate in the recovery reaction is not clear. Since other sulfur compounds, such as sodium sulfide and, to some extent, cysteine, also gave similar results, reversible oxidation-reduction of sulfhydryl and disulfide groups is suggested. Disulfides are known to be reduced by excess thiol compounds, sulfite and cyanide (69). One might propose that, under certain conditions, such chemical reactions might reverse the bactericidal effects of monochloramine or other powerful oxidizing agents. Green and Stumpf (28) have implicated sulfhydryl enzymes in their studies with chlorine, but they were not able to reactivate these enzymes with thiols.

The chapters that follow describe experiments designed to test many aspects of this problem in order to gain some insight into the mechanisms involved. A general discussion later will bring all of the data together, at which point certain hypotheses will be presented.

The results in the section clearly indicate that under specific conditions, bacteria will recover from treatment with monochloramine. The fact that such a phenomenon is not always reproducible suggests that the mechanisms are highly sensitive

and subject to numerous variables, few of which are known.

Whether aftergrowths in streams receiving sewage result from revival of injured cells or from growth of survivors, or both, cannot be proved. Evidence suggests that both mechanisms may be in force.

V. RELATIVE REACTIVITIES OF MONOCHLORAMINE WITH ORGANIC MATERIALS

There have been few reports in the literature on the chemical reactions of monochloramine with organic compounds (20, 21, 47). From a fundamental point of view, such information would be extremely helpful in investigating the mode of action of monochloramine. A purely chemical study of monochloramine would provide many years of interesting research but, however, it would be beyond the initial aims of this investigation. Therefore, the qualitative and semi-quantitative experiments in this section were pursued only to the extent that they might suggest certain cellular sites of reactivity with monochloramine pertinent to this study. Considerable evidence, both in the literature (28) and in this preliminary investigation, has pointed to enzymes as being vulnerable to the action of chlorine. For this reason, amino acids, the building blocks of protein, were initially studied. Later studies included the genetically important purine and pyrimidines, several of the simple sugars and, finally, bacterial cells.

A. RESULTS

1. L-Alanine

No information was found in the literature which dealt specifically with the reaction between monochloramine and the amino acids. One of the simplest amino acids, alanine, was selected as a prototype for a study of the reactions of amino acids with monochloramine. Typical results of a nitrogen analysis during the reaction of approximately 1:1 molar concentrations of

alanine (4.16×10^{-4} M) and monochloramine (4.39×10^{-4} M) are given in table 5-1. It is interesting to note that ammonia nitrogen increased with time until essentially all of the amino nitrogen was present in this form. There was little or no nitrate or nitrite formed during this reaction. Note that the values of Kjeldahl and ammonia nitrogen are higher than those computed for the total molar concentrations of alanine and monochloramine. This is due to the excess ammonium sulfate in the monochloramine solution. (Monochloramine itself yields a positive NH_3 test upon direct Nesslerization.)

In order to ascertain the presence of other end products, 10^{-4} M quantities of alanine and monochloramine were added to the reaction tube of the aeration system. After 60 minutes the acidified p-hydroxydiphenyl gave a deep purple color indicating the presence of acetaldehyde (53). The test for α -keto acids, however, was negative.

The UV spectrum of the mixture was determined in order to gain further information on the intermediates formed in this reaction. Absorbance of the 1:1 molar concentrations (4.39×10^{-4} M) of alanine and monochloramine in a 5-cm quartz cell were measured at various wave lengths and at appropriate intervals of time (figure 5-1). Alanine absorbs weakly in the region between 220-300 m μ , having a maximal molar absorptivity of 17 at 220 m μ . Molar absorptivities of monochloramine are given in table 5-2 for selected wave lengths. Molar absorptivities of the mixture, calculated by Beer's equation, decreased with time at 245 m μ but increased with

TABLE 5-1
TYPICAL RESULTS
FOR

MONOCHLORINATION OF ALANINE

$\text{NH}_2\text{Cl} - 4.39 \times 10^{-4} \text{ M}$
 $\text{Alanine} - 4.16 \times 10^{-4} \text{ M}$

Time Min.	Reducible Chlorine Residual* Molar $\times 10^4$	Kjeldahl Molar $\times 10^4$	Nitrogen		
			NH_3 Molar $\times 10^4$ (Nitrogen)	NO_2^-	NO_3^-
0	4.39 (31.2 mg/L)	10.0 (14.0 mg/L)	5.62***	0	Trace**
41	3.89	-	6.00	-	-
71	3.64	-	6.29	0	Trace
106	2.88	-	6.61	-	-
136	2.62	-	7.46	0	Trace
190	1.96	-	8.47	-	-
233	1.64	9.92	9.29	0	Trace
271	1.41	-	9.71	-	-
315	1.12	-	9.78	-	-
403	0.89	-	10.05	0	Trace
471	0.69	10.08	10.02	0	Trace

* Residuals by Starch-Iodide

** Trace - Less than .003 mg/L as N.

*** Initial NH_3 from Monochloramine (Residual + Excess $(\text{NH}_4)_2\text{SO}_4$)

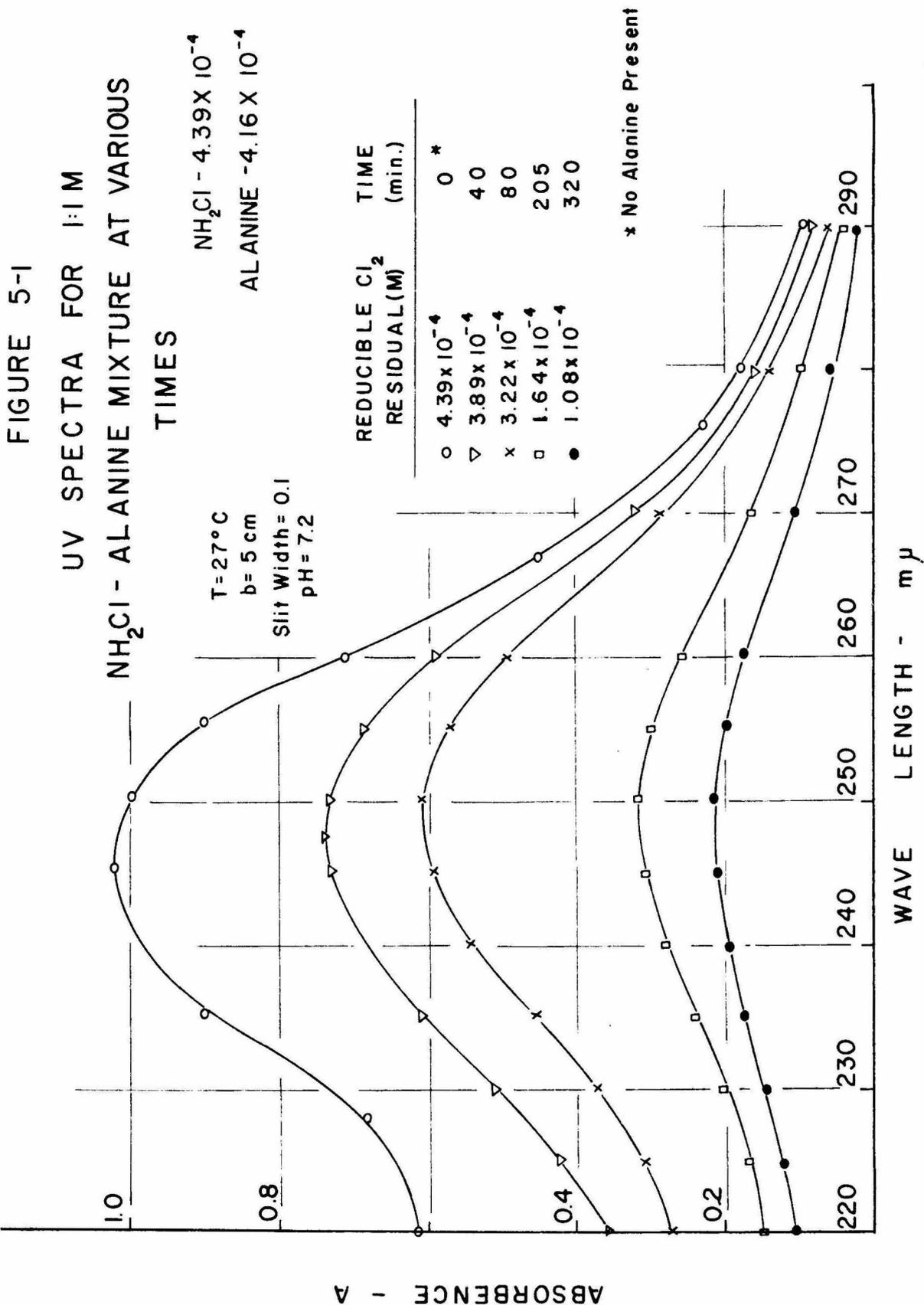
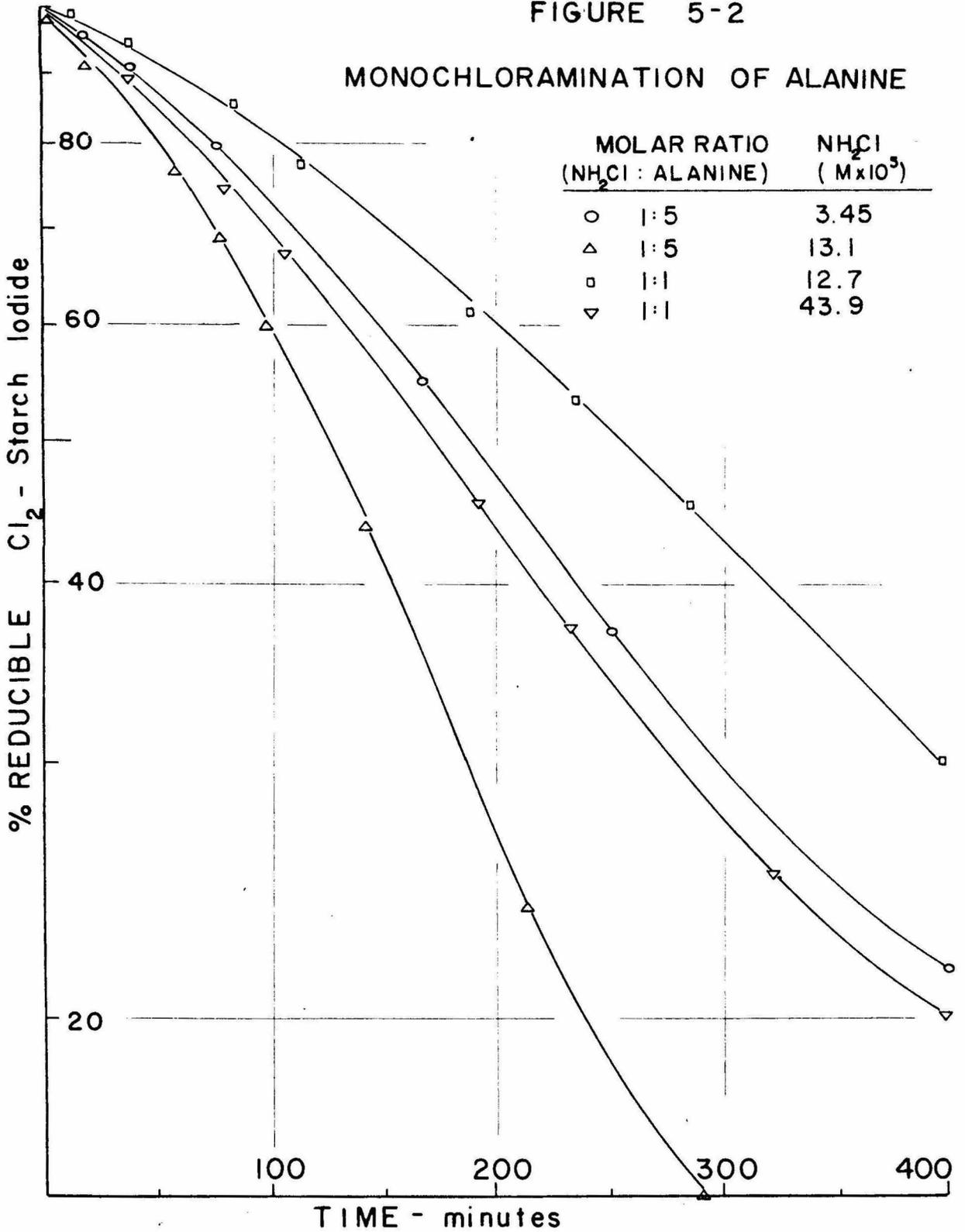


TABLE 5-2
MOLAR ABSORPTIVITIES
OF MONOCHLORAMINE

<u>Wave Length</u> <u>mμ</u>	<u>Molar Absorptivities</u> <u>liter moles⁻¹ cm⁻¹</u>
300	20
280	76
260	320
245	458
240	440
230	275
222	220

FIGURE 5-2

MONOCHLORAMINATION OF ALANINE



time in the direction of 250 m μ . This indicates that a shift in the spectrum toward the longer wave lengths occurred.

Several concentrations of monochloramine were reacted with alanine at various molar ratios in order to characterize this reaction better (figure 5-2). Two pieces of information were gained from this analysis: (a) the reaction rate is dependent upon the concentration of alanine; and (b) the reaction rate is not first-order with respect to monochloramine. In order to check whether this reaction is second-order, $\log \frac{b}{a} \frac{(a-x)}{(b-x)}$ vs time was plotted, where a and b are the initial molar concentrations of monochloramine and alanine, respectively, and x is the number of moles per liter which reacted in time, t. Again, however, the curve was not linear, indicating that the reaction was more complex. This is not surprising since one might conclude from these brief tests that at least one intermediate is present in this reaction.

2. L-Cysteine

The reaction of cysteine and monochloramine was studied in a nitrogen atmosphere since cysteine oxidizes rapidly in the presence of oxygen. A typical reaction curve shown in figure 5-3 reveals an initial rapid decrease in monochloramine, followed by a slower rate of loss. An UV spectral analysis of the mixture, figure 5-4, illustrates that at least one intermediate was formed in this reaction, having a maximum peak at 250 m μ and a minimum peak at 235 m μ . The absorption spectrum of cysteine at its initial concentration is also

FIGURE 5-3

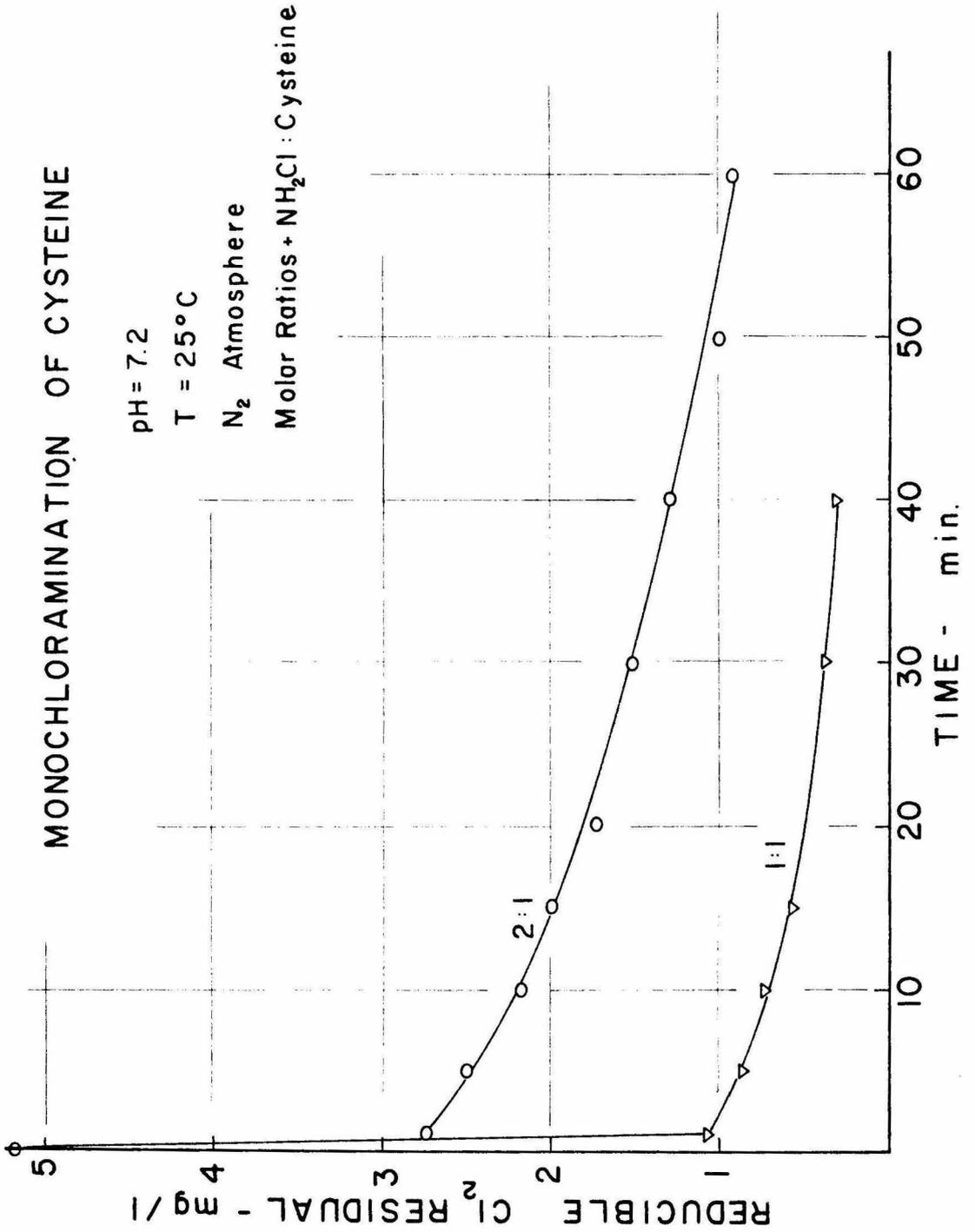


FIGURE 5-4

UV SPECTRA
1:1 MOLAR $\text{NH}_2\text{Cl} : \text{CYSTEINE}$ MIXTURE

TIME AFTER ADMIXTURE - 1 min.

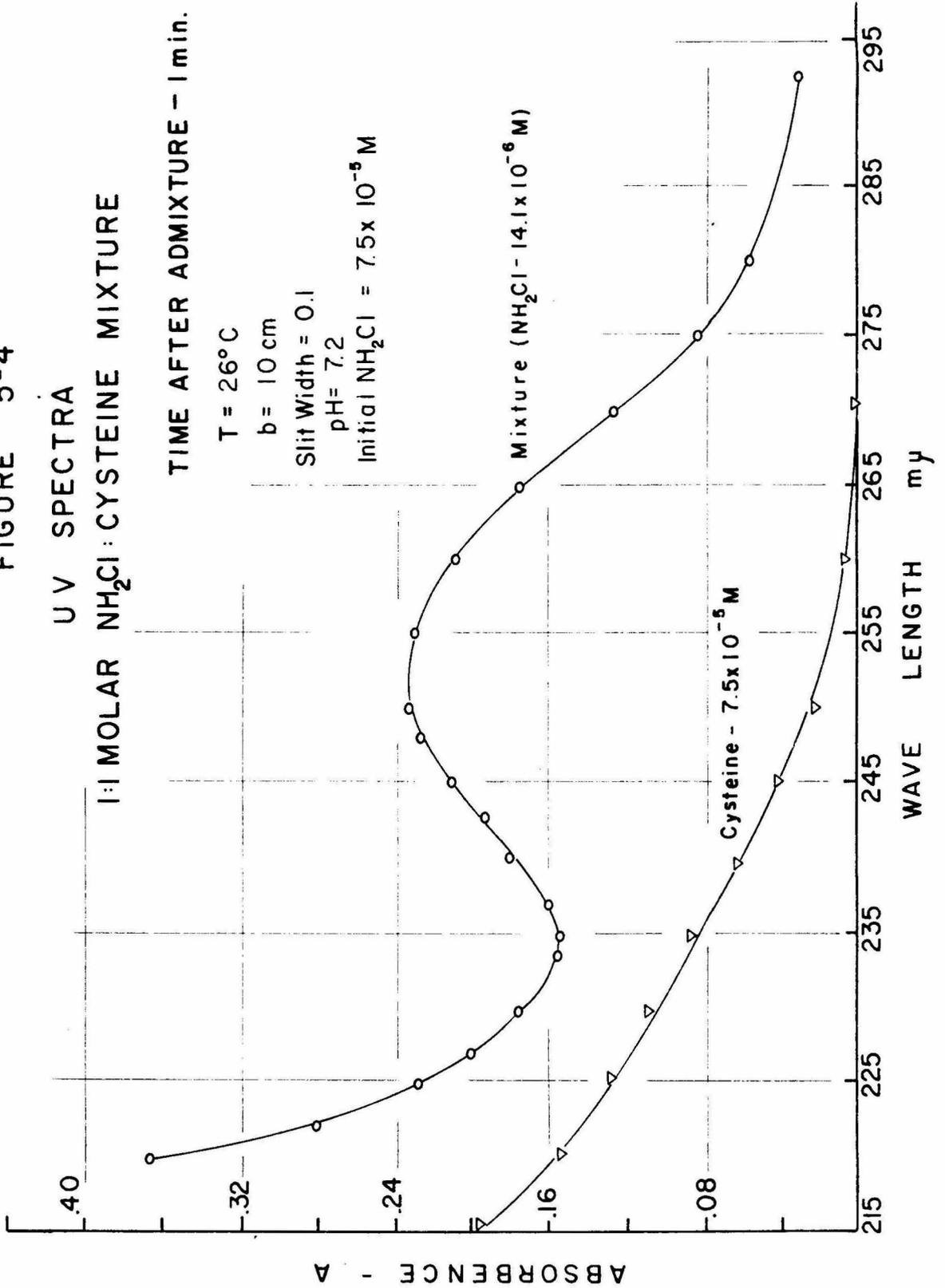
$T = 26^\circ\text{C}$

$b = 10 \text{ cm}$

Slit Width = 0.1

$\text{pH} = 7.2$

Initial $\text{NH}_2\text{Cl} = 7.5 \times 10^{-5} \text{ M}$



presented in this figure.

The reaction of 1:1 molar concentrations (7.5×10^{-5}) of cysteine and monochloramine indicates that all -SH is lost almost immediately upon mixing these compounds (table 5-3). Additions

TABLE 5-3

SH CONCENTRATIONS IN NH_2Cl TREATED CYSTEINE

Control (1.5×10^{-4} M SH)		1:1 M (7.5×10^{-5} M NH_2Cl)		1:2 M (7.5×10^{-5} M NH_2Cl)	
T (Min)	SH (μ Moles)	T (Min)	SH (μ Moles)	T (Min)	SH (μ Moles)
0	157	0	74	0	153
1	158	1	0	1	70
10	150	10	0	10	66

In N_2 atmosphere at 26°C , pH 7.2 (phosphate buffer)

of up to 6 molar excesses of sodium thiosulfate one minute after mixing did not reverse this reaction (table 5-4).

TABLE 5-4

SH CONCENTRATIONS IN NH_2Cl TREATED CYSTEINE
WITH 6 MOLAR EXCESS THIOSULFATE

Control (1.5×10^{-4} M SH)		1:1 M (7.5×10^{-5} NH_2Cl)		1:2 M (7.5×10^{-5} NH_2Cl)	
T (Min)	SH (μ Moles)	T (Min)	SH (μ Moles)	T (Min)	SH (μ Moles)
0	152	0	72	0	157
→ 1	154	→ 1	0	→ 1	74
2	153	2	0	2	73
10	150	10	0	10	65
30	146	30	0	30	62

In N_2 atmosphere at 26°C , pH 7.2 (phosphate)
Sodium Thiosulfate added →

3. L-Cystine · HCl

The reactions of L-cystine·HCl and monochloramine at pH 7.2 were studied at several molar ratios (figure 5-5). As with cysteine, there was a rapid initial reduction of monochloramine followed by a slower rate. The second reaction rate was not first- or second-order, and the cystine concentration was a factor in both reaction sequences. A disulfide analysis of a 1:1 molar mixture (3.5×10^{-5} M) of monochloramine and cystine indicated that all of the disulfide bonds were lost shortly after mixing. On the other hand, at a 1:2 molar mixture (3.5×10^{-5} M NH_2Cl) only 50% of the disulfide bonds were lost (table 5-5).

TABLE 5-5

SS CONCENTRATIONS IN NH_2Cl TREATED CYSTINE

Control (7.0×10^{-5} M SH)		1:1 Molar (NH_2Cl 3.5×10^{-5} M)		1:2 M (NH_2Cl 3.5×10^{-5} M)	
T	SS	T	SS	T	SS
(Min)	(μ Moles SH)	(Min)	(μ Moles SH)	(Min)	(μ Moles SH)
0	72	0	76	0	143
1	70	1	5	1	70
10	74	10	0	10	72

Treated at 25° C, pH 7.2 (phosphate buffer)

Reported in μ moles SH = 1/2 μ moles SS

4. DL-Methionine

Methionine, like the other sulfur-containing amino acids, exerts a rapid initial demand on monochloramine, followed by a slower reaction rate (figure 5-6). It is interesting to note,

FIGURE 5-5
MONOCHLORAMINATION OF CYSTINE

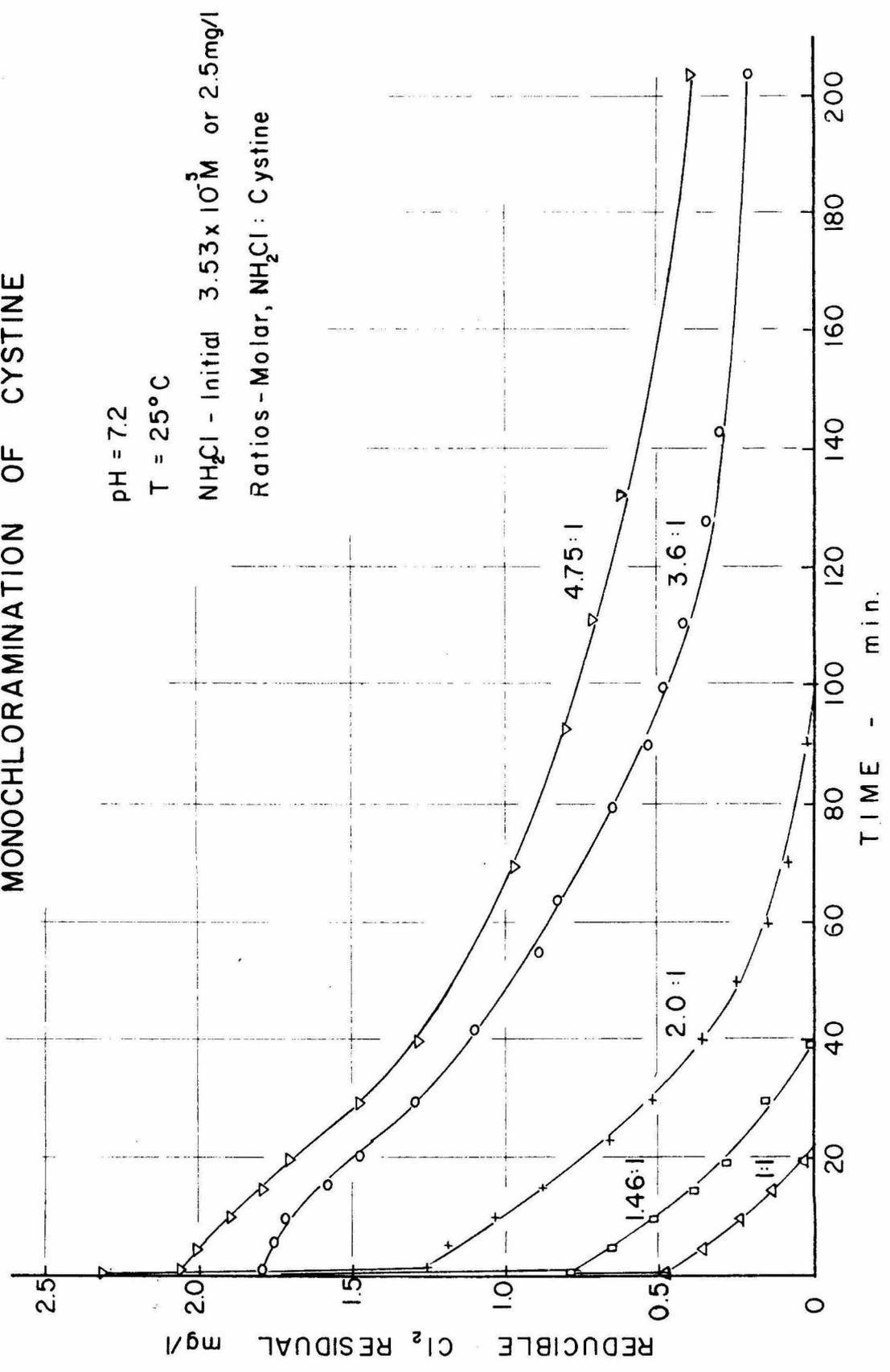


FIGURE 5-6

NH_2Cl REACTION WITH DL-METHIONINE

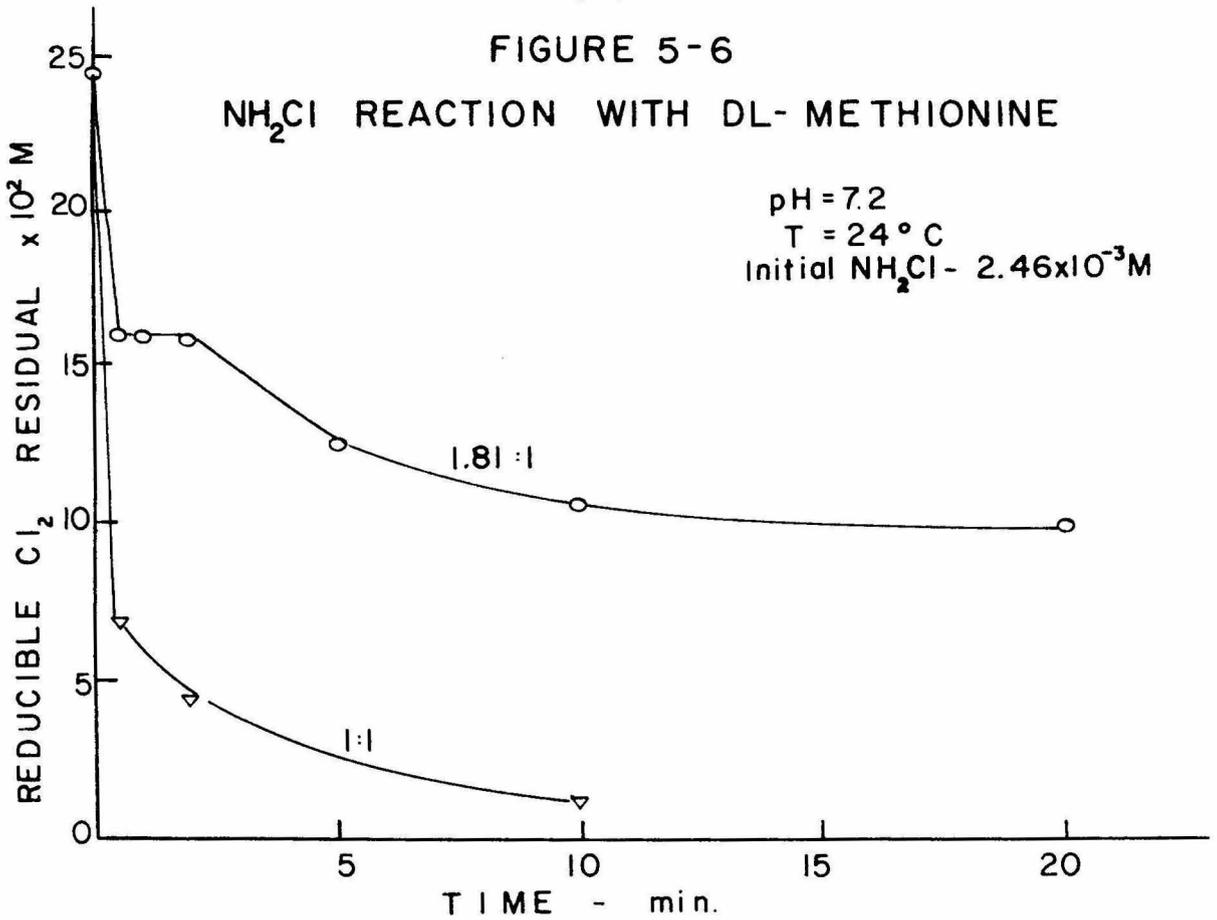
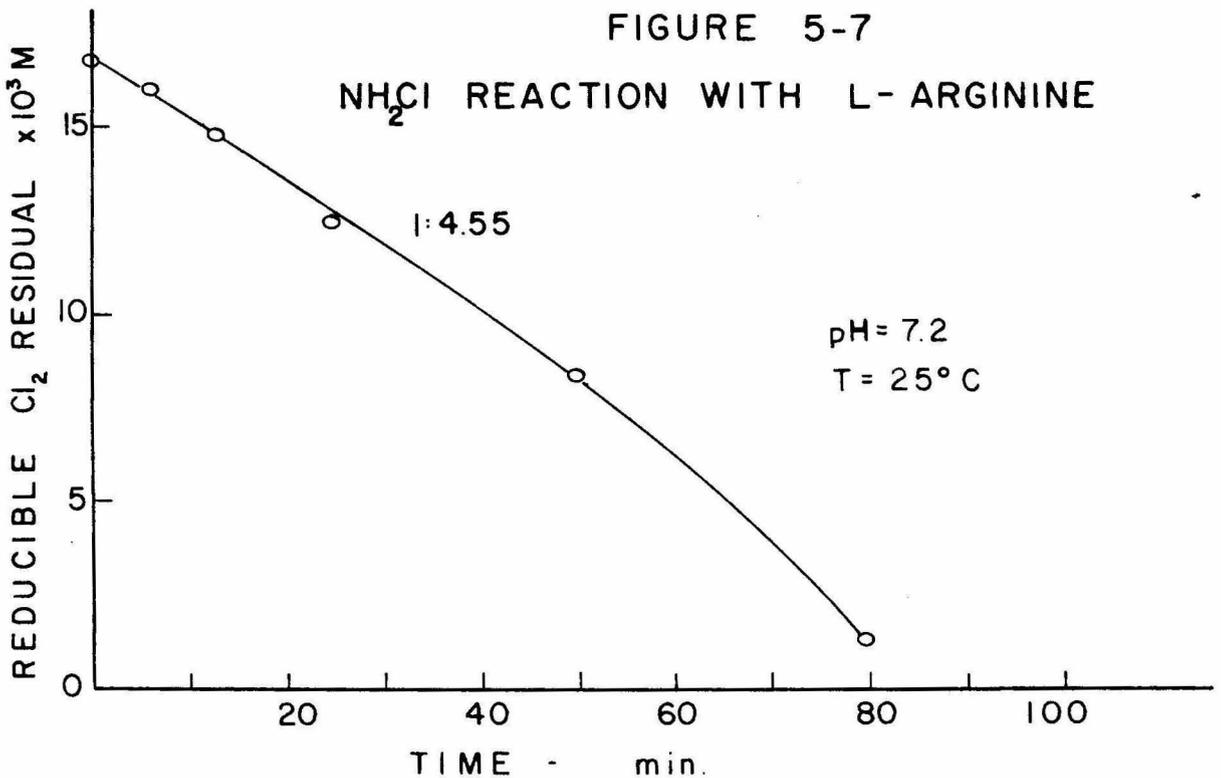


FIGURE 5-7

NH_2Cl REACTION WITH L-ARGININE



however, that in a 1:1 molar mixture of monochloramine and DL-methionine only about 75% of the residual chlorine is reduced in the initial reaction.

5. Other Amino Acids

The reaction rates of L-arginine·HCl, L-histidine and DL-tryptophan with monochloramine appear in figures 5-7, 5-8, and 5-9. With arginine a 1:4.55 molar ratio (NH_2Cl :arginine) was employed; the molar ratios of L-histidine and DL-tryptophan systems were 1:9.6. The reaction rates of these compounds with monochloramine were relatively slow, and were neither first- nor second-order. A deep pink color was detected in the mixture of tryptophan and monochloramine 15 minutes after admixture.

6. Purines and Pyrimidines

The rates of reaction between monochloramine and the purines, adenine sulfate and guanine sulfate, and the pyrimidines, uracil, thymine and cytosine, were not of the first-order and were relatively slow (figures 5-10 to 5-14). Guanine was tested in ammonia water at pH 11.3, since it was insoluble at the lower pH values of buffers used with the other compounds.

7. Sugars

Neither glucose, lactose nor ribose were found to react with monochloramine in 2 hours at room temperature (figure 5-15).

8. Escherichia coli B Cells

To 50-ml of E. coli B cell suspension, prepared as usual and adjusted to a density of 10^{10} cells/ml (14 mg/ml), 30 ml of demand-free water, 4 ml of a 25% glucose solution and 16 ml

FIGURE 5-8

NH_2Cl REACTION WITH DL-TRYPTOPHAN

pH = 7.2
T = 25°C

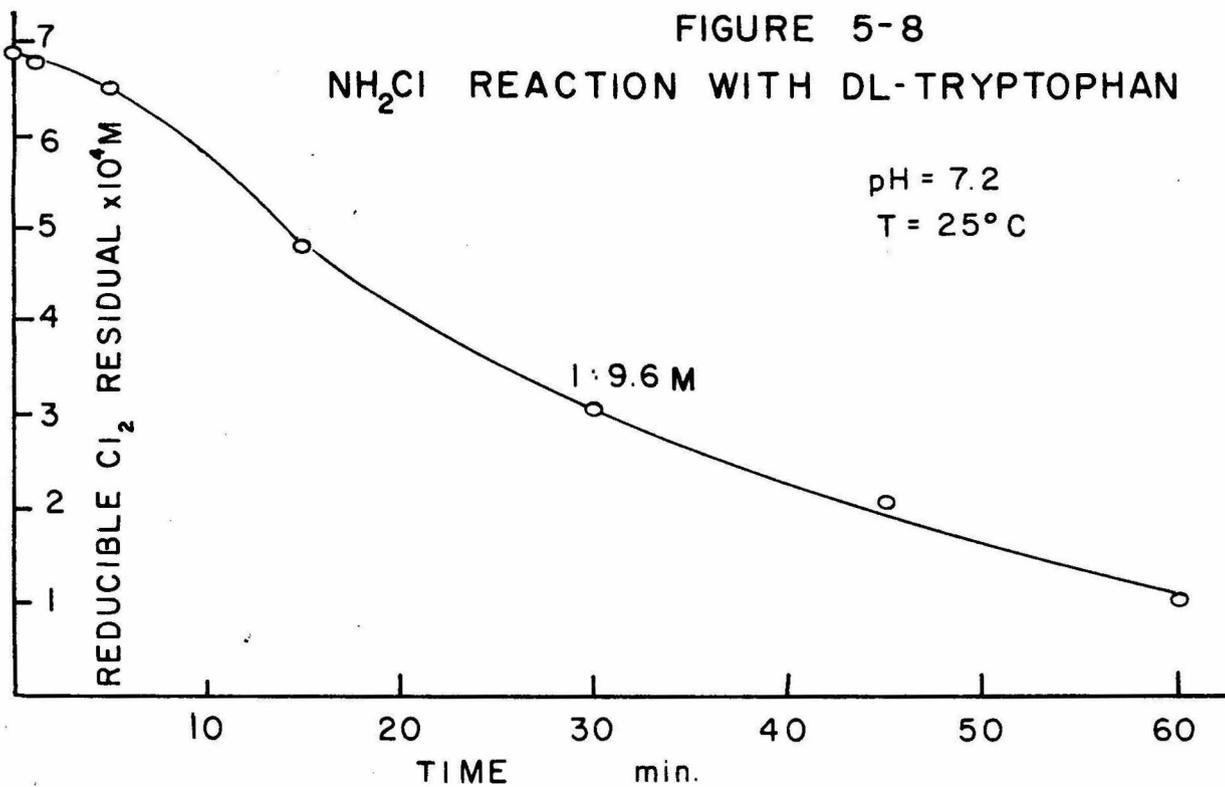


FIGURE 5-9

NH_2Cl REACTION WITH L-HISTIDINE

pH = 7.2
T = 25°C

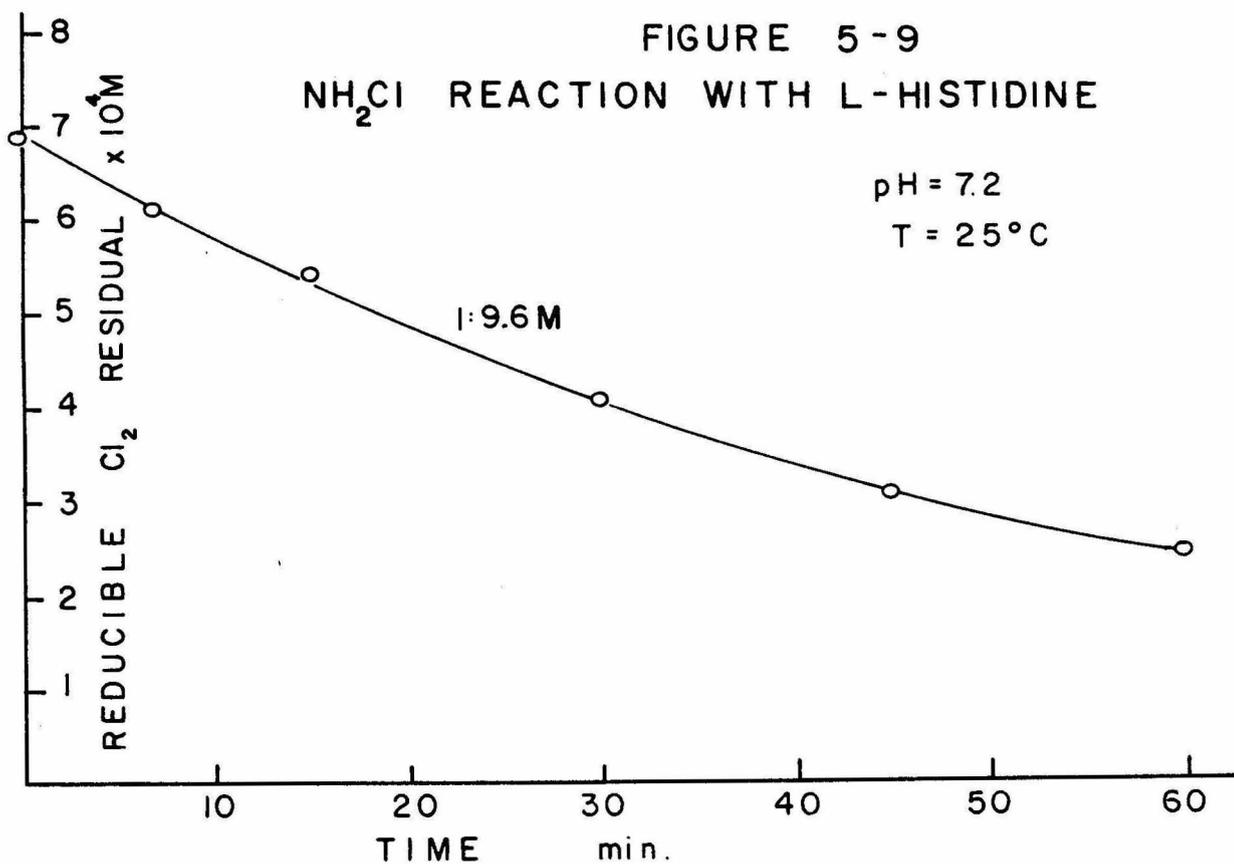


FIGURE 5-10
 NH_2Cl REACTION WITH THYMINE

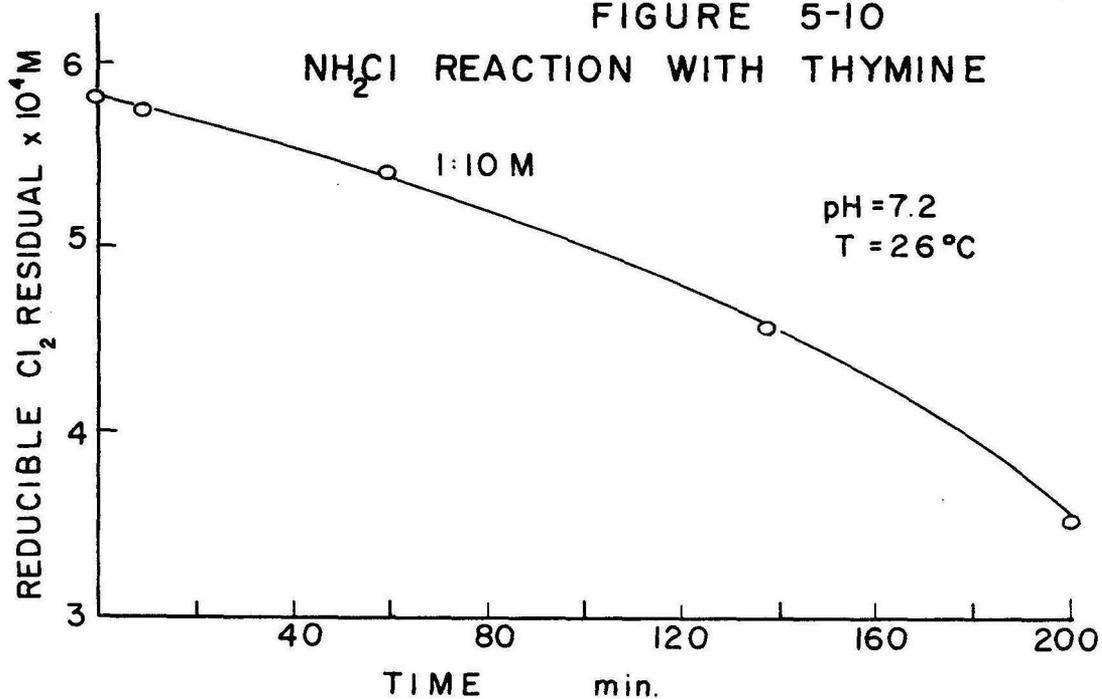


FIGURE 5-11
 NH_2Cl REACTION WITH URACIL

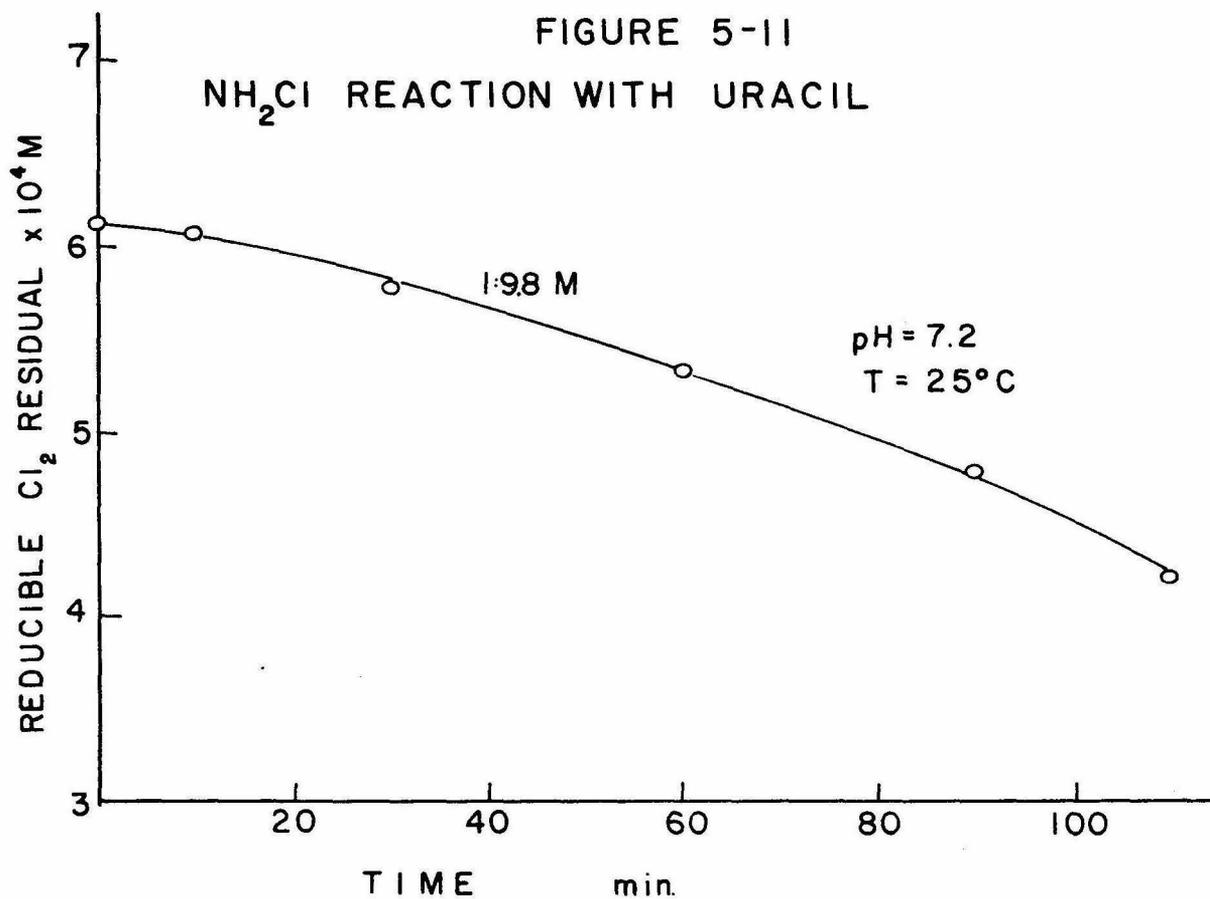


FIGURE 5-12
NH₂Cl REACTION WITH CYTOSINE

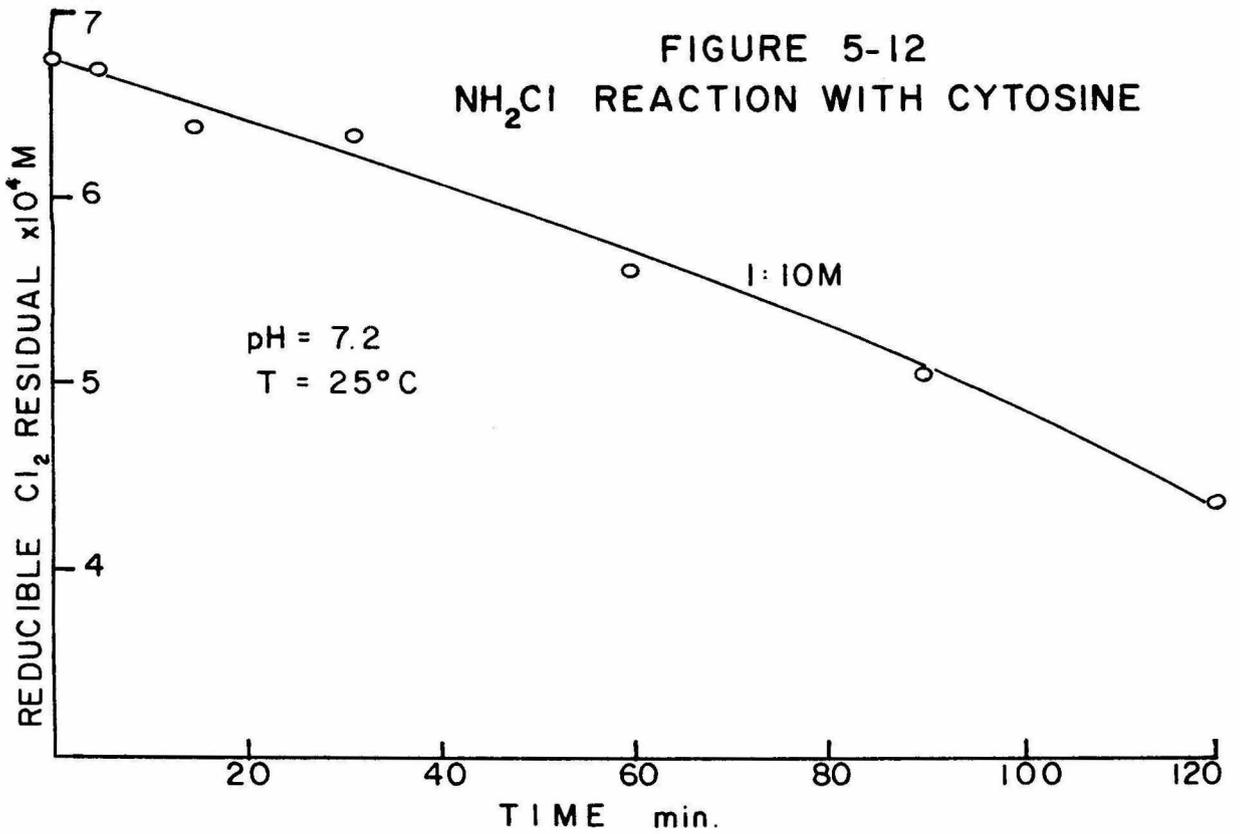


FIGURE 5-13
NH₂Cl REACTION WITH ADENINE·SO₄

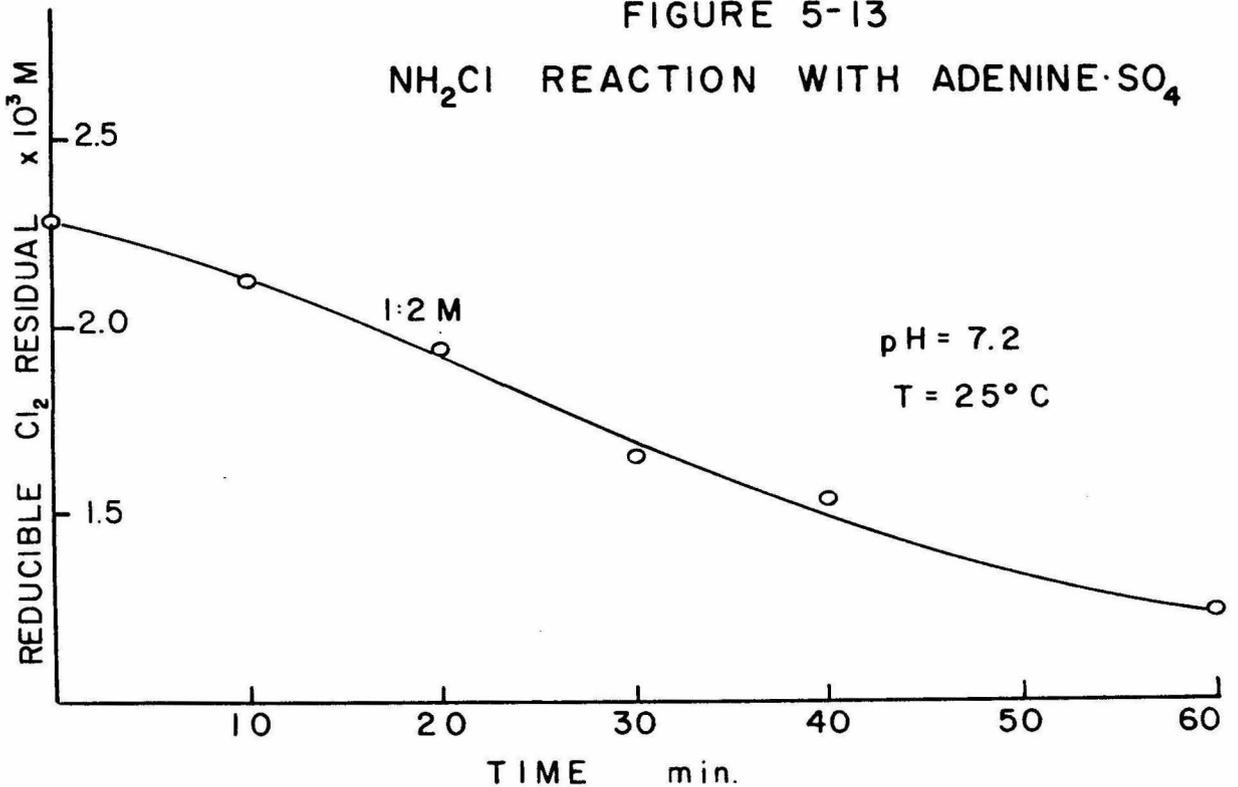


FIGURE 5-15

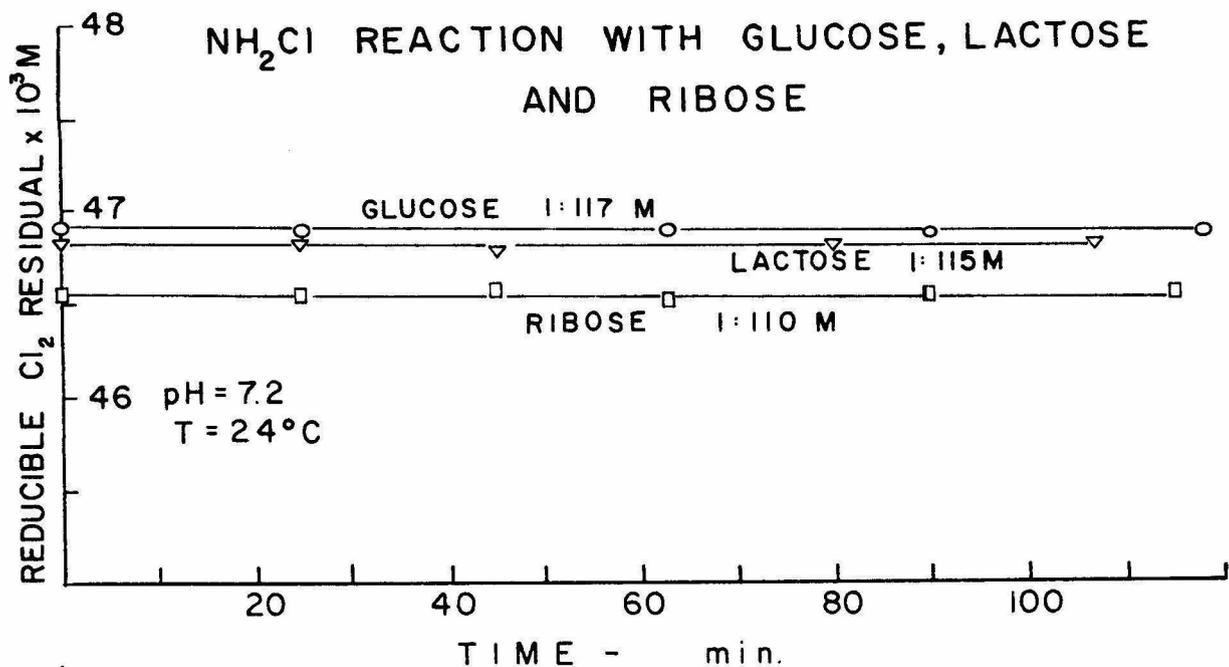
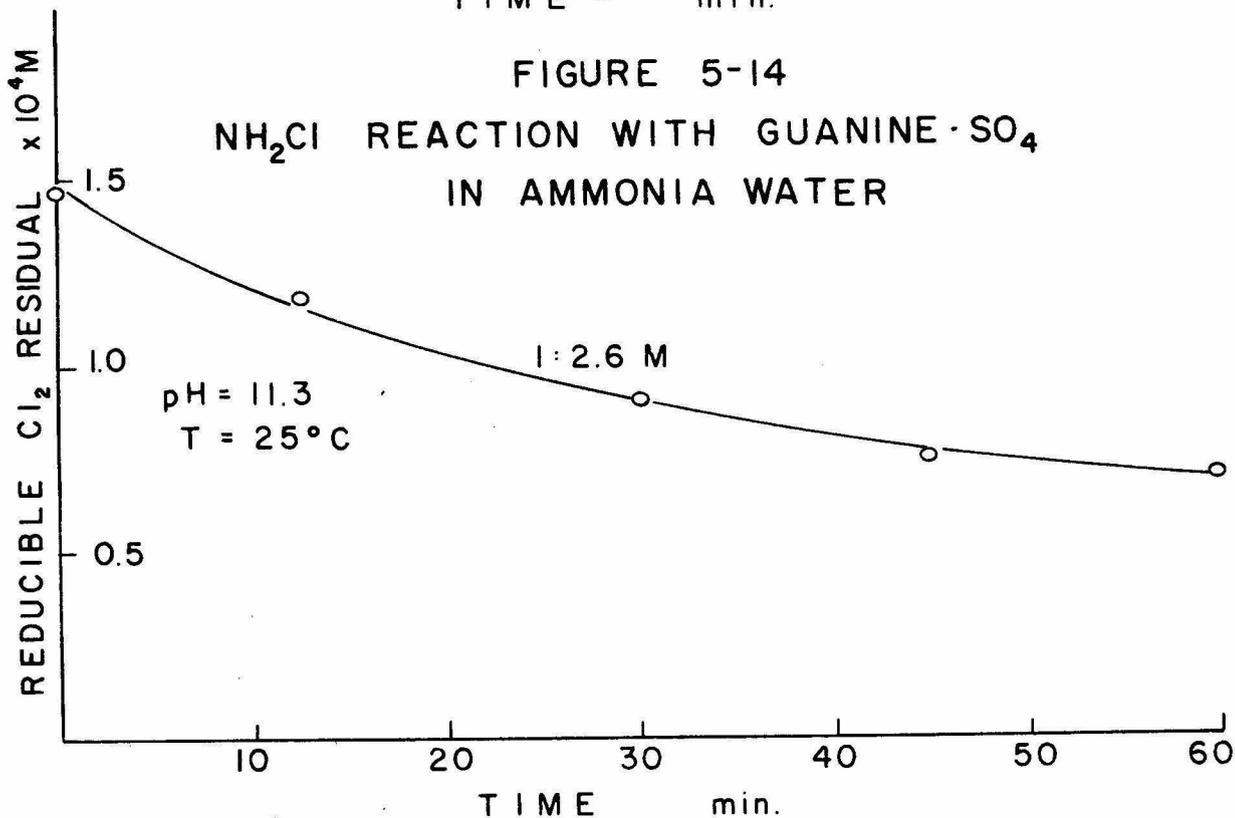


FIGURE 5-14

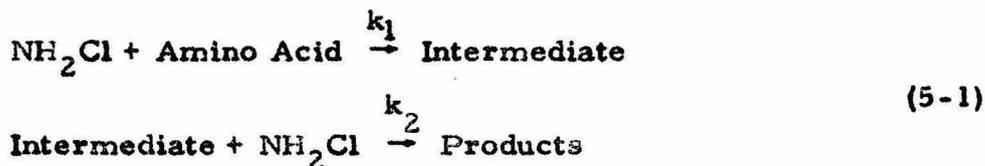


2.66×10^{-3} M NH_2Cl were added. Monochloramine was assayed at 1, 5, 10, 20, 30, 40, 60 and 120 minutes by means of polarographic techniques (figure 5-16). The resultant reaction rate, similar to that demonstrated by the sulfur-containing amino acids, is very rapid initially and then falls off to a much slower rate.

B. REACTION RATES OBSERVED

The preceding experimental data roughly illustrate the relative reactivities of some of the simple organic molecules with monochloramine. However, the molar ratios of monochloramine to the compound tested were not identical in each case, since it was desirable to obtain reaction rates which were measurable within a reasonable time. Thus, it is difficult to compare the reaction rates of the different compounds directly. The data indicate that the reactions were not of first- or second-order and probably involved several reaction systems, thereby complicating the calculation of reaction rates. Nevertheless, one can speculate over the mechanism of these reactions in an attempt to estimate rate constants. Three of the most probable systems are outlined below:

a. Competitive-Consecutive Second-Order Reaction

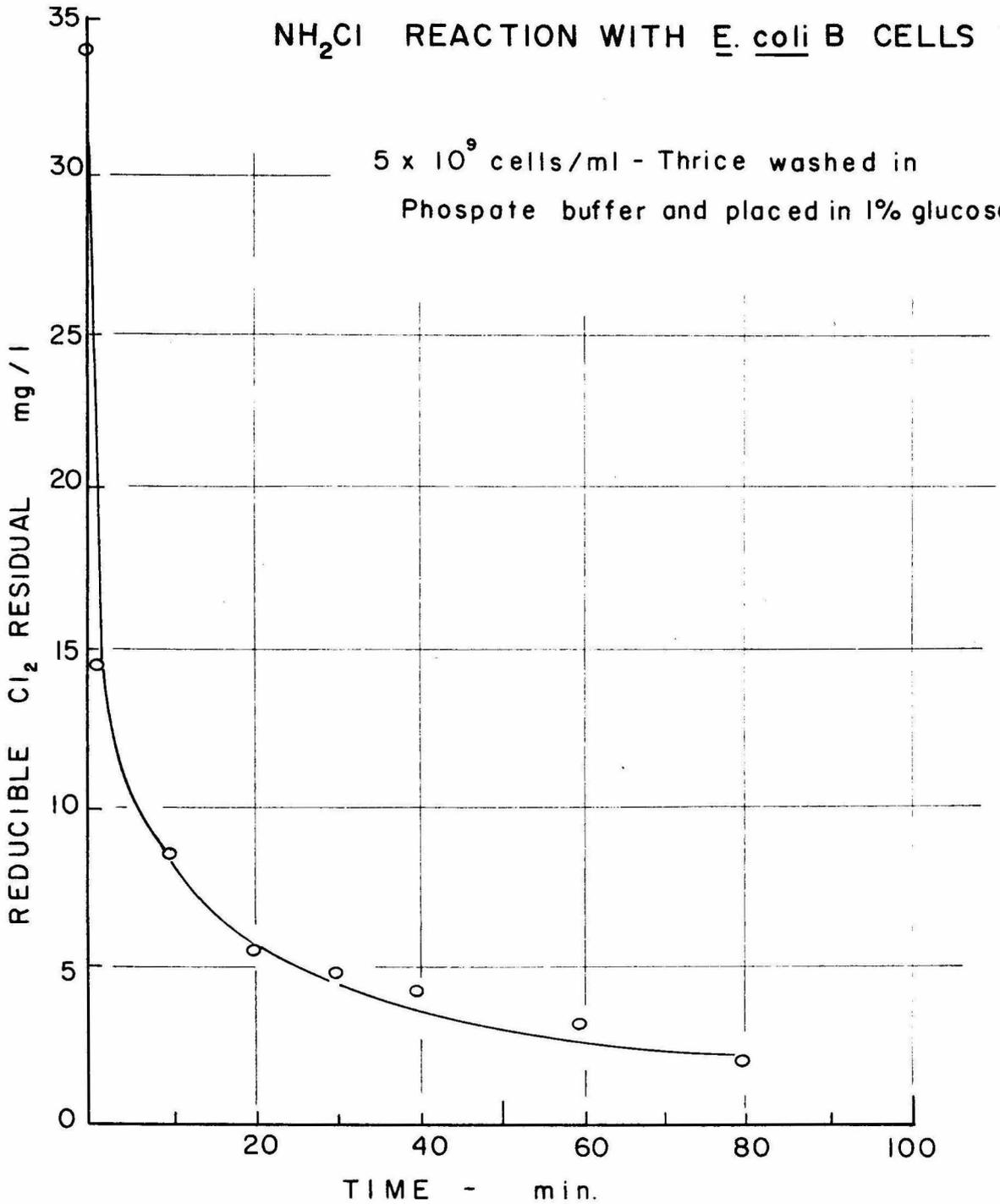


If the concentrations of monochloramine, the amino acid, a

FIGURE 5-16

NH_2Cl REACTION WITH E. coli B CELLS

5×10^9 cells/ml - Thrice washed in
Phosphate buffer and placed in 1% glucose



hypothetical intermediate product and the final products are assigned the symbols A, B, C and D, respectively, the differential forms of these equations become:

$$\frac{dA}{dt} = -k_1AB - k_2AC$$

$$\frac{dB}{dt} = -k_1AB$$

$$\frac{dC}{dt} = k_1AB - k_2AC$$

$$\frac{dD}{dt} = k_2AC$$

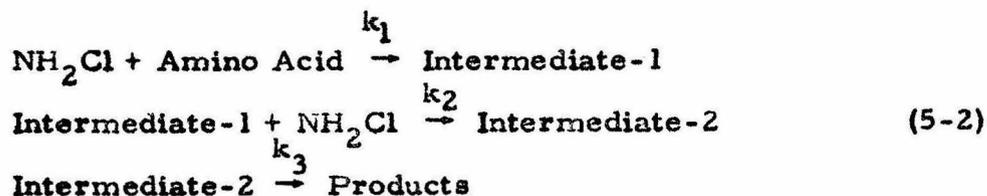
In our experimental system the initial values, A_0 , B_0 , C_0 and D_0 , are known, and D is evaluated according to the equation:

$$A_0 - (A+C) = 2D$$

$$\therefore D = \frac{1}{2} [A_0 - (A+C)]$$

The expression $(A + C)$ represents the chlorine residual remaining in time, t . We assume that C is a chlorinated intermediate in which chlorine is still in the +1 oxidation state.

b. Competitive-Consecutive Second-Order with Spontaneous Hydrolysis to Products



Again let the letters A through C have the meanings as described above, but D will represent the concentration of a second

intermediate and E will be the concentration of the end product from spontaneous hydrolysis of D.

The differential equations for this system are as follows:

$$\frac{dA}{dt} = -k_1AB - k_2AC$$

$$\frac{dB}{dt} = -k_1AB$$

$$\frac{dC}{dt} = k_1AB - k_2AC$$

$$\frac{dD}{dt} = k_2AC - k_3D$$

$$\frac{dE}{dt} = k_3D$$

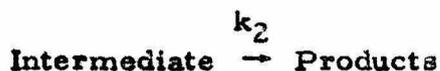
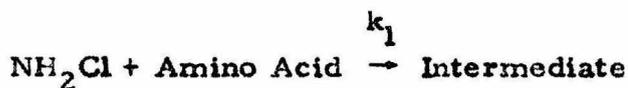
All initial conditions are known (A_0 through E_0) and E would be computed from the equation

$$A_0 - (A+C+D) = 2E$$

$$\therefore E = \frac{1}{2} [A_0 - (A+C+D)]$$

The expression $(A + C + D)$ represents the chlorine residual remaining where both C and D also contain chlorine in the +1 oxidation state.

c. Second-First Order Reaction



(5-3)

The differential equations for this sequence are

$$\frac{dA}{dt} = -k_1AB$$

$$\frac{dB}{dt} = -k_1 AB$$

$$\frac{dC}{dt} = k_1 AB - k_2 C$$

$$\frac{dD}{dt} = k_2 C$$

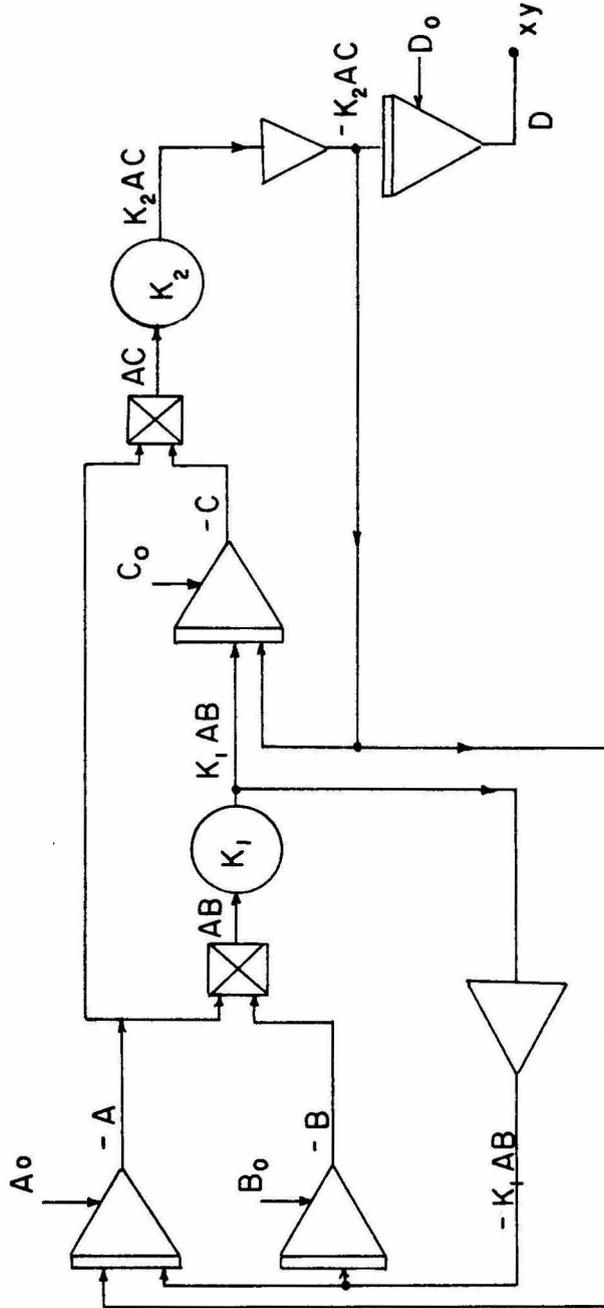
The values of A_0 through C_0 are known and D is determined by

$$D = A_0 - (A + C)$$

The systems of differential equations described above appear simple in themselves but are difficult to solve. Thus, the calculation of k values (by numerical methods) would be extremely laborious. Thankfully, the analog computer was used to solve these equations very rapidly with programs developed for each of the three systems (figures 5-17, 5-18, 5-19). In such a computer analysis, the initial conditions are fed into the program and a curve of D versus time is plotted using the experimental data. The k values are then adjusted until the x-y scribe follows these experimental points.

For these analyses an Electronics Associates - Pace 231 R analog computer with a 30 inch by 30 inch x-y plotter was used. This instrument is ideally accurate at 0.01%, which is far in excess of the required accuracy considering the nature of the experimental data. The initial analysis was made with alanine, the compound studied in the greatest detail. The k values observed were checked against several different experimental curves in order to insure selection of the proper programmed system.

FIGURE 5-17
ANALOG PROGRAM - EQUATION 5-1



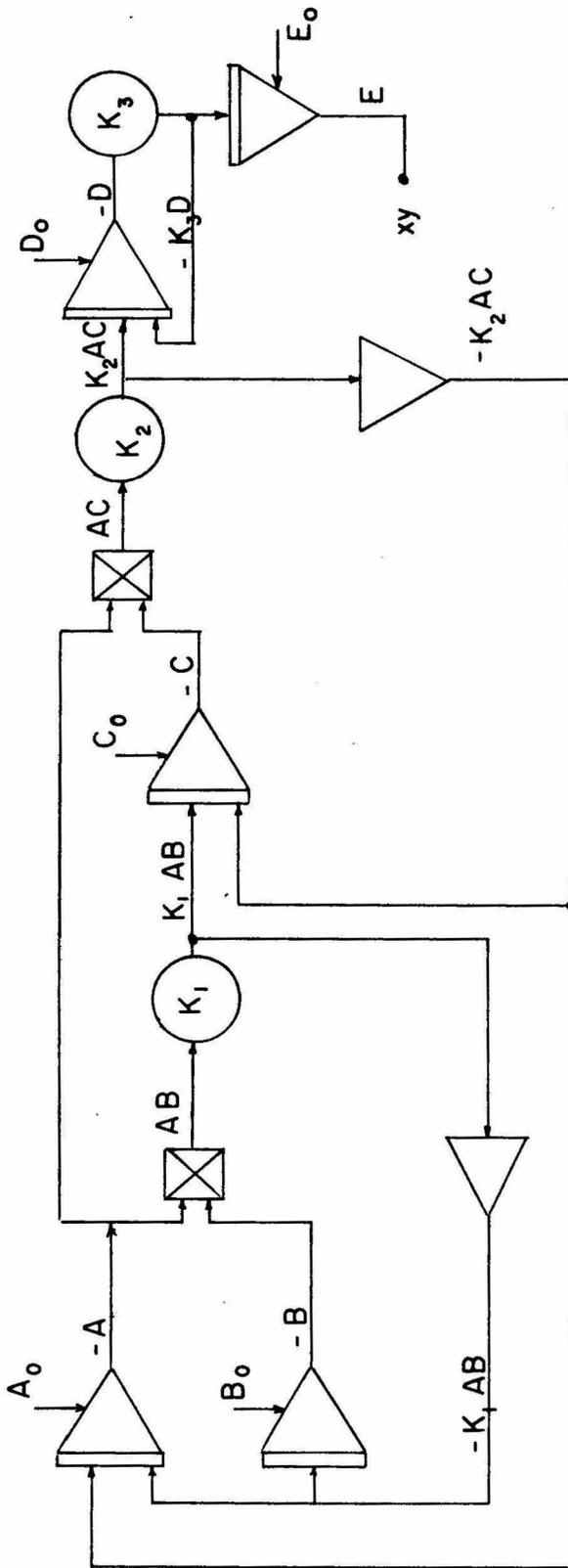
$$\frac{dA}{dt} = -k_1 AB + k_2 AC$$

$$\frac{dC}{dt} = k_1 AB - k_2 AC$$

$$\frac{dB}{dt} = -k_1 AB$$

$$\frac{dD}{dt} = k_2 AC$$

FIGURE 5-18
 ANALOG PROGRAM - EQUATION 5-2



$$\frac{dA}{dt} = -k_1 AB + k_2 AC$$

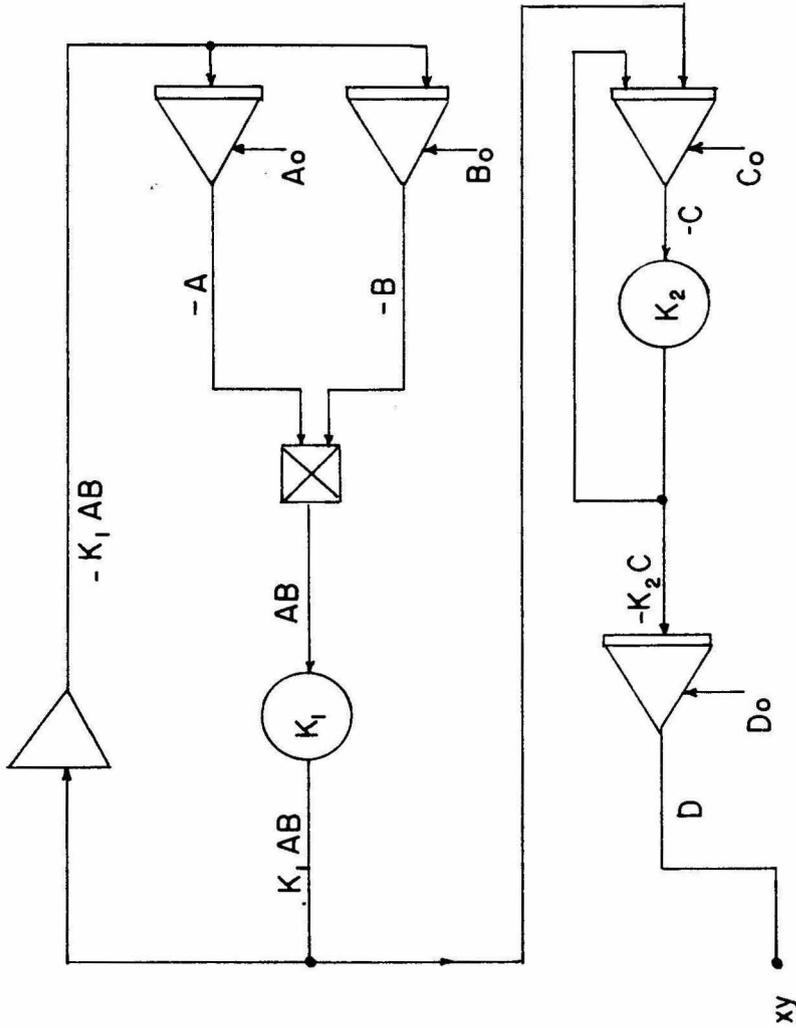
$$\frac{dB}{dt} = -k_1 AB$$

$$\frac{dC}{dt} = k_1 AB - k_2 AC$$

$$\frac{dD}{dt} = k_2 AC - k_3 D$$

$$\frac{dE}{dt} = k_3 D$$

FIGURE 5-19
ANALOG PROGRAM-EQUATION 5-3



$$\frac{dC}{dt} = k_1 AB - k_2 AC$$

$$\frac{dD}{dt} = k_2 C$$

$$\frac{dA}{dt} = -k_1 AB$$

$$\frac{dB}{dt} = -k_1 AB$$

Neither equations 5-1 nor 5-2 appeared to apply in this analysis, for no combination of k values could be found to satisfy the experimental data. The equations 5-3 did give excellent approximations of the observed data and, thereby, were used for all computations of observed rate constants. A sample of the computer analysis made for alanine is shown in figure 5-20, a photograph of the 36" × 36" x-y graph sheet.

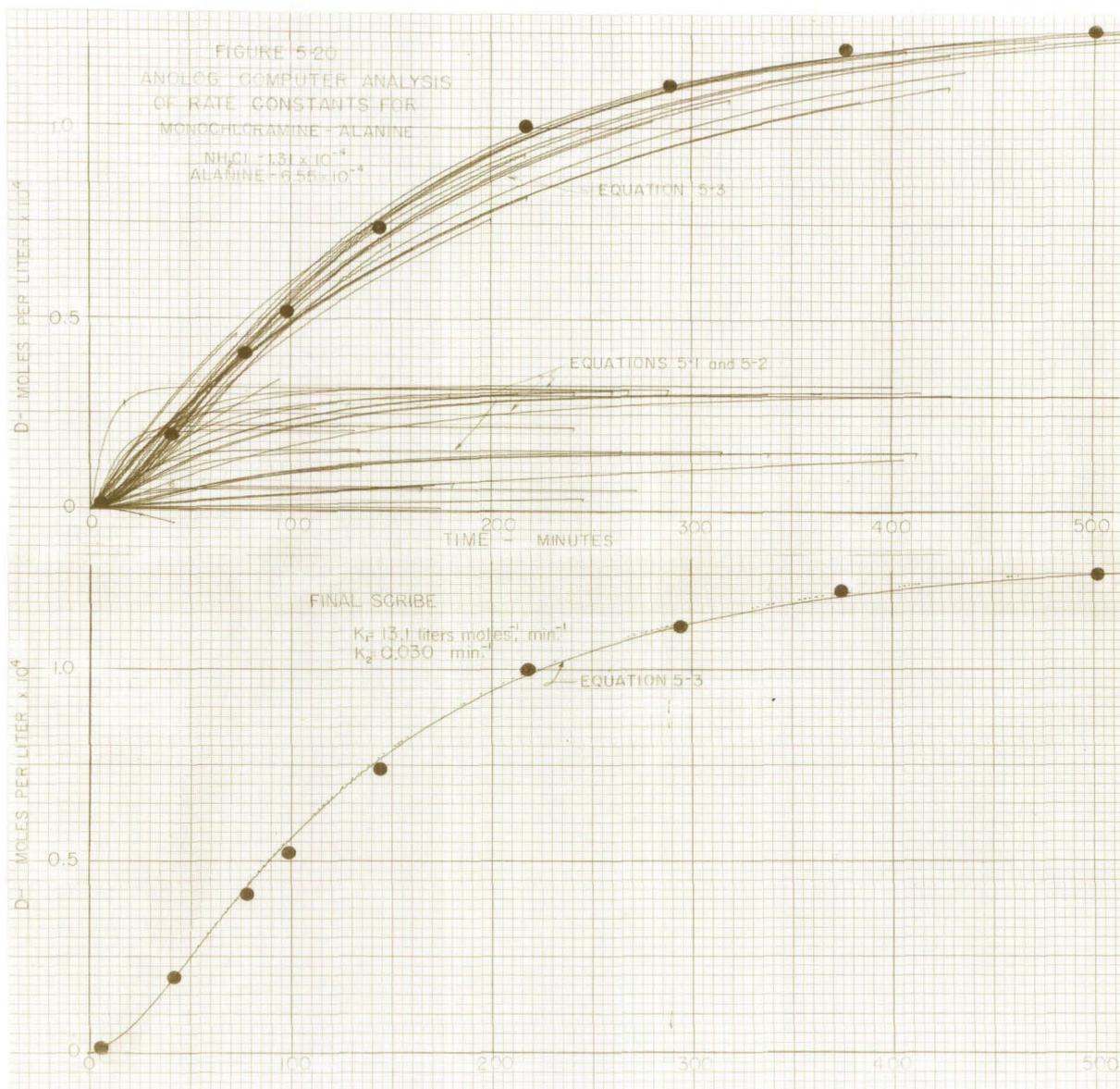
The results of the analysis of observed constants using the equation 5-3 system are given in table 5-6. It is important to note that these are only observed constants based on the apparent reaction sequences for monochloramine and alanine, and do not necessarily represent true rate values.

C. DISCUSSION

The rate of reduction of monochloramine by alanine is neither a first- nor second-order reaction but, rather, a complex reaction sequence involving at least one intermediate compound. The information obtained in these experiments is not sufficient to deduce all the steps in this reaction, but it does suggest a certain general pattern. From the nitrogen studies two conclusions can be made: (a) alanine is probably deaminated in its reaction with monochloramine; and (b) nitrogen is not oxidized in the reaction since it is converted from amino nitrogen (oxidation state -3) to ammonia nitrogen (oxidation state -3). It is important to point out, however, that monochloramine yields a positive ammonia test by direct Nesslerization, a property which might also be exhibited by a

TABLE 5-6
OBSERVED REACTION RATES
FOR NH_2Cl REACTIONS

Compound	K_1 obs min^{-1} liter moles $^{-1}$	K_2 obs min^{-1}
L-Alanine	13.1	.030
L-Histidine	15.1	.023
DL-Tryptophan	14.8	.048
L-Arginine HCl	23.6	.059
Uracil	0.64	.059
Thymine	0.38	.025
Cytosine	1.13	.021
Adenine- SO_4	5.7	.031
Guanine- SO_4	4.4	.060
Glucose	Nil	Nil
Pentose	Nil	Nil
Lactose	Nil	Nil



chloro-derivative of alanine. Distillation of the monochloramine-alanine mixture gave similar results in the ammonia assay. It might be argued that a chloro-derivative would not distill over and, thus, such evidence would indicate only that free ammonia exists. The chlorine molecule may exert enough force on the amino nitrogen, however, to weaken the N-C bond to such a point that this group would distill off and subsequently give a positive test for ammonia.

It is interesting to note that the rate of formation of ammonia nitrogen paralleled the rate of reduction of monochloramine up to about 190 minutes (figure 5-21). After this time ammonia was formed at a rate more rapid than reduction of the chlorine residual, monochloramine, so that all the alanine amino nitrogen was "converted" to ammonia nitrogen when about 25% of the chlorine residual activity remained. Such findings might be explained if there were formed a chloro-derivative (which might yield a positive ammonia-nitrogen test), independently of and at a more rapid rate than the reduction of monochloramine. A more obvious suggestion is that alanine can be deaminated only after the molecule is sufficiently chlorinated, and this deamination is independent of the amount of monochloramine remaining.

The UV spectral analysis of the monochloramine-alanine reaction demonstrated a shift in the maximum absorption peak toward 250 m μ to accompany a reduction in molar absorptivity. Molar absorptivities calculated for the monochloramine-alanine mixture are tabulated in table 5-7 along with selected values for

FIGURE 5-21
PER CENT NH_2Cl REDUCED & NH_3 FORMED
VS TIME IN REACTION OF 1:1 M NH_2Cl
AND ALANINE

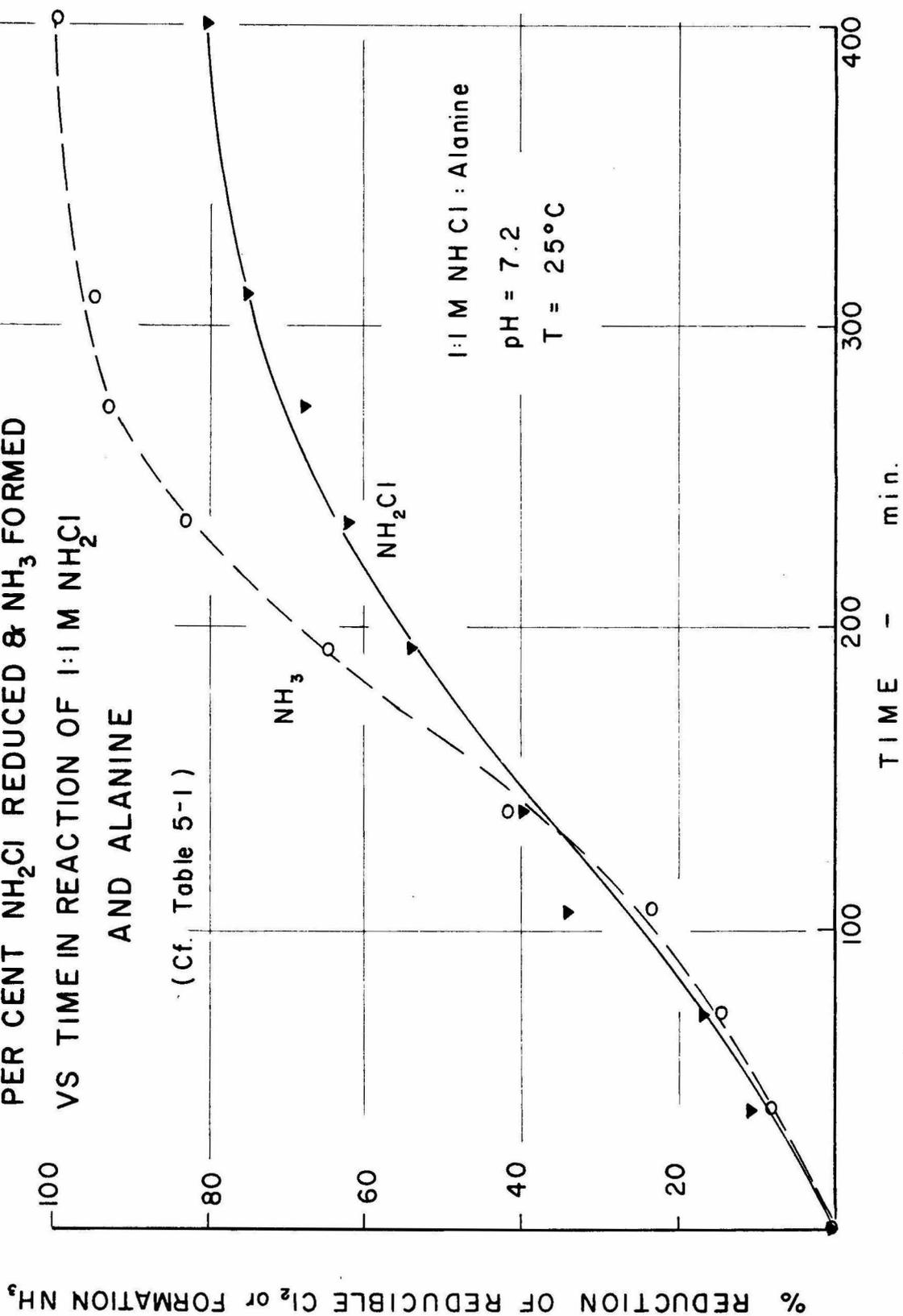


TABLE 5-7
CALCULATED MOLAR ABSORPTIVITIES
FOR NH₂Cl-ALANINE REACTION

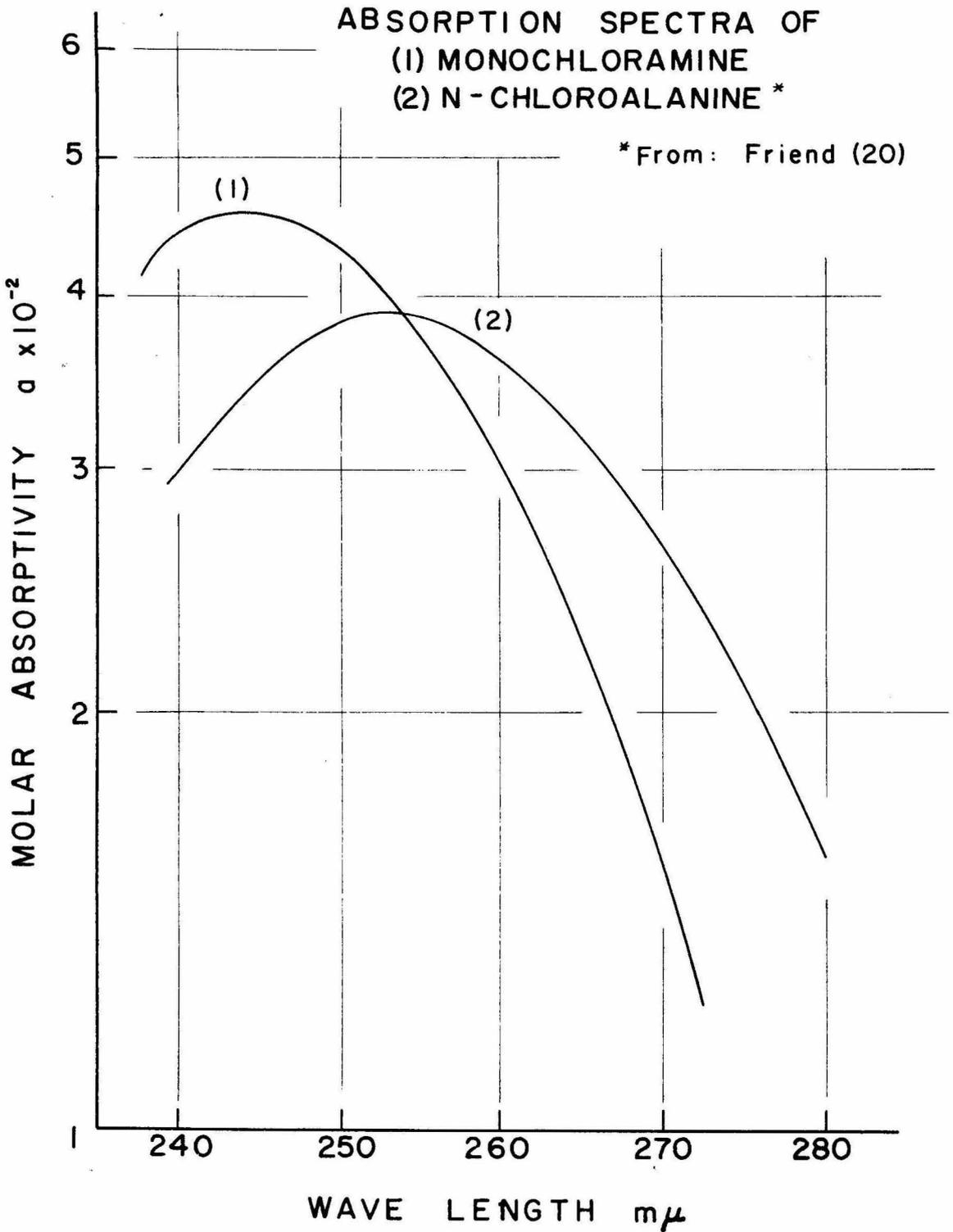
Wave length m μ	NH ₂ Cl	N-Chloroalanine*	Mixture**		
	a	a	40 min	80 min	205 min
245	458	348	357	345	350
255	410	382	380	381	378
280	62	156	98	120	155

* From Friend (20)

** Calculated from data, figure 5-1

monochloramine and N-chloroalanine. This indicates that the chlorine residuals, as given by the starch-iodide titration, were the result of monochloramine plus an intermediate in which chlorine retained its +1 oxidation state. The spectra of many of the chloro-derivatives of amino acids have been elucidated by Friend (20). Chloroalanine has a maximum molar absorptivity of 382 at 255 m μ (figure 5-22). Friend has also shown that amino acids react more rapidly with chlorine than does ammonia at neutral pH values. This would suggest that the amino group of alanine and other amino acids might have a greater attraction for Cl⁺¹ than for the ammonia molecule. Thus, it is conceivable that alanine is chlorinated at the amino group yielding N-chloroalanine. This hypothesis is supported by (a) the UV spectra obtained for this mixture, (b) the computation of molar absorptivity for the mixture and (c) the nitrogen studies discussed above.

FIGURE 5-22



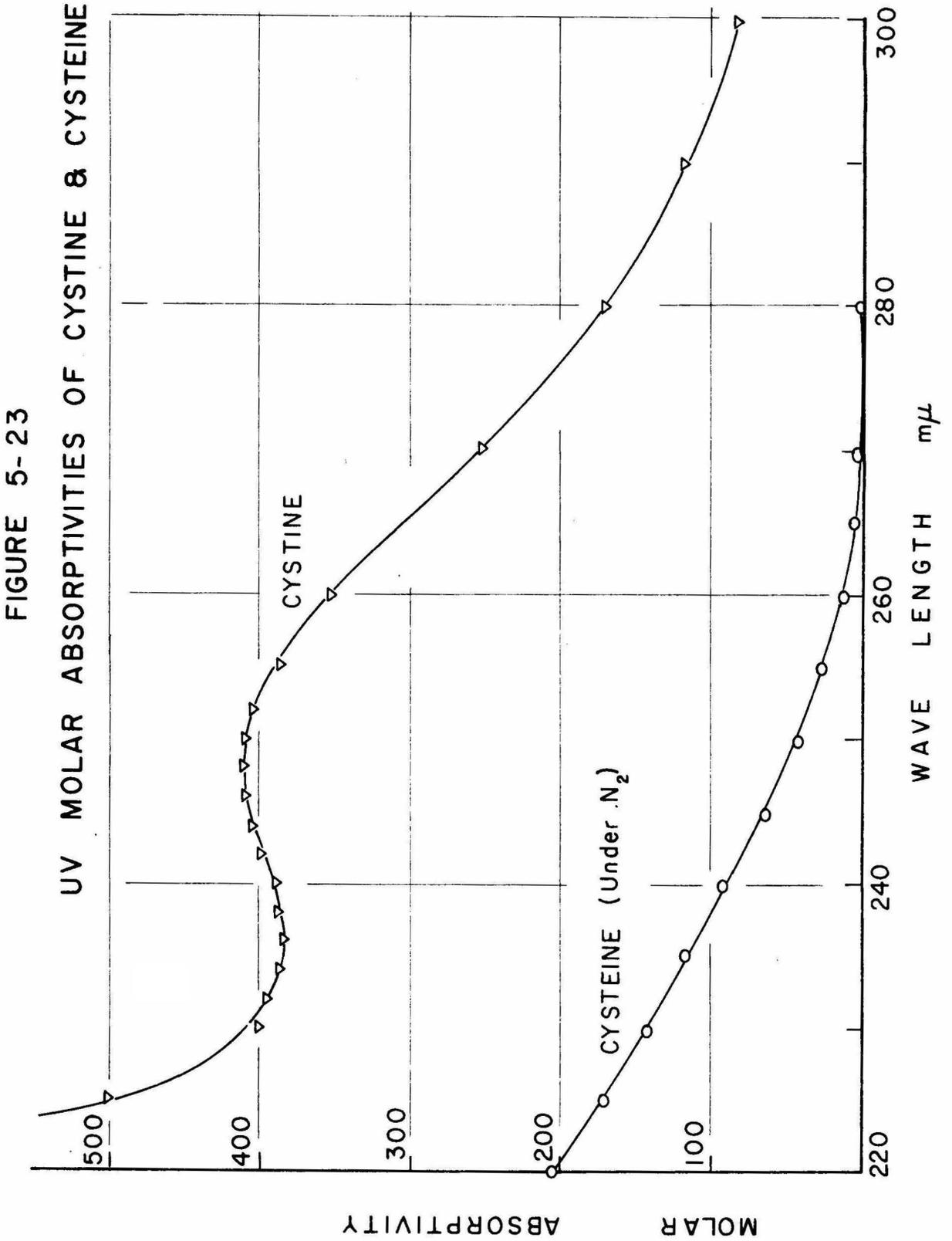
moles⁻¹, min⁻¹ and 0.030 min⁻¹, respectively. A computation of the half time of the first- and second-order reactions was made in accordance with the following equations:

$$\text{First-order: } T_{\frac{1}{2}} = \frac{1}{k_2} \ln 2 \quad (5-5)$$

$$\text{Second-order: } T_{\frac{1}{2}} = \frac{1}{k_1(B_0 - A_0)} \ln \frac{2B_0 - A_0}{B_0} \quad (5-6)$$

The values of $T_{\frac{1}{2}}$ for a 1:5 molar ratio NH_2Cl :alanine ($\text{NH}_2\text{Cl} - 1.31 \times 10^{-4} \text{ M}$) are 1380 seconds for the first-order reaction and 86 seconds for the second-order. For a 1:1 molar solution with the same monochloramine concentrations, the second-order $T_{\frac{1}{2}}$ increased to 235 seconds. These computations illustrate the relative importance of both reaction constants. In comparison to the monochloramination of alanine, Friend found the specific rate constant for the chlorination of alanine to be 5.4×10^9 liter moles⁻¹, min⁻¹ at 25° C.

All of the sulfur containing amino acids were extremely reactive with monochloramine. Spectral analysis of the cysteine-mono-chloramine reaction indicated the presence of an intermediate which did not contain chlorine in a +1 oxidation state, i. e., mono-chloramine was present in only trace amounts. Initially it was thought that the spectrum was that of cystine (figure 5-23). The molar absorptivity of cystine at 250 m μ is 411 and that of cysteine is 41. If one assumes that 2 moles of cysteine would be oxidized to 1 mole of cystine by monochloramine, then $C_{\text{SH}} + 2 C_{\text{SS}} = M_c$, where M_c is the initial concentration of cysteine. One may also



assume that the total absorbance, A, would be equal to the sum of the absorbencies of monochloramine, cysteine and cystine.

Therefore, $A_T = abc_{\text{NH}_2\text{Cl}} + abc_{\text{SS}} + abc_{\text{SH}}$. Solving the equations as follows:

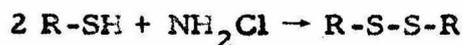
$$2 C_{\text{SS}} + C_{\text{SH}} = 7.5 \times 10^{-5}$$

at 250 m μ $0.234 = \text{Negligible NH}_2\text{Cl} + 411 \times 10 \times C_{\text{SS}} + 41 \times 10 \times C_{\text{SH}}$

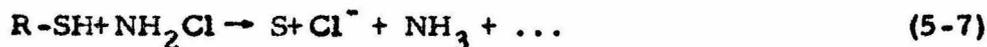
$$\therefore C_{\text{SS}} = 6.1 \times 10^{-5} \text{ M}$$

$$C_{\text{SH}} = -4.1 \times 10^{-5} \text{ M}$$

One sees that such an assumption is not valid. A similar analysis at the trough (235 m μ) gives an equivalent solution. Therefore, it may be said that the reaction



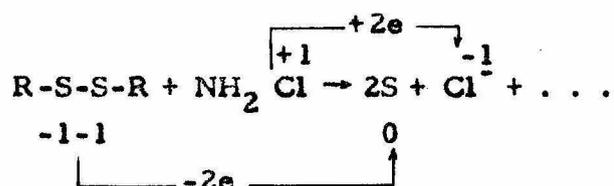
is not valid. When SH groups were analyzed it was shown that monochloramine oxidizes sulfhydryl to the free sulfur state (oxidation state 0). The equation then becomes



An examination of oxidation states indicates that such an equation is valid. The residual reducible chlorine found in the experiment using 1:1 molar ratios probably resulted from a loss in SH groups by oxidation with oxygen in the air prior to treatment. The spectra observed may have been the result of an unidentified product in the

reaction, or, perhaps a chloro-derivative formed by a reaction of the excess monochloramine with amino acid fragments. When a 1:2 molar mixture of monochloramine and cysteine was analyzed, 50% of the residual chlorine was found to be lost, and about 50% of the SH groups was oxidized. This further supports the mechanism of reaction proposed in equation 5-7.

An examination of the NH_2Cl -time curves for cystine (figure 5-5) indicate that 1 mole of cystine reacts with 1 mole of monochloramine as follows:



This is substantiated by the disulfide analysis (table 5-4) of 1:1 and 1:2 molar mixtures of these compounds. Essentially all SS was lost in the 1:1 molar solution, whereas only 50% of the SS bonds were oxidized at a 1:2 molar ratio.

Methionine, which contains a methyl-thio ether ($-\text{SCH}_3$) group, was as reactive with monochloramine as cysteine and cystine. However, at a 1:1 molar ratio only about 75% of the chlorine residual was initially reduced; 35% was lost at a 1.8:1 molar ratio. It is difficult to postulate a reaction mechanism for the monochloramine-methionine system. The sulfur in methionine is in an oxidation state of -2, similar to that of cysteine. Conceivably monochloramine would reduce the sulfur of methionine to an oxidation state of 0 with somewhat more difficulty than the sulfur in

sulphydryl groups. However, since the rates of reaction of monochloramine with both cysteine and methionine are very rapid, the experimental analysis failed to show a difference. In both cases, monochloramine reduction was slow after the initial reaction, indicating either (a) that the +1 chlorine was tied up on the molecule so that it was unavailable for further sulfur oxidation or (b) that sulfur had been oxidized to a stable oxidation state. The methyl group may play an important role in this reaction, although there is a tendency for oxidizing agents to attack the sulfur atom preferentially (70).

A close examination of the observed rates for the amino acids indicates that the guanidino group of arginine chlorinates most rapidly, whereas the indole group of tryptophan and the imidazolyl group of histidine do not greatly affect the rate of reaction with monochloramine. It is important to note, however, that the reaction constants, being derived from an idealized reaction mechanism (see equation 5-3, p. 112), may not reflect the true mechanism of these reactions. The hydrolysis rate constants (k_2 obs), which appear to play the greater role in these reactions (in terms of overall rates), indicate that the chloro-derivatives of arginine and tryptophan hydrolyze considerably faster than alanine, whereas chloro-histidine hydrolyzes more slowly. The pinkish color imparted to the tryptophan-monochloramine mixture is probably the reaction found by Kühne (71) to be given by indole in the presence of chlorine water. This strongly

supports the theory that these molecules hydrolyze in the process of monochloramination. The low k_2 value observed for histidine may indicate that the chlorinated nitrogen atoms of the imidazolyl ring are relatively stable, whereas the chlorinated nitrogen atoms in the guanidino group of arginine are much more easily hydrolyzed.

The purine bases, adenine and guanine, both chlorinated and hydrolyzed relatively slower than the amino acids. This might be expected owing to the high stability of the heterocyclic nitrogen ring by virtue of resonance. It is difficult to compare these two compounds, since guanine·SO₄ was chlorinated at pH 11.3. The pyrimidines are even less reactive than the purines, probably because they contain fewer nitrogen atoms. Cytosine chlorinated somewhat faster than either thymine or uracil, perhaps because of the amino group in the number 6 position. It is difficult to explain the reasons for uracil's higher constant for hydrolysis.

The sugars are apparently very unreactive with monochloramine as well as with free chlorine (22). The reducing group at position 1 of glucose, lactose and ribose may require a more vigorous agent for oxidation than monochloramine.

The monochloramine demand of cells is surprisingly high, presumably much of it residing in the protein component. Approximately 15% of the dry weight of E. coli is nitrogen (72). Therefore, 7 grams of dry cells per liter contain about 1.05 grams of nitrogen per liter, or a molarity of nitrogen of 7.5×10^{-2} . If one assumes that all cell nitrogen is readily available to react

with monochloramine, the molar ratio of monochloramine to nitrogen in this test would be 1:157 ($4.78 \times 10^{-4} : 7.5 \times 10^{-2}$). If one assumes an average k_1 value for this nitrogen (as amino nitrogen) of 25 liter mole⁻¹, min⁻¹, the $T_{\frac{1}{2}}$ (equation 5-6) for the second-order reaction would be 22 seconds. However, the reducible chlorine residual would not change in this time, as chlorine remains in the +1 oxidation state. The first-order reaction governs the rate of reduction of reducible chlorine, and using a k_2 of .030 min⁻¹, the $T_{\frac{1}{2}}$ by equation 5-5 is about 1400 seconds. On the other hand, about 1.1% of the dry weight of E. coli cells is sulfur (72); therefore, the sulfur concentration of the cell suspension was 0.077 g/liter or 4.81×10^{-3} M, and the molar ratio (NH₂Cl:S) was 1:10. We have seen that molar ratios of 1:1 (NH₂Cl:S) reduce chlorine residuals very rapidly. On the basis of this analysis, one might conclude that all of the initial monochloramine demand of cells resides in sulfur containing compounds. With resting cells suspended in phosphate buffer, the vast majority of the monochloramine must have reacted with intracellular sulfur, strongly suggesting that permeation of the cell by monochloramine proceeds very rapidly.

One factor which was not evaluated in these investigations is the effect of pH on the rates of reaction. Since the neutral monochloramine molecule has greater bactericidal effects at lower pH values than higher ones, a study of the effect of pH on chemical reaction rates might have proved valuable. Since the sulfur-containing compounds, which are deemed most important, were far too reactive with monochloramine to measure with the methods and conditions

employed in this work, the development of measuring techniques to differentiate effects of pH would have carried this investigation beyond its original scope.

One of the most important conclusions that may be drawn from this phase of the investigation is that sulfur-containing organic compounds are highly reactive with monochloramine. There seems to be little doubt that monochloramine preferentially reacts with molecular sites containing sulfur. Contrary to statements made by Ingols (19), however, the oxidation of sulfhydryl proceeds beyond the disulfide state, for stable elemental sulfur (oxidation state of zero) appears. Disulfide, itself, is also easily oxidized to the sulfur state. All attempts to reverse these reactions met with failure, suggesting that the in vivo effects of monochloramine may not be reversible, despite hypotheses to the contrary (19, 38). However, sulfhydryl enzymes and other sulfur-containing molecules may not be the only protoplasmic constituents which react with monochloramine. Some biochemicals may act reversibly with monochloramine. One may speculate further and suggest that many important cellular functions, which are controlled by sulfur-containing compounds, can be affected by monochloramine. The cofactors, coenzyme A, reduced lipoic acid amide and thiamin pyrophosphate play important roles in pyruvic acid metabolism. Coenzyme A also plays important roles in the citric acid cycle (succinyl CoA), in the synthesis of porphyrins (cytochromes), and in the β -oxidation of fatty acids. The disulfide bond (SS) plays an important role in tertiary protein structure. In the few instances

where SS bonds are known to occur in enzymes, cleavage of all of these bonds resulted in the loss of enzyme activity (69).

Amino nitrogen, as well as other forms of reduced nitrogen, are considerably less sensitive to monochloramination than sulfur. This does not exclude them from playing an important role in inhibition of cell function caused by monochloramine. The chloro-derivatives formed may, in themselves, cause lethal damage of cells. This is especially true for the purine and pyrimidine bases which play a vital role in protein synthesis and genetic coding.

The second-order reaction rates of chlorine with organic nitrogenous compounds are of the order of 8 to 9 magnitudes greater than those of monochloramine (20). This implies that monochloramination of waste waters might be more feasible than chlorination because more than 50% of the chloramines formed in waste water through free chlorination are organic chloramines of little or no bactericidal efficiency (20). It also indicates that monochloramine should be considerably more efficient in its reaction with cells by virtue of fewer non-lethal, non-specific side reactions.

It is interesting to note that the unit weight of monochloramine initially demanded per unit weight of cells is constant (0.0026), at least within the range of from 0.07 to 7 mg dry weight of cells. This figure is further substantiated by the work of Friberg (15). The comparable ratio for chlorine is approximately 0.013 mg Cl_2 per mg cells. The differences between chlorine and monochloramine

demands in these computations is only of the order of magnitude of 1 as compared to a value of 8 for the respective second-order reaction rates. This may be a reflection of the rapidity of both the monochloramine-sulfur and the chlorine-sulfur reaction, neither of which could be measured by techniques employed in this investigation. It may also suggest that the hydrolysis of the N-chloro-derivative is the rate-determining reaction of the bactericidal kinetics within the cell. Thus, since it is hypothesized that both monochloramine and free chlorine yield similar N-chloro-derivatives, the rate constant -- which, if first-order, is not dependent upon concentration -- would be the same.

VI. EFFECTS OF MONOCHLORAMINE TREATMENT ON ENZYME FUNCTION OF RESTING CELLS

One of the most direct approaches in studying the mode of action of disinfectants is an investigation of the effects of the agent on enzyme activity. Although the choice of enzymes is almost limitless, certain criteria were used to govern the choice of specific systems. One of the better known and easier systems to study is the energy-yielding oxidation-reduction reactions of the cell. For the facultative anaerobe, Escherichia coli, these may include both the respiratory (aerobic) and fermentative (anaerobic) systems. The primary energy-yielding systems of E. coli (figures 6-1 and 6-2) consist of two distinct metabolic pathways: (a) the Embden-Meyerhoff system, an anaerobic pathway; and (b) the citric acid cycle, an aerobic pathway. These systems are interconnected through pyruvic acid which, in the absence of oxygen, may (a) be reduced to lactic acid, (b) undergo a phosphoroclastic reaction yielding acetyl CoA and formate, or (c) be decarboxylated to acetaldehyde which is transferred by several cofactors to acetyl CoA (figure 6-3). In the presence of oxygen, pyruvate is converted to acetyl CoA and CO₂, whereby DPNH is oxidized via the cytochrome system. It is significant to mention at this point that the oxidation of glucose 6-phosphate to 6-phosphogluconate, which in turn is converted to pentose phosphates, is a significant oxidative pathway for carbohydrate metabolism in E. coli (73, 74).

An examination of these pathways reveals a large variety of enzymes, many of which possess sulfhydryl groups. The

FIGURE 6-1 PATHWAYS OF FERMENTATION OF GLUCOSE FOR E. coli EMBDEN - MEYERHOFF SYSTEM

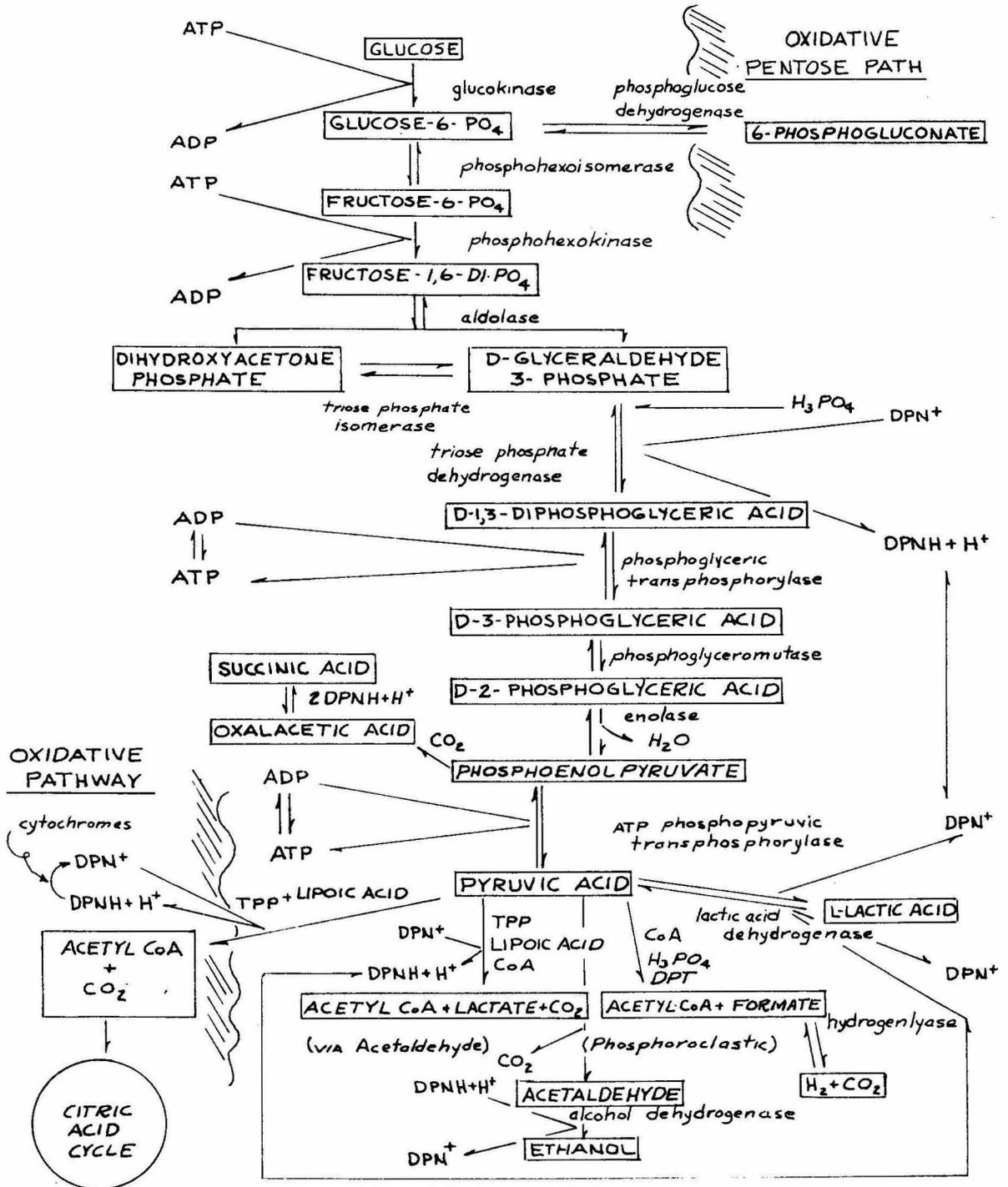


FIGURE 6-2
PATHWAY OF AEROBIC BREAKDOWN OF CARBOHYDRATE
BY E. coli
CITRIC ACID CYCLE

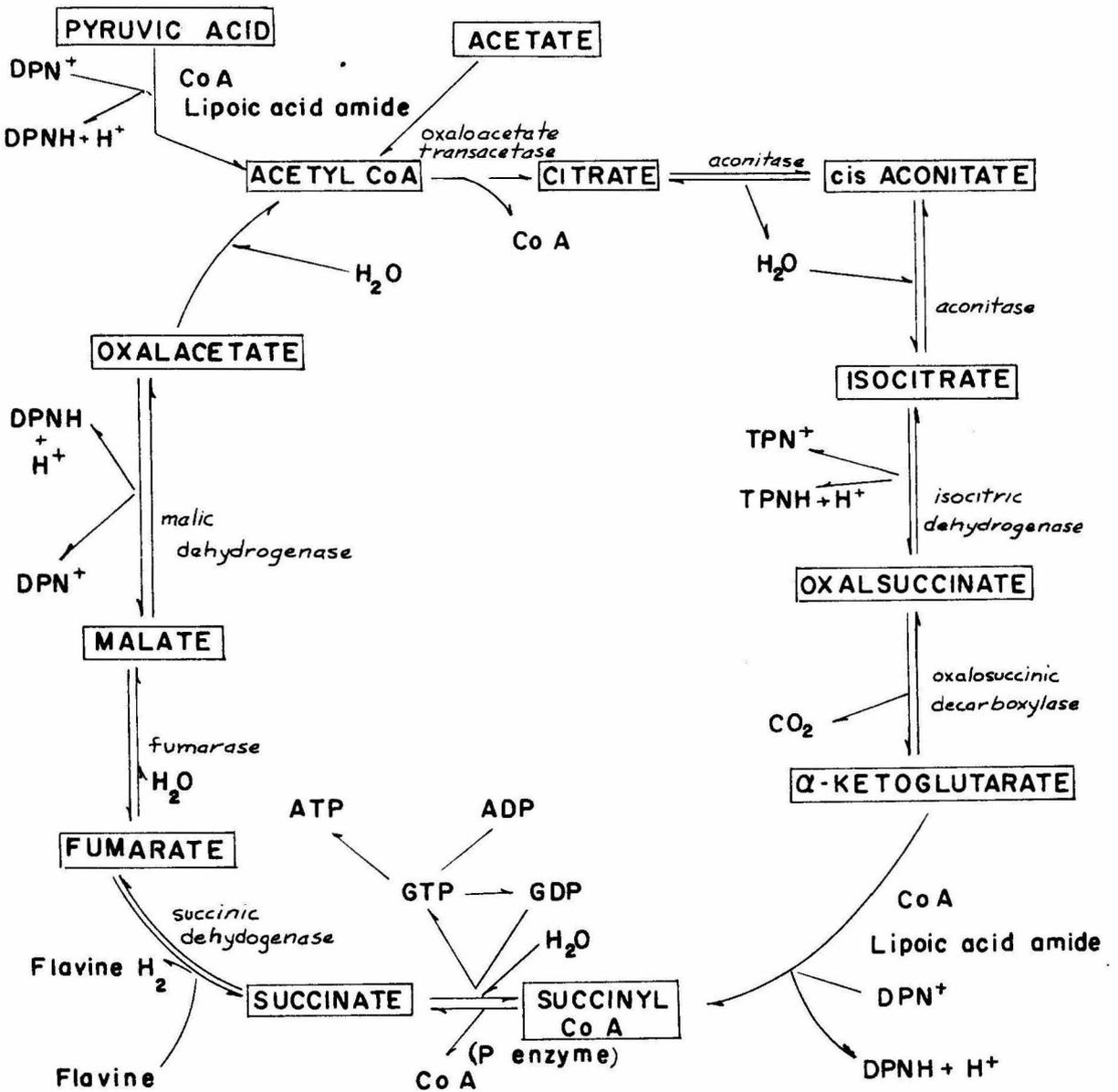
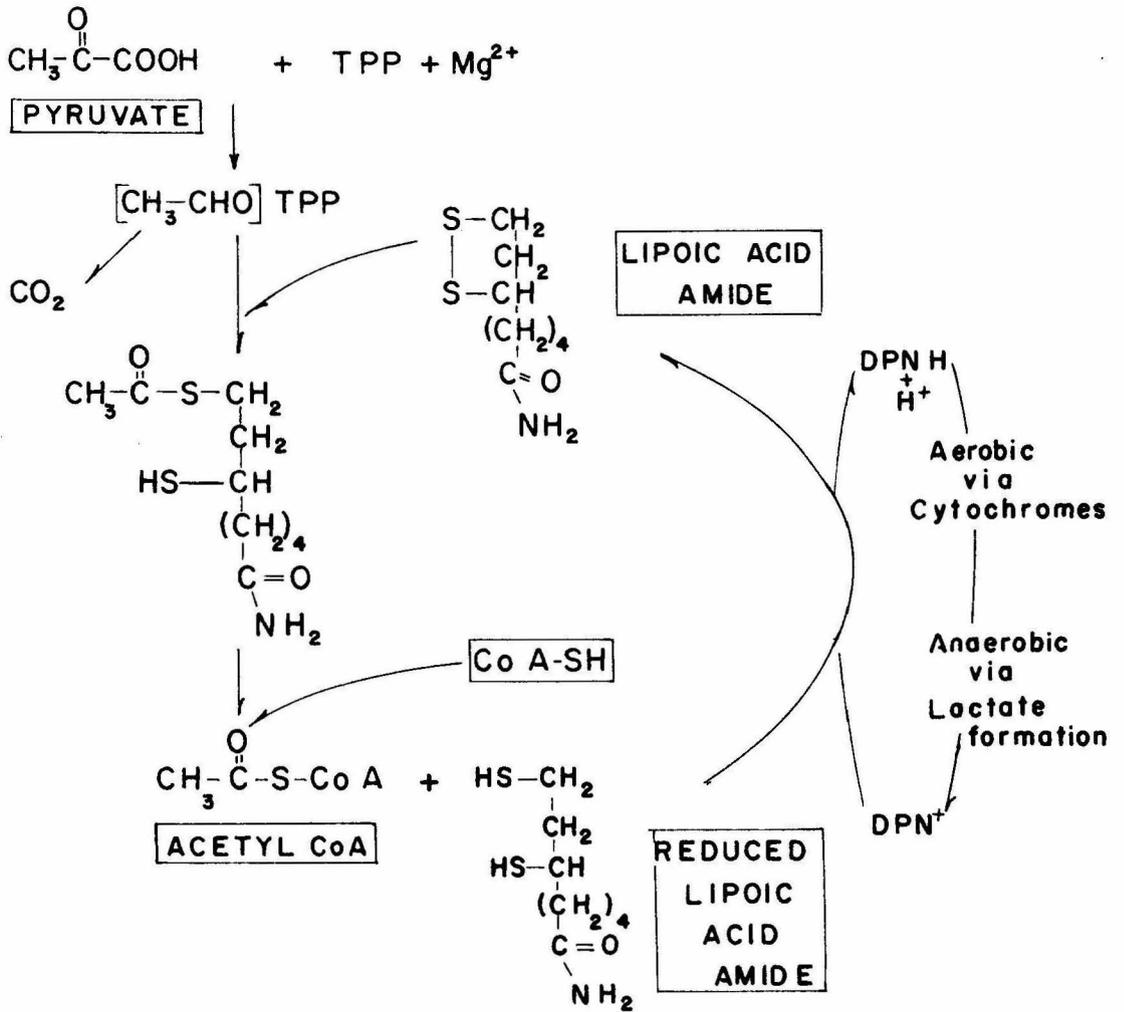


FIGURE 6-3

CONVERSION OF PYRUVATE TO ACETYL-CoA



aldolase-triose phosphate dehydrogenase system, for example, has been implicated as the critical site of chlorine attack because of the reactivity between sulfhydryl groups and chlorine (17). In order to gain some idea of the gross effects of monochloramine on these pathways, glucose oxidation and fermentation by monochloraminated resting cells was studied. Other oxidizable substrates included in these studies were (a) pyruvate, an intermediate at the end of the Embden-Meyerhoff system, (b) acetate, which enters the citric acid cycle after being activated, and (c) succinate, an intermediate in the citric acid cycle. The fermentation of several intermediates of the Embden-Meyerhoff system studied included glucose, fructose-1, 6-diphosphate and sodium pyruvate.

The effects of monochloramine on specific dehydrogenases of E. coli were tested. These included formic dehydrogenase, lactic acid dehydrogenase, succinic dehydrogenase, and triose phosphate dehydrogenase, several of which are known to contain sulfhydryl groups.

In addition to studying the effect of monochloramine on enzymes in oxidation-reduction systems, the effect of this inhibitor on the induction of β -D-galactosidase was chosen as a measure of interference with protein synthesis. The techniques for assaying β -D-galactosidase have been well established and the system has been studied in great detail (58, 60, 75, 76).

A. RESULTS - RESPIRATION

1. Glucose

The first manometric studies with glucose (generally

0.0556 M) were carried out by tipping monochloramine into flasks 10 to 15 minutes after the glucose tip. Representative flasks were removed 15 minutes thereafter for plate counts. A plot of cumulative μ l of oxygen utilized per mg dry weight of cells versus time was made for each trial (figures 6-4 through 6-10). In some cases endogenous rates were plotted along with total respiration rates to indicate the effects of monochloramine on endogenous activity (tests 3, 5 and 8). In other figures, cumulative glucose respiration was corrected for endogenous respiration. Respiration rates (Q_{O_2}) were computed from the initial linear portions of the curves and tabulated (table 6-1). There was some variation in control Q_{O_2} values from experiment to experiment, ranging from 10 to 14.

The intensity of monochloramination was varied in each experiment in order to encompass the entire range of effects on respiration. Note that in order to reduce respiration rates below 70%, monochloramine doses which killed more than 99% of the cells were required (table 6-1).

It can be seen (figures 6-6 through 6-8) that the higher doses of monochloramine lowered the rates of oxidation. The new rate remained steady for awhile (approximately 1.5 hours) and then increased significantly, although not to the extent that the Q_{O_2} equalled that of the untreated cells. In test 12 (figure 6-9) plate counts were made of the contents of all 18 Warburg vessels at suitable intervals (table 6-2). There is little doubt that treated cells were growing in the buffered glucose substrate, whereas control cells showed essentially no increase in population.

FIGURE 6-4

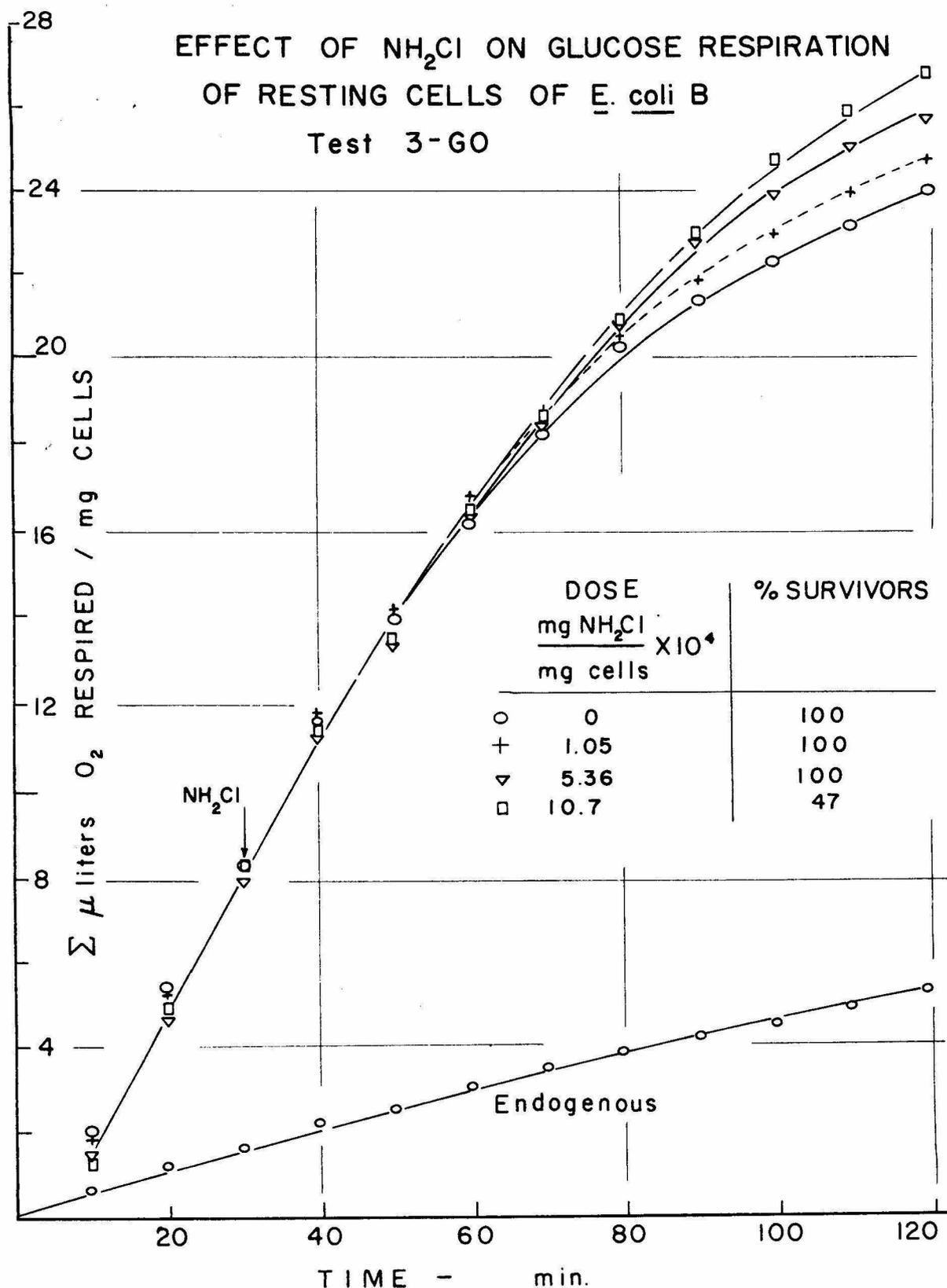


FIGURE 6-5
EFFECT OF NH_2Cl ON GLUCOSE RESPIRATION
OF RESTING CELLS OF *E. coli* B
Test 5 GO

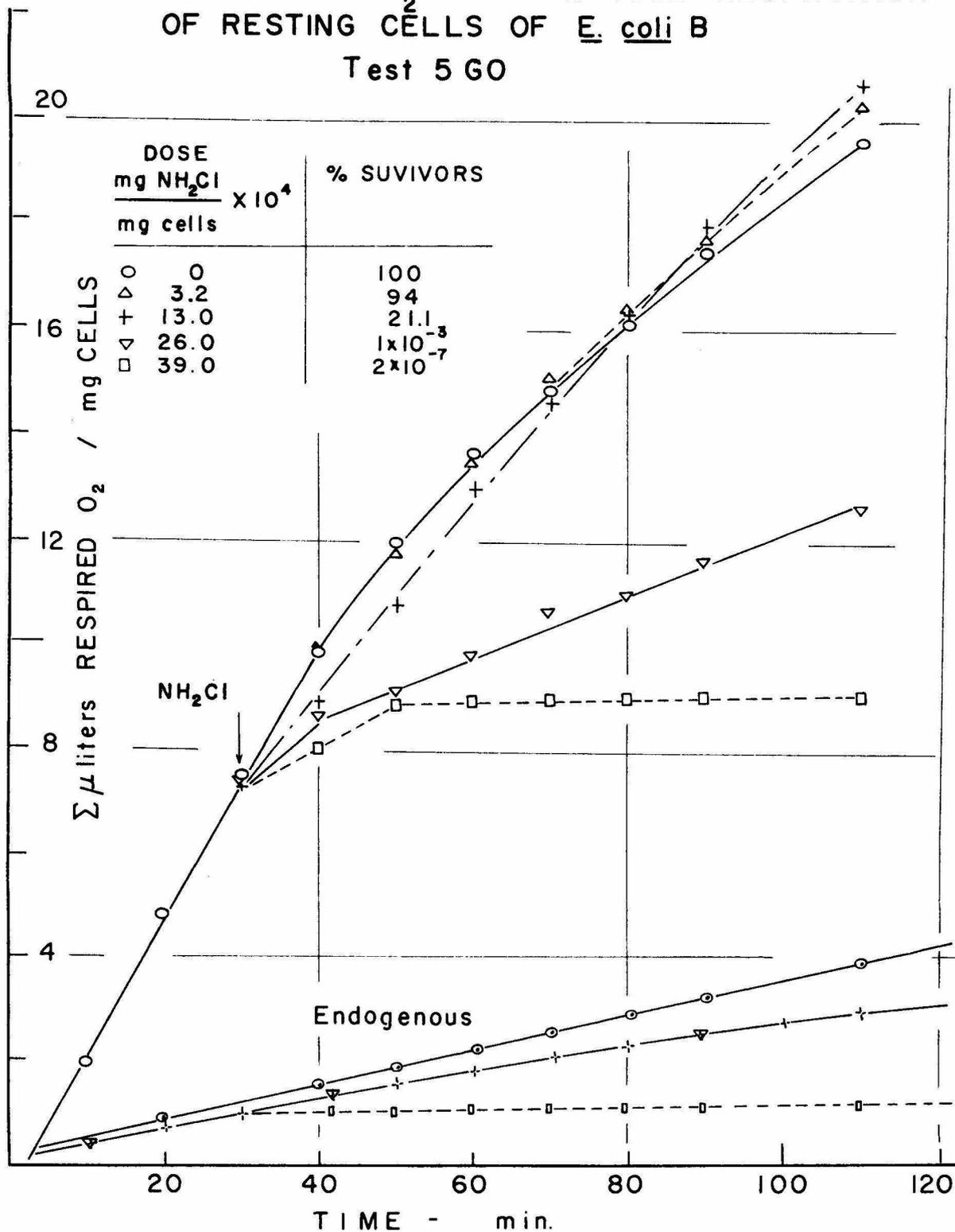


FIGURE 6-6

EFFECT OF NH_2Cl ON GLUCOSE RESPIRATION OF RESTING CELLS OF E. coli B

Test 8 GO

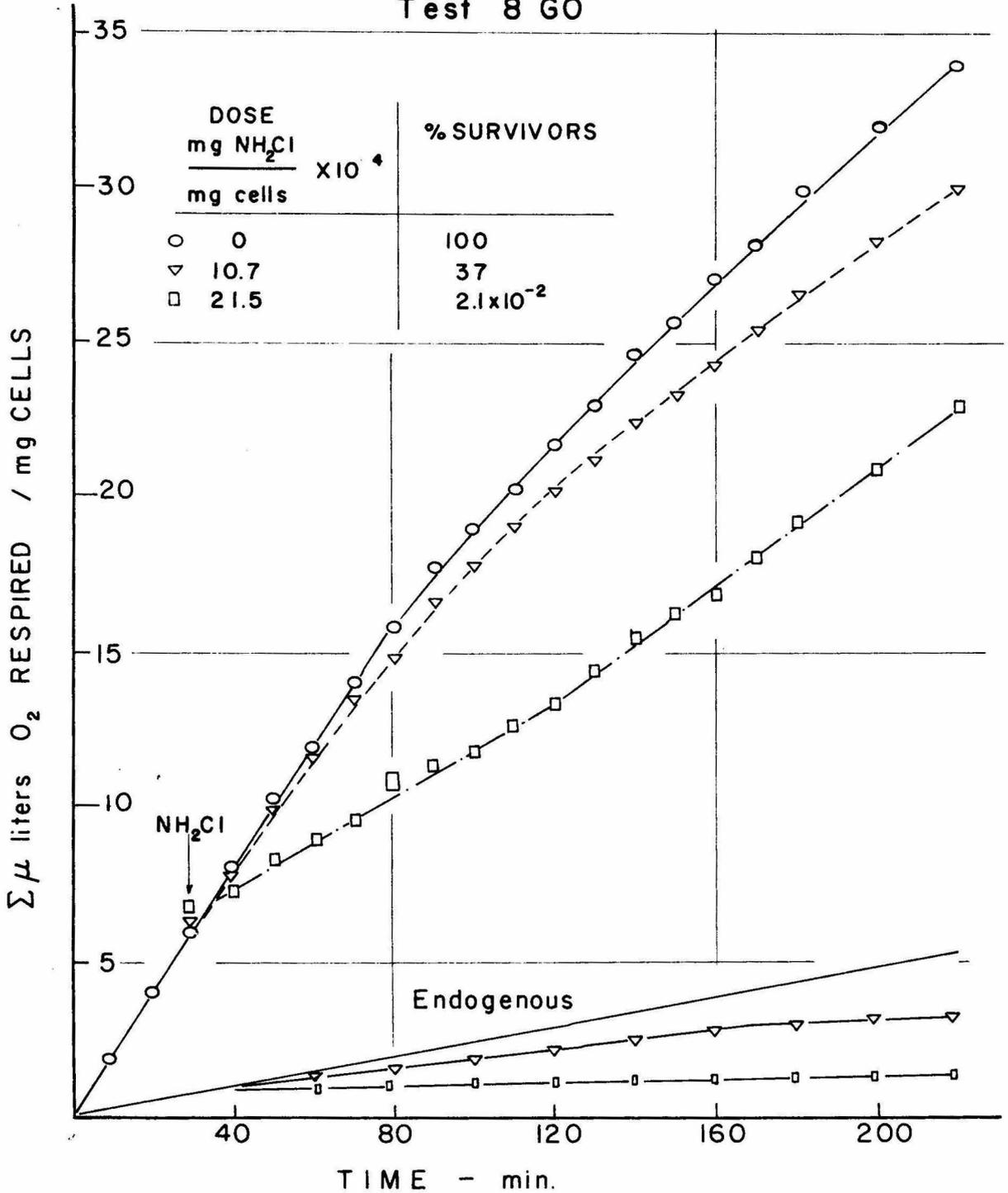


FIGURE 6-7

EFFECT OF NH_2Cl ON GLUCOSE
RESPIRATION OF RESTING
CELLS OF E. coli B

Test 9 GO

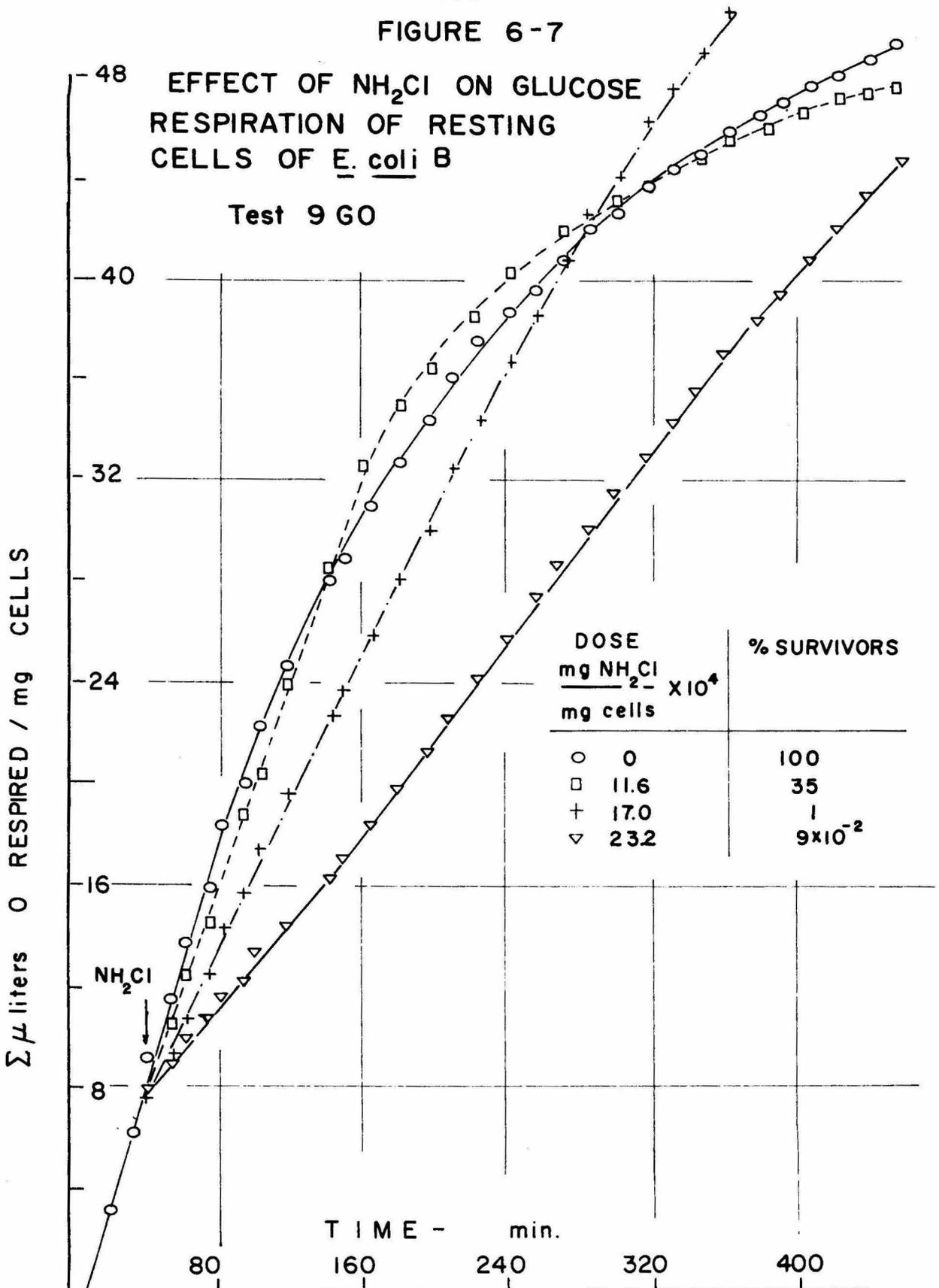


FIGURE 6-8

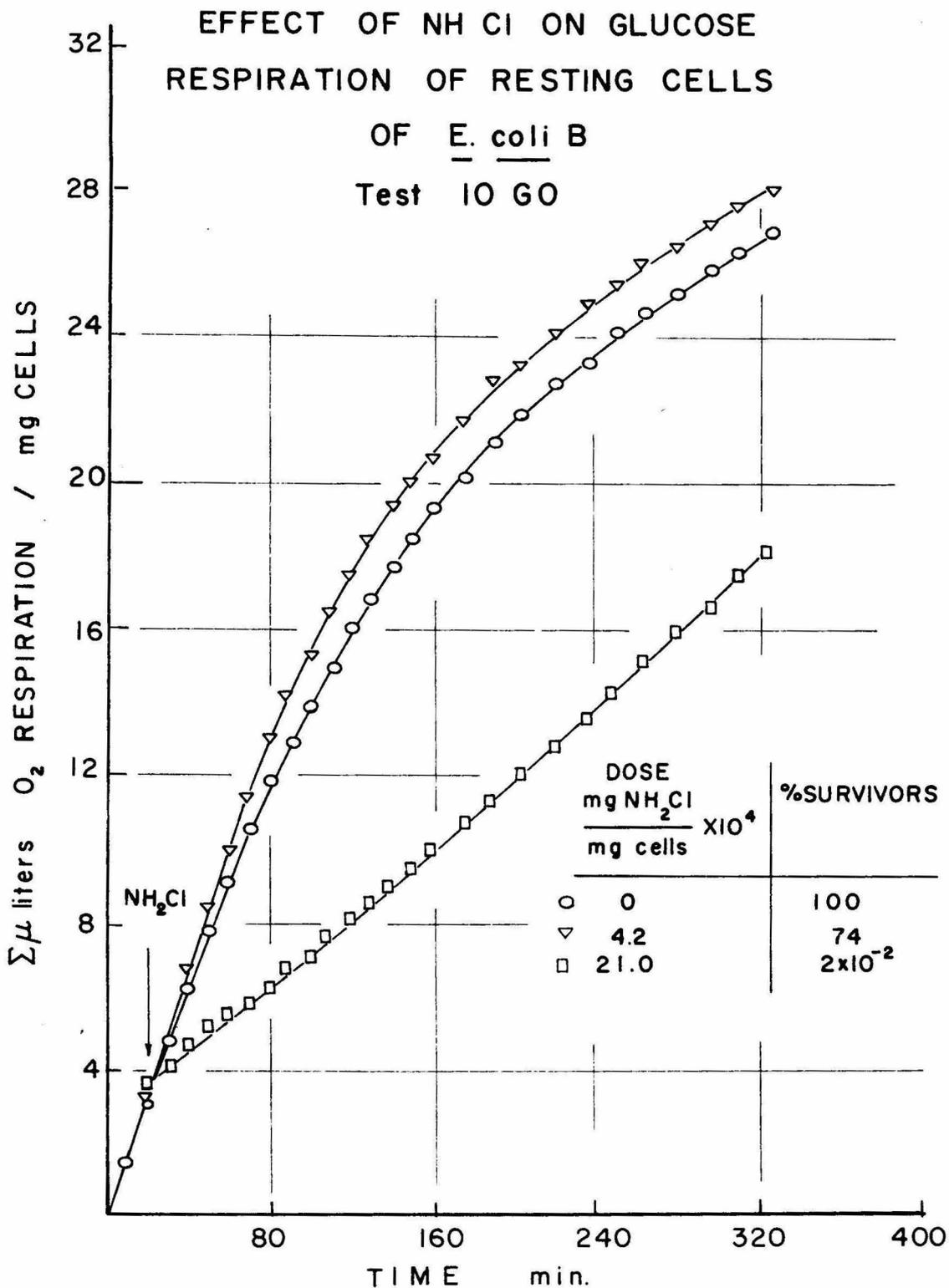


FIGURE 6-9

EFFECT OF NH_2Cl ON GLUCOSE RESPIRATION
OF RESTING CELLS OF E. coli B

Test 12 GO

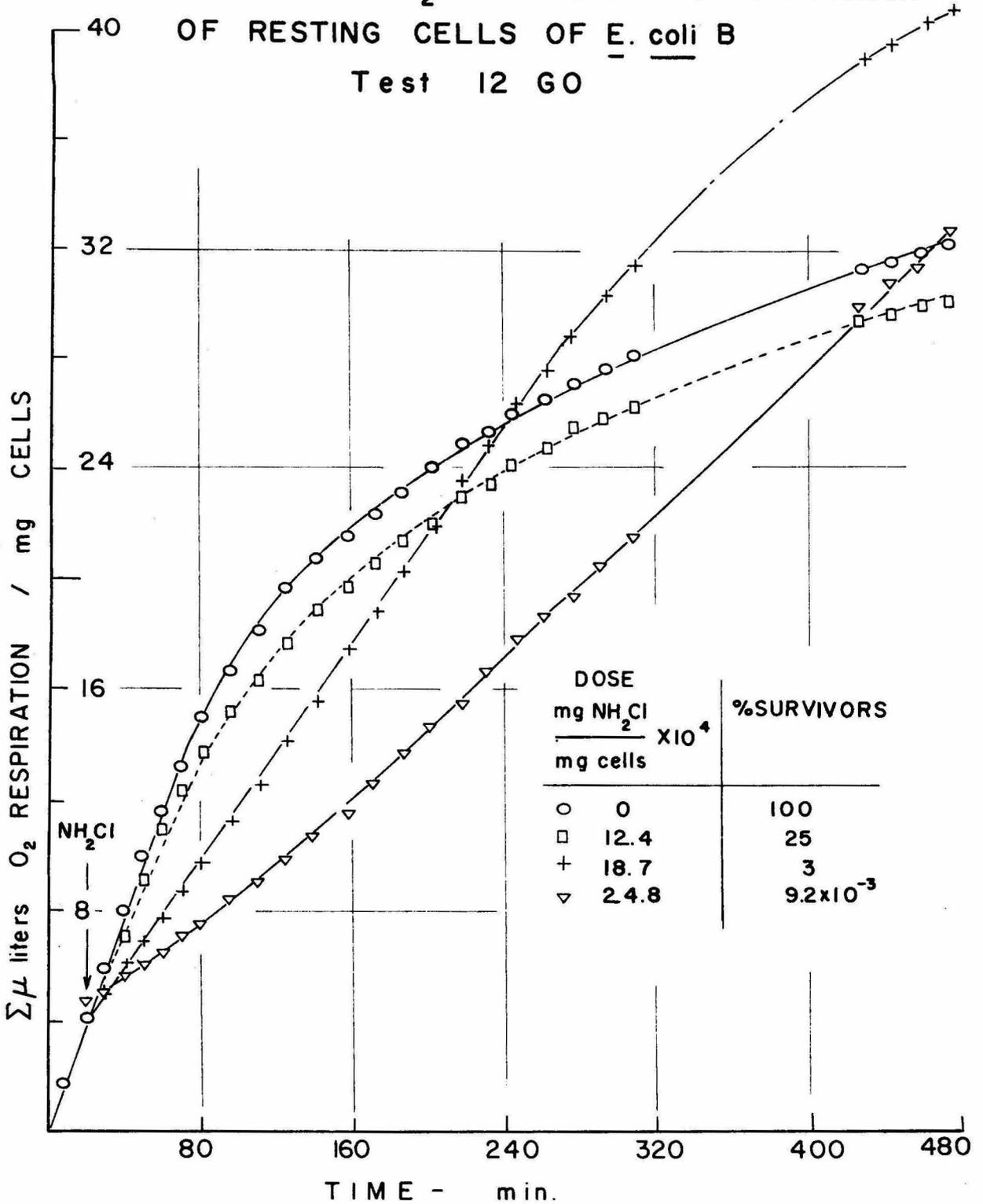


FIGURE 6-10

EFFECT OF NH_2Cl ON
GLUCOSE RESPIRATION
OF RESTING CELLS
OF E. coli B

Thiosulfate tests
11-AGO

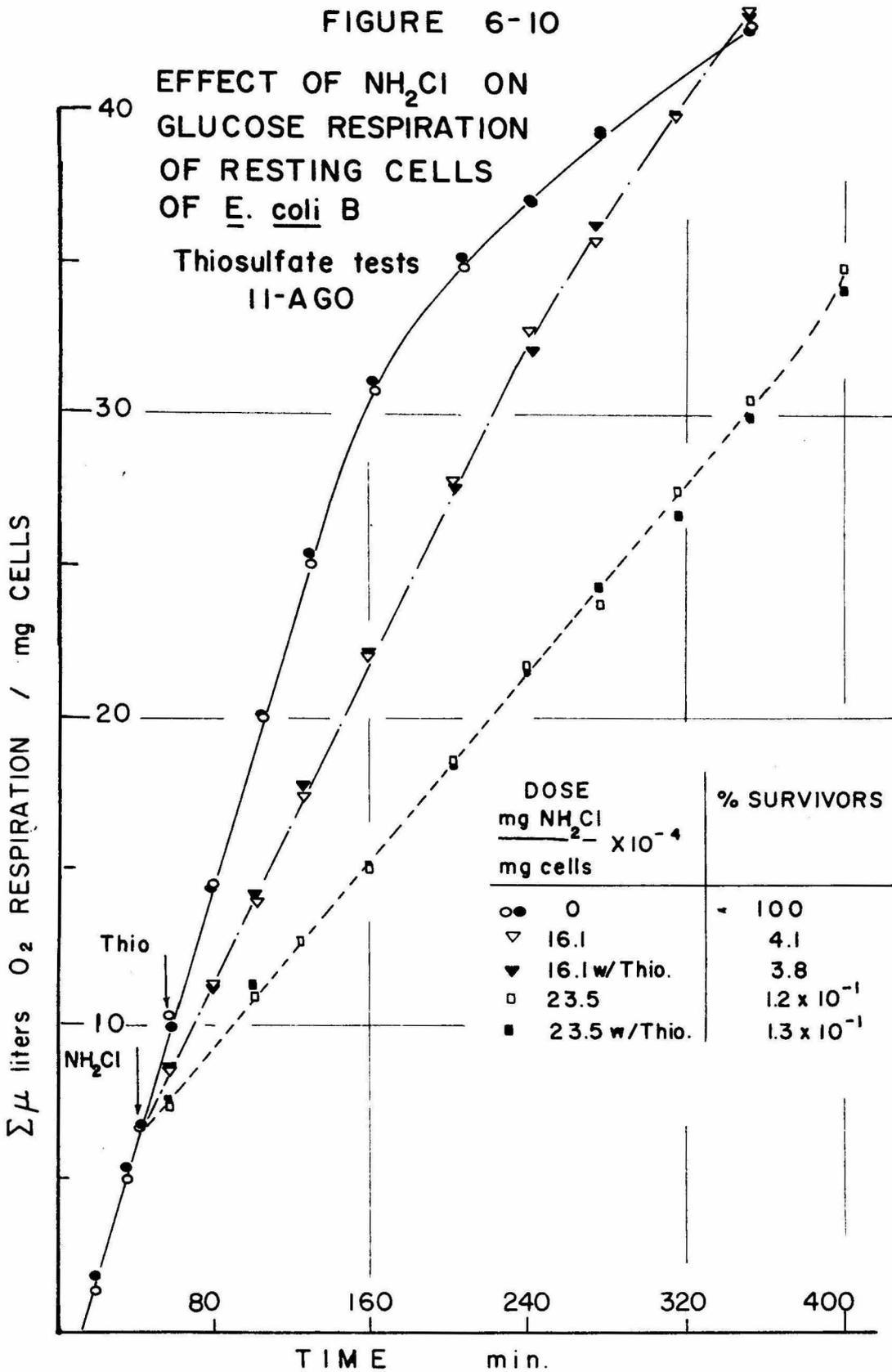


TABLE 6-1

EFFECT OF NH_2Cl ON GLUCOSE OXIDATION
OF RESTING CELLS OF E. COLI B. (Treated in Flasks).

Experiment No.	Treatment $\frac{\text{mgNH}_2\text{Cl}}{\text{mg cells}} \times 10^4$	QO_2 $\mu\text{l/mg/hr.}$	% Activity Remaining	% Survivors
3·GO	0	14.1	-	-
	1.05	14.1	100	100
	5.36	14.1	100	100
	10.7	14.1	100	47
5·GO	0	14.2	-	-
	3.2	14.2	100	94
	13.0	9.7	68	21
	26.0	3.0	21	1×10^{-3}
	39.0	0.0	0	2×10^{-7}
8·GO	0	10.2	-	-
	10.7	9.2	90	37
	21.5	4.8	47	2.1×10^{-2}
9·GO	0	13.0	-	-
	11.6	11.2	86	35
	17.0	8.2	63	1
	23.2	4.4	34	9×10^{-2}
10·GO	0	10.2	-	-
	4.2	10.2	100	74
	21.0	3.1	30	2×10^{-2}
12·GO	0	12.0	-	-
	12.4	9.6	80	25
	18.7	6.4	53	3
	24.8	2.9	24	9.2×10^{-3}

TABLE 6-1 (Cont'd)

Experiment No.	Treatment $\frac{\text{mgNH}_2\text{Cl}}{\text{mg cells}} \times 10^4$	Q_{O_2} $\mu\text{l/mg/hr.}$	% Activity Remaining	% Survivors
11-A-GO	0	14.8	-	-
	0 w/thio	14.8	-	-
	16.1	8.9	60	4.1
	16.1 w/thio	8.9	60	3.8
	23.5	4.9	33	0.12
	23.5 w/thio	4.9	33	0.15

TABLE 6-2
 TEST 12 G.O
 PLATE COUNTS FROM VESSELS
 THROUGHOUT WARBURG TRIAL (cf. Fig. 6-10)

Time (min)	Control	1.24×10^{-3} mgNH ₂ Cl <u>mg cells</u>	1.87×10^{-3} mgNH ₂ Cl <u>mg cells</u>	2.48×10^{-3} mgNH ₂ Cl <u>mg cells</u>
0	93×10^8	23×10^8	28×10^7	86×10^4
50	-	17×10^8	25×10^7	89×10^4
100	97×10^8	30×10^8	47×10^7	14×10^5
200	-	32×10^8	66×10^7	37×10^5
400	101×10^8	40×10^8	84×10^7	97×10^5

Time = 0 at 15 min. following NH₂Cl tip - no neutralization

All counts from Warburg vessels removed during run

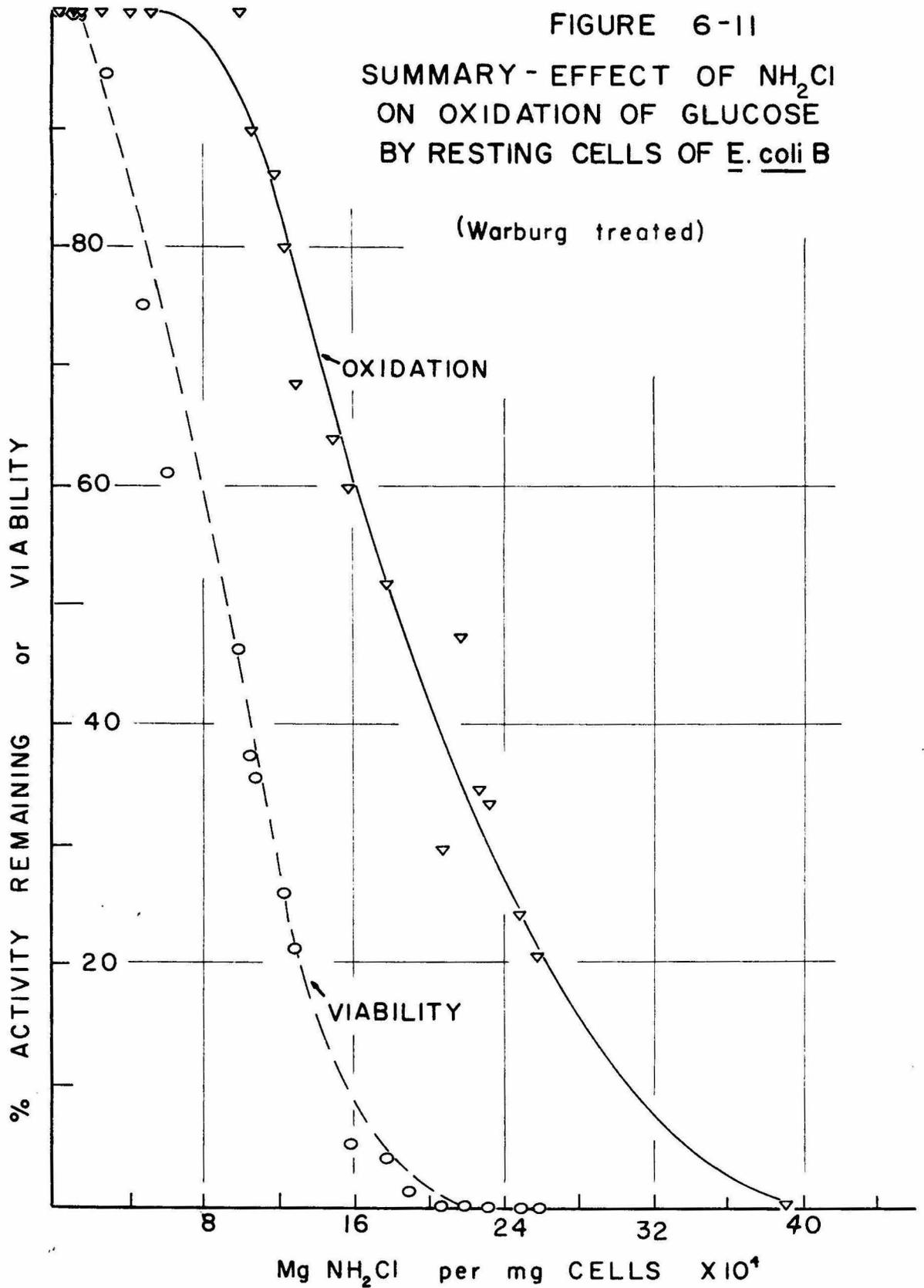
Test 11 A (figure 6-10) was designed to evaluate the effects of adding sodium thiosulfate to monochloramine treated cell suspensions oxidizing glucose. Six-fold molar excesses of sodium thiosulfate were used initially. Glucose was added directly to cell suspensions, and the sidearms were filled with appropriate dilutions of monochloramine and sodium thiosulfate. Thiosulfate was tipped 10 minutes after the monochloramine tip. Note that additions of sodium thiosulfate had no effect on respiration rates. Analogous results were obtained with lower stoichiometric excesses of thiosulfate (2 to 5 fold) tipped in 1 to 5 minutes after monochloramination.

The results of the preceding seven experiments are tabulated (table 6-1) and are graphically represented in figure 6-11. These data clearly show that cell viability is much more sensitive than glucose oxidation to the effects of monochloramine. Also the effect is not strictly linear and does not assume the appearance of a first-order reaction.

The respiratory quotient ($R.Q. = CO_2 \text{ produced} / O_2 \text{ consumed}$) of E. coli was not appreciably affected by monochloramine. In one experiment, for example, the R.Q. of untreated cells was 1.08, whereas a monochloramine dose of 21 mg monochloramine per mg cells lowered the R.Q. to 0.972. This dose was sufficient to kill over 99.9% of the cells and reduce the oxidative activity to about 49%.

Certain difficulties, inherent in the aforementioned approach, became apparent. One question had to do with the high pH of the monochloramine solution which was tipped into the cell-substrate suspension. Checks with indicators proved the buffer capacity of

FIGURE 6-11
SUMMARY - EFFECT OF NH_2Cl
ON OXIDATION OF GLUCOSE
BY RESTING CELLS OF E. coli B



the system to be adequate, although there was some question concerning the incomplete mixing which might have occurred instantaneously after the tip. It was not desirable to neutralize the monochloramine solution prior to inoculation into the Warburg flask arm, since at the neutral pH range and in the presence of light the monochloramine solution might lose some of its strength. Moreover, with only two sidearms on the flasks, one containing monochloramine and the other with thiosulfate, it was not possible to adjust the pH after treatment with monochloramine. Furthermore, the cells remained in continued contact with thiosulfate and reaction products between thiosulfate and monochloramine during manometric measurements. Accordingly, cell suspensions were treated with monochloramine, neutralized with thiosulfate, washed and then tested for their oxidative capacities in the Warburg vessels. This "pretreatment" method provided closer control than carrying out the reaction in the closed system.

A series of four tests were made with such "pretreated" cell suspensions. Tests 13 (figure 6-12) and 16 (figure 6-13) were extensions of the thiosulfate studies made previously (figure 6-10). Cells were treated for 15 minutes with monochloramine at 30° C, neutralized with excess sodium thiosulfate, diluted with buffer, centrifuged and resuspended to the original volume. Control samples were not neutralized with thiosulfate. Figures 6-12 and 6-13 indicate that thiosulfate had no effect on respiration and that apparently dilution and washing of the cells with buffer was as effective as thiosulfate in neutralizing excess monochloramine.

FIGURE 6-12

EFFECT OF NH_2Cl ON GLUCOSE RESPIRATION OF RESTING CELLS OF *E. coli* B

PRETREATED
TEST 1360

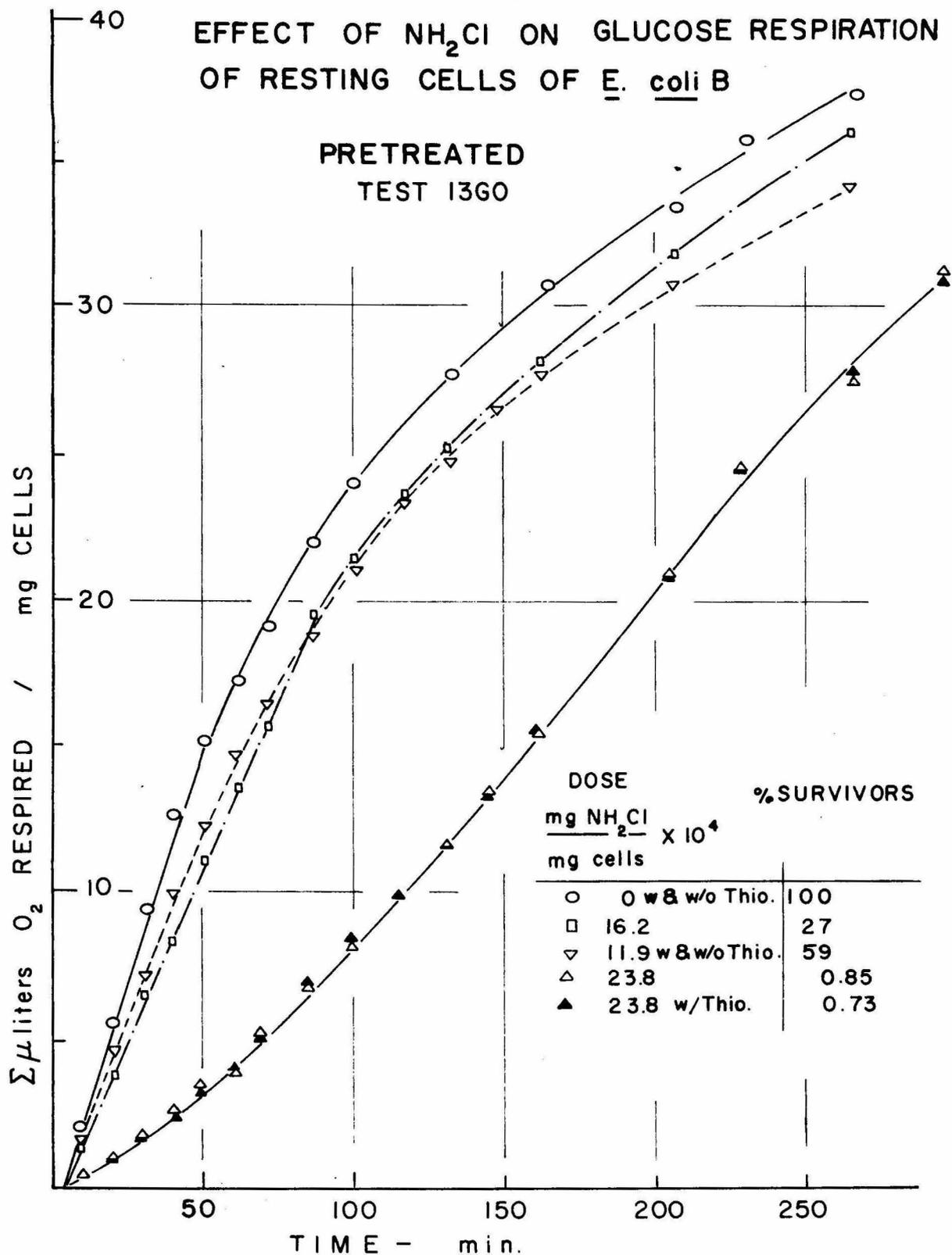
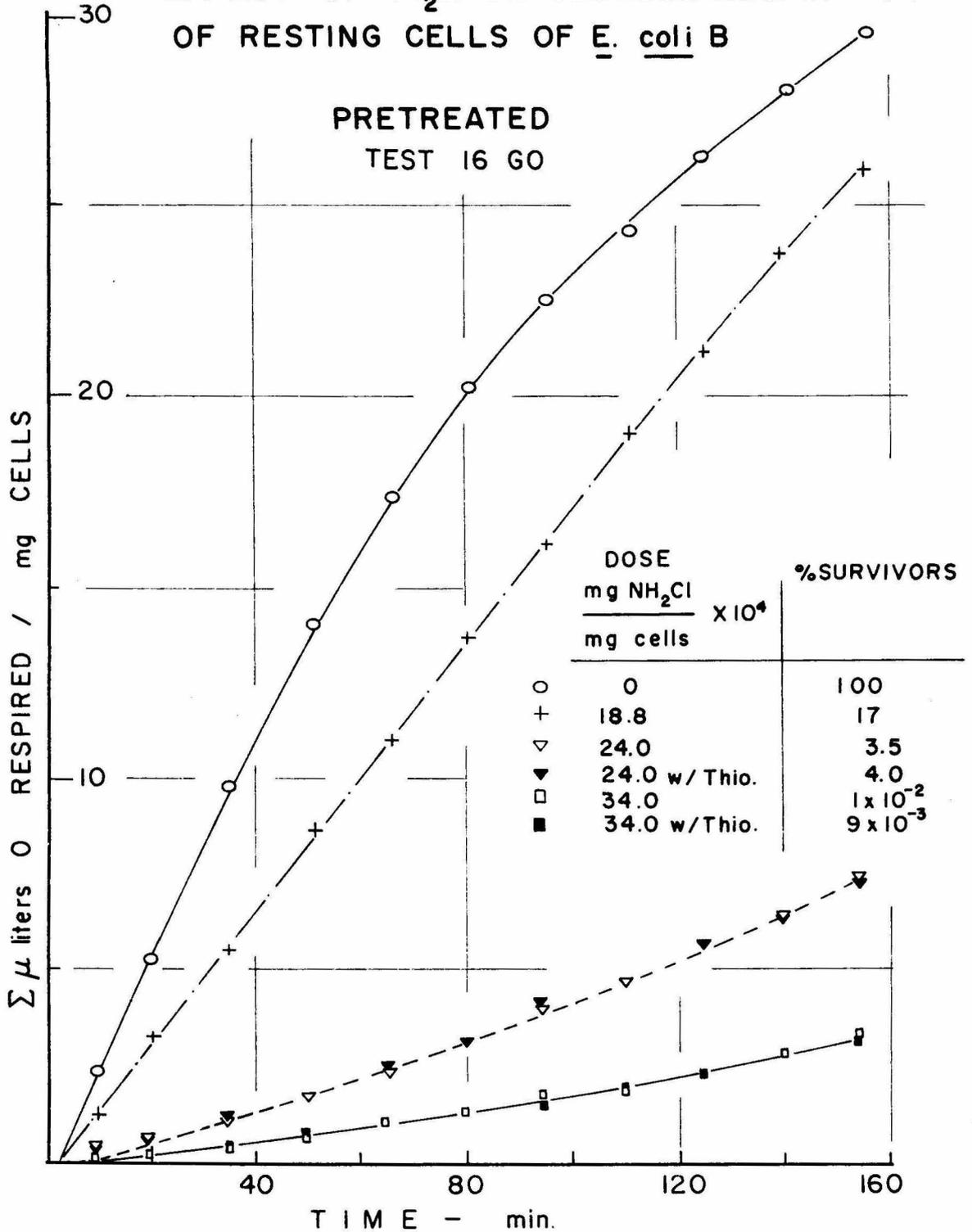


FIGURE 6-13

EFFECT OF NH_2Cl ON GLUCOSE RESPIRATION OF RESTING CELLS OF E. coli B



In the early part of the respiration studies it was found that doses of monochloramine required for a given per cent kill could not be extrapolated from the curves of Butterfield and Wattie (6) who used much more dilute cell suspensions than did we. At the cell densities used in our study, less monochloramine per cell was required to give a kill equivalent to that predicted from Butterfield's data. In tests 14 and 15 (figures 6-14 and 6-15) two parallel cell suspensions were prepared and added to flasks. Prior to dosing with the same weight of monochloramine, one flask was diluted so as to give a final suspension 1/100 that of the other. Following an exposure period of 10 minutes at 37° C, both preparations were neutralized with thiosulfate, centrifuged and the cells in each suspended to identical densities for measuring rates of respiration of glucose. The results (figures 6-14 and 6-15) showed that the respiration rate of the dilute cell suspensions was less sensitive to monochloramine than the concentrated suspensions. Plate counts verified that the dilute suspensions had a higher survival rate than the undiluted ones.

As was observed with cell suspensions treated with monochloramine in the Warburg vessels, the respiration rate of suspensions treated, washed and placed in the vessels increased slightly (cf. curve E, figure 6-14 and curves D and E, figure 6-15) over the measured period. Plate counts made before, and 155 minutes after, inoculation into Warburg flasks (table 6-3) increased appreciably (10-fold or better in two instances) over the 155 minute interval in the glucose-phosphate substrate. Note that the sample

TABLE 6-3

TESTS 15 AND 16 (cf. figure 6-13)

PLATE COUNTS BEFORE AND AFTER

WARBURG RUNS PRETREATED

Treatment $\frac{\text{mg NH}_2\text{Cl}}{\text{mg cells}} \times 10^4$	Before Inoculation	155 min. after Inoculation	Treatment $\frac{\text{mg NH}_2\text{Cl}}{\text{mg cells}} \times 10^4$	Before Inoculation	155 min. after Inoculation
Control	67×10^8	66×10^8	Control	74×10^8	81×10^8
2.17×10^{-3}	53×10^7	88×10^8			
2.71×10^{-3}	43×10^6	45×10^7	2.42×10^{-3}	23×10^7	10×10^8
4.35×10^{-3} (conc.)	29×10^4	36×10^4	2.42×10^{-3}	34×10^7	9.5×10^8
4.35×10^{-3} (1:100 Dilute)	44×10^4	51×10^6	3.41×10^{-3}	21×10^5	56×10^5
			3.41×10^{-3} (w/thio)	43×10^4	20×10^5

FIGURE 6-14

EFFECT OF NH_2Cl ON GLUCOSE RESPIRATION
OF RESTING CELLS OF E. coli B

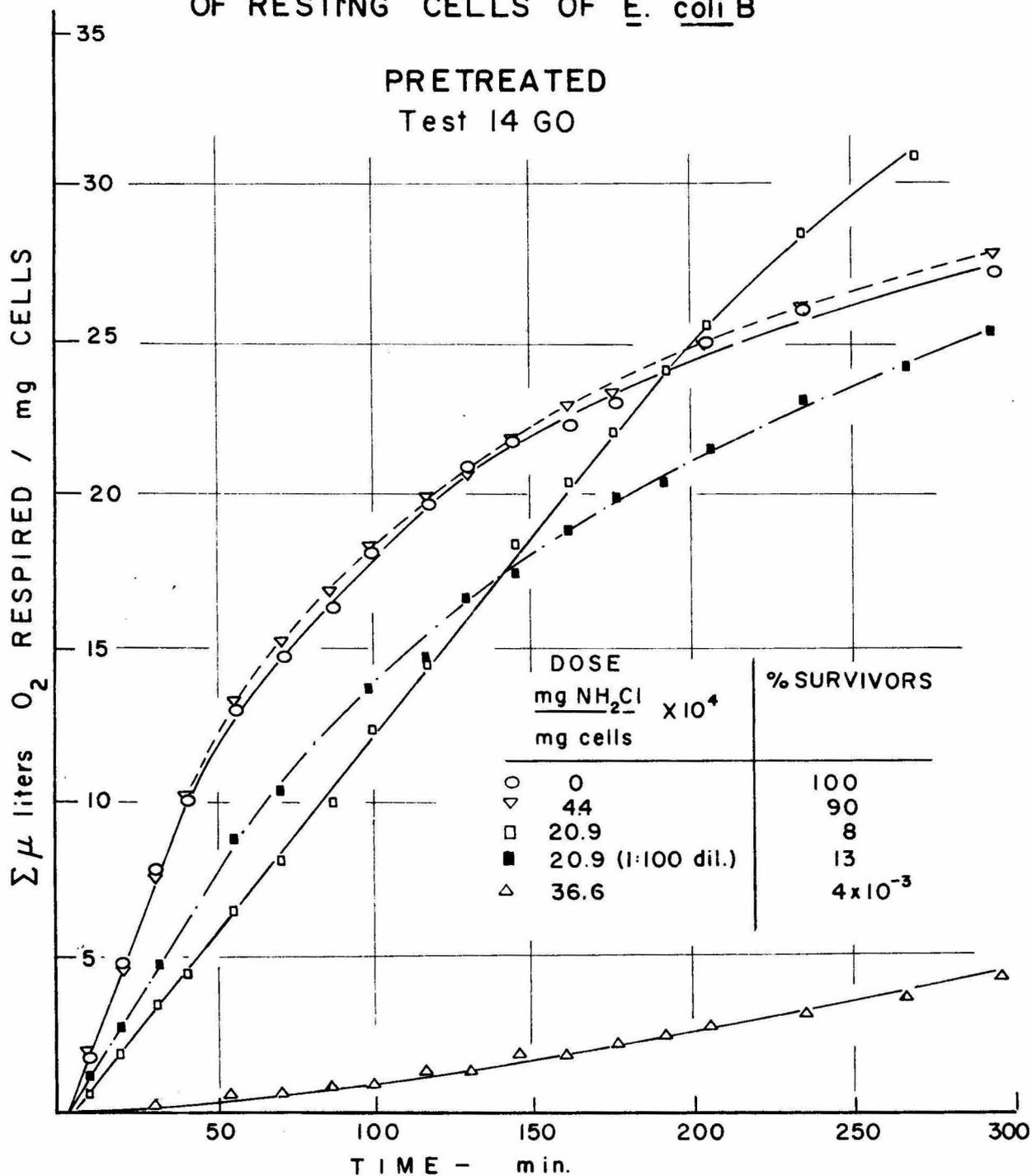
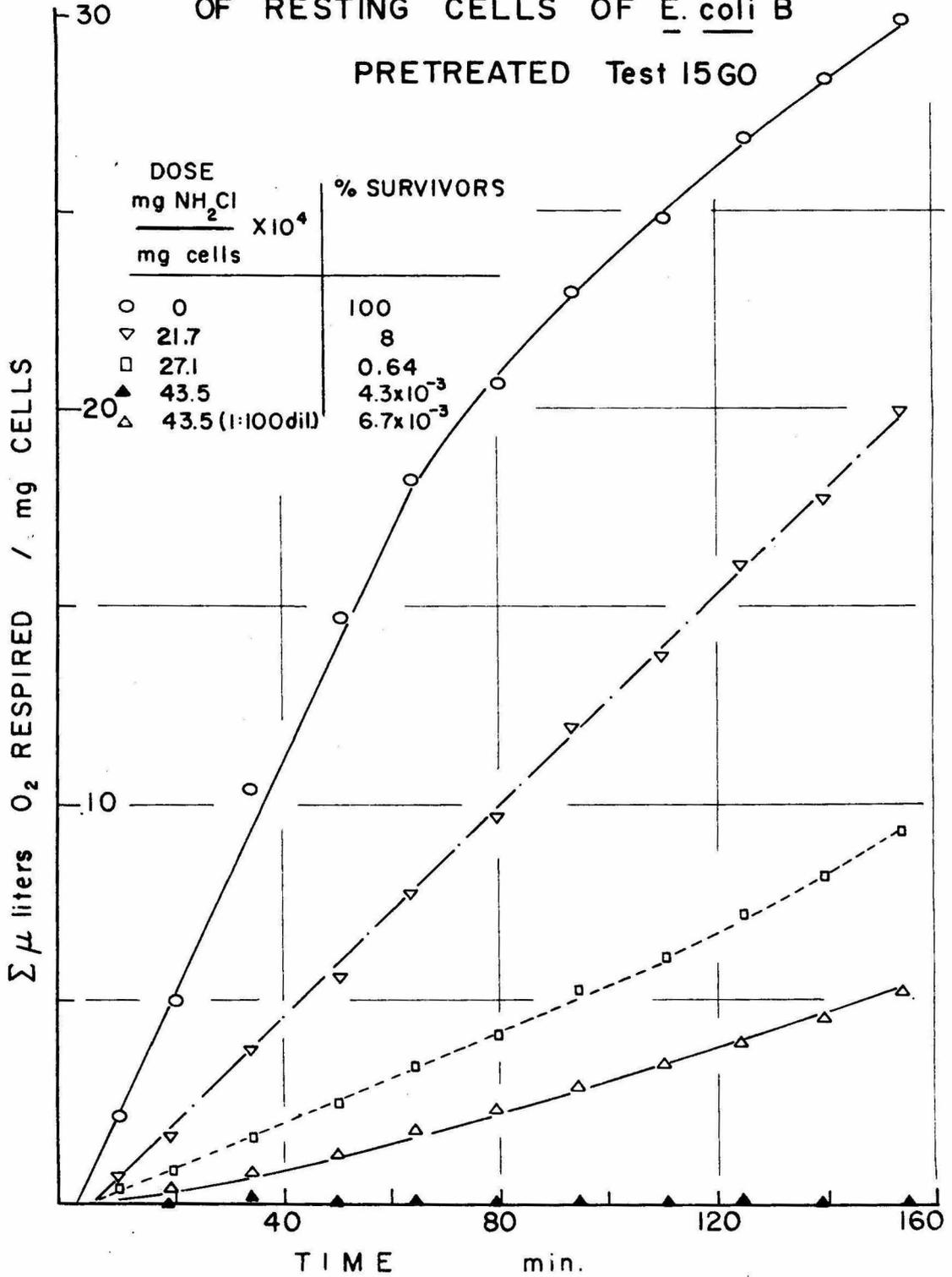


FIGURE 6-15

EFFECT OF NH_2Cl ON GLUCOSE RESPIRATION
OF RESTING CELLS OF E. coli B

PRETREATED Test 15G0



diluted prior to monochloramination recovered at a more rapid rate than the concentrated sample.

The results of the four tests in which cells were treated outside of the Warburg flasks are tabulated in table 6-4 and graphically presented in figure 6-16. The Q_{O_2} values in these experiments were somewhat higher than for those of cells treated in the Warburg apparatus. Also, at comparable monochloramine doses per cent kills were lower in the former experiments, but reductions in respiration effected by the disinfectant were similar.

The role of cell permeability in the action of monochloramine was studied by testing the susceptibility of respiration of cell-free suspensions to chloramination. Table 6-5 indicates that sonic lysates of E. coli B oxidized glucose to a very limited extent (20 to 40% of the total oxidative activity). However, cell viability was reduced from 10 to 16%. Additions of ATP did not increase the oxidative activity of the lysates. Assuming that the cells in the lysate oxidized glucose at a rate proportional to the surviving fraction, it is possible to assign a Q_{O_2} value to the lysate (table 6-5). On the basis of these assumptions, reductions in lysate activity were computed and compared with reductions in activities of intact cells. Note that both preparations were comparable in their sensitivity to monochloramine.

2. Succinate

The effects of monochloramine on succinate oxidation were studied with 0.8% (0.056 M) sodium succinate and a monochloramine tip. Plate counts were made 10 and 120 minutes after the monochloramine tip from selected Warburg vessels. The results of

TABLE 6-4

EFFECT OF NH_2Cl ON GLUCOSE OXIDATION
OF RESTING CELLS OF E. COLI B. (PRETREATED).

Exp. No.	Treatment $\frac{\text{mg NH}_2\text{Cl}}{\text{mg cells}} \times 10^4$	Qo_2 $\mu\text{l O}_2/\text{mg/hr.}$	% Activity Remaining	% Survivors
13-GO	0	14.1	-	-
	11.9	11.3	80	59
	16.2	9.7	69	27
	23.8	3.7	26	0.85
14-GO	0	18.0	-	-
	4.4	18.0	100	90
	20.9	8.5	47	8
	20.9 (100:1)	11.9	66	13
	36.6	1.1	6	4×10^{-3}
15-GO	0	19.2	-	-
	21.7	8.1	42	8
	27.1	3.1	16	0.64
	43.5	0	0	4.3×10^{-3}
	43.5 (100:1)	1.6	8.3	6.7×10^{-3}
16-GO	0	18.0	-	-
	18.8	9.7	54	17
	24.0	3.4	19	3.5
	34.0	1.1	6	1×10^{-2}

FIGURE 6-16

SUMMARY- EFFECT OF NH_2Cl
ON OXIDATION OF GLUCOSE
BY RESTING CELLS OF E. coli B

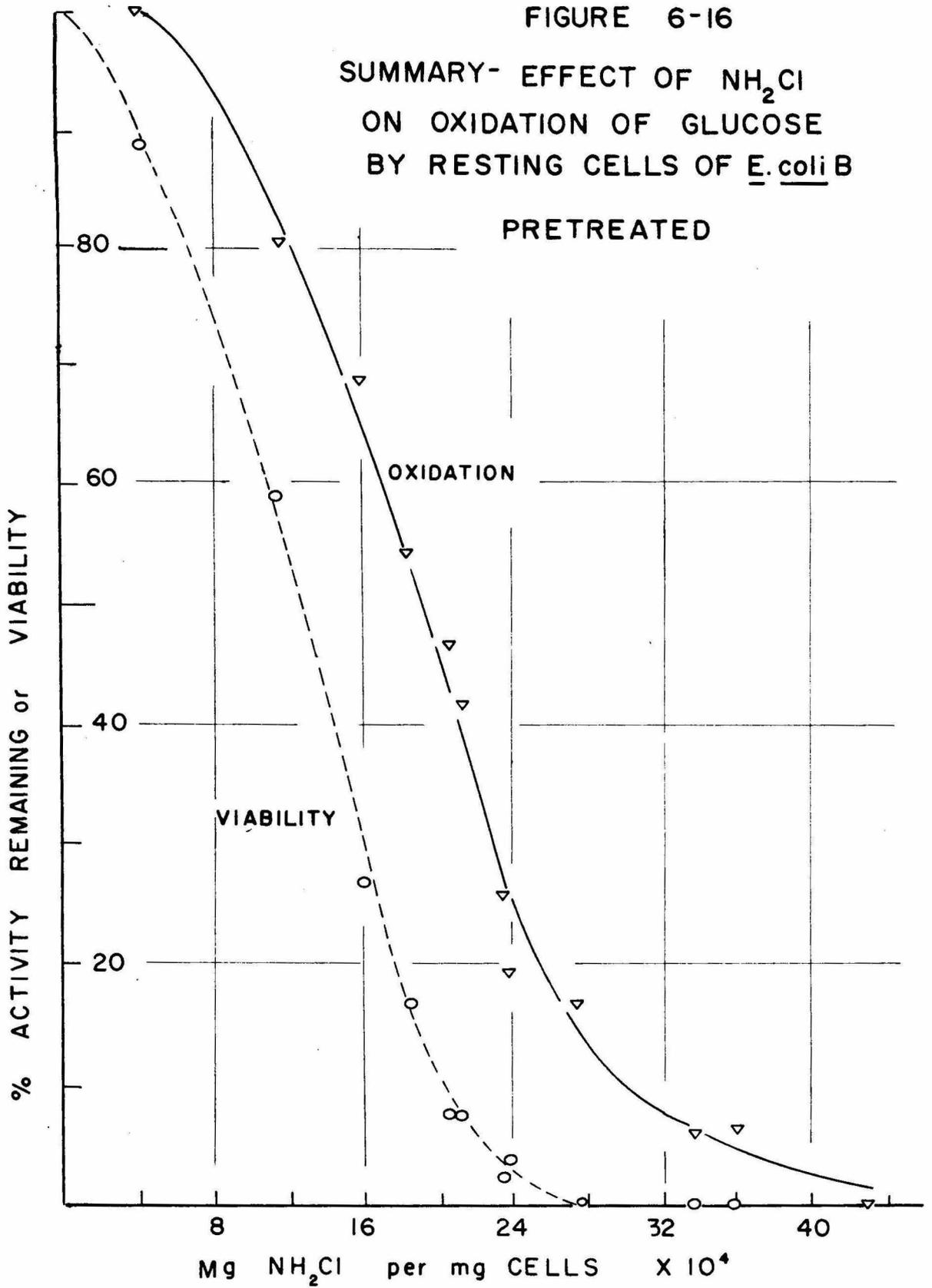


TABLE 6-5

EFFECT OF NH₂Cl ON OXIDATION
OF GLUCOSE BY CELL FREE SYSTEM

Treatment mg NH ₂ Cl mg cells × 10 ⁴	Pl. count	Non-sonicated		Pl. Ct.	Sonicated	
		Qo ₂ (μl O ₂ /mg/hr.)	% Act. Remain- ing		Qo ₂ ** (μl/mg/hr.)	% Act. in Cell* Free System
<u>Test 17</u> control	83 × 10 ⁸	14.8	-	87 × 10 ⁷	3.0	-
13	-	11.0	74	-	2.08	65
29	-	22.2	15	-	0.39	11
<u>Test 18</u> control	62 × 10 ⁸	14.5	-	99 × 10 ⁷	6.1	-
15.5	-	9.4	65	-	4.6	85
<u>Test 19</u> control	55 × 10 ⁸	19.1	-	40 × 10 ⁷	2.9	-
24.0	-	5.5	29	-	0.79	26

** Based on original cell population

* Calculation of activity in cell free system:

Test 17 - % viability in lysate = $\frac{8.7}{8.3} = 10.5\%$

% activity in lysate = $\frac{3.0}{14.8} = 20.1\%$

Division of Qo₂ control among cells and lysate

Qo₂ cells = .105 × 14.8 = 1.48 49% of total activity

Qo₂ lysate = 3.0 - 1.48 = 1.52 51% of total activity

Division of Qo₂ treated among cells and lysate

Qo₂ cells = .74 × 1.48 = 1.10

Qo₂ lysate = 2.08 × 1.10 = .98

∴ % act. in lysate = $\frac{0.98}{1.52} = .65 = \underline{\underline{65\%}}$

three tests are illustrated in figures 6-17, 6-18 and 6-19. Respiration rates, Q_{O_2} , were computed from the initial linear portions of the curves. There is evidence in all three tests of recovery of respiration in the monochloramine treated suspensions, although succinate oxidation was very sensitive to monochloramine. Note that the control in test 3 (figure 6-19) showed evidence of increased respiration rate after about 90 minutes. Plate counts made at intervals after monochloramination indicate that very slight increases in cell population occurred in the flasks with lower monochloramine levels. No population increases were detected in flasks dosed with monochloramine levels above 15×10^{-4} mg monochloramine per mg cells.

Cell lysates were prepared as previously described, and the effects of monochloramine on these lysates were compared with effects on cell-free systems. Although only 4% of the viable population survived, 97% of the succinic oxidase activity remained. Note that Asnis et al. (77) showed that sonic lysates of E. coli retained their ability to oxidize succinate. The results of this test (table 6-6) indicate that the response of succinate oxidation in cell-free systems was almost identical to that of intact cells.

The results of the tests with succinate oxidation are summarized in table 6-7 and in figure 6-20. Note that viability is considerably more sensitive to monochloramine than is succinic acid oxidation. The reduction in succinic acid oxidation was less than that of glucose oxidation, but the per cent kill in the former tests

FIGURE 6-17

EFFECT OF NH_2Cl ON OXIDATION OF SODIUM SUCCINATE BY RESTING CELLS OF E. coli B

TEST 1-S0

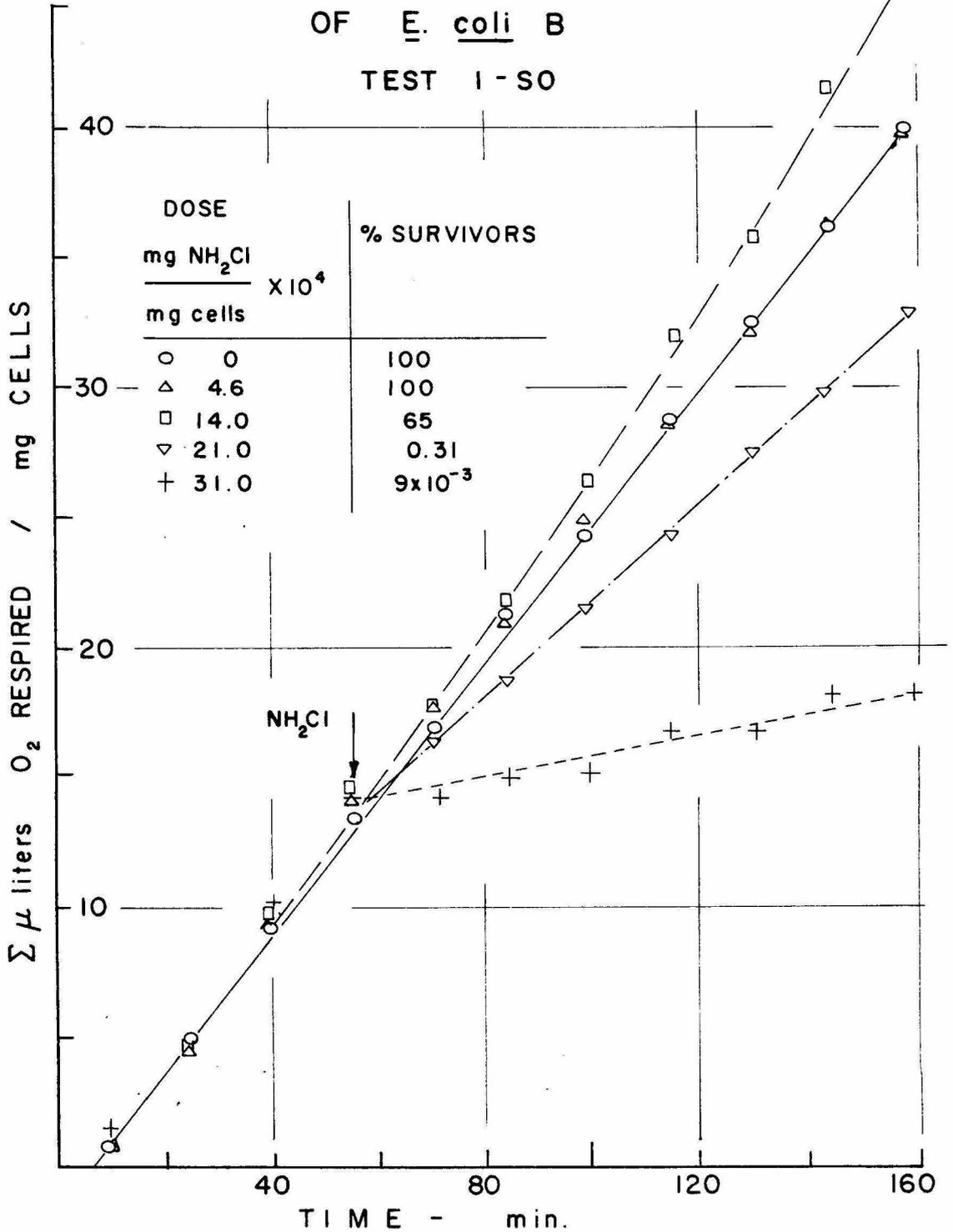


FIGURE 6-18

EFFECT OF NH_2Cl ON OXIDATION
OF SODIUM SUCCINATE BY
RESTING CELLS OF E. coli B
TEST 2-SO

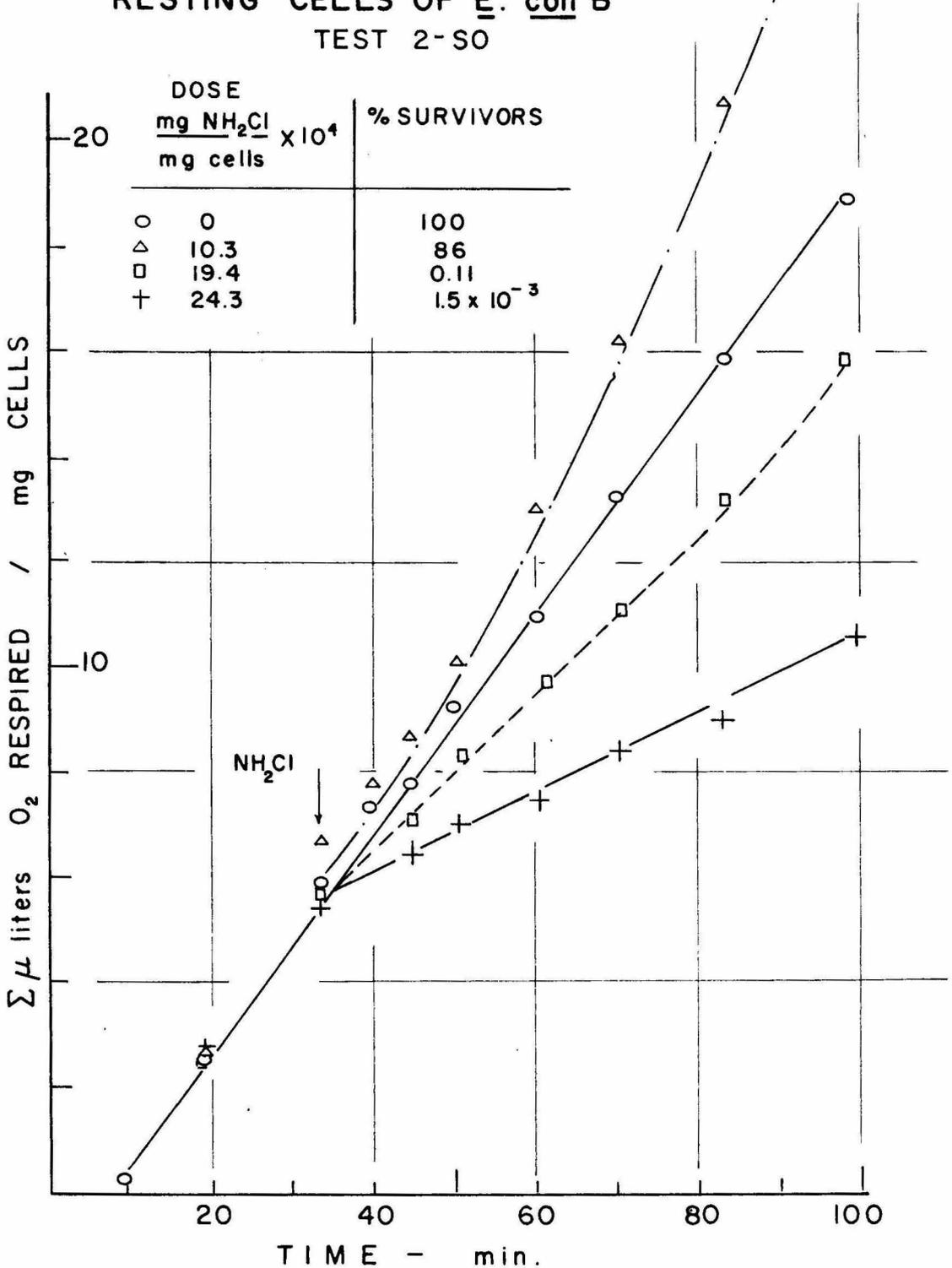


FIGURE 6-19

EFFECT OF NH_2Cl ON OXIDATION OF
SODIUM SUCCINATE BY RESTING
CELLS OF *E. coli* B
TEST 3-S0

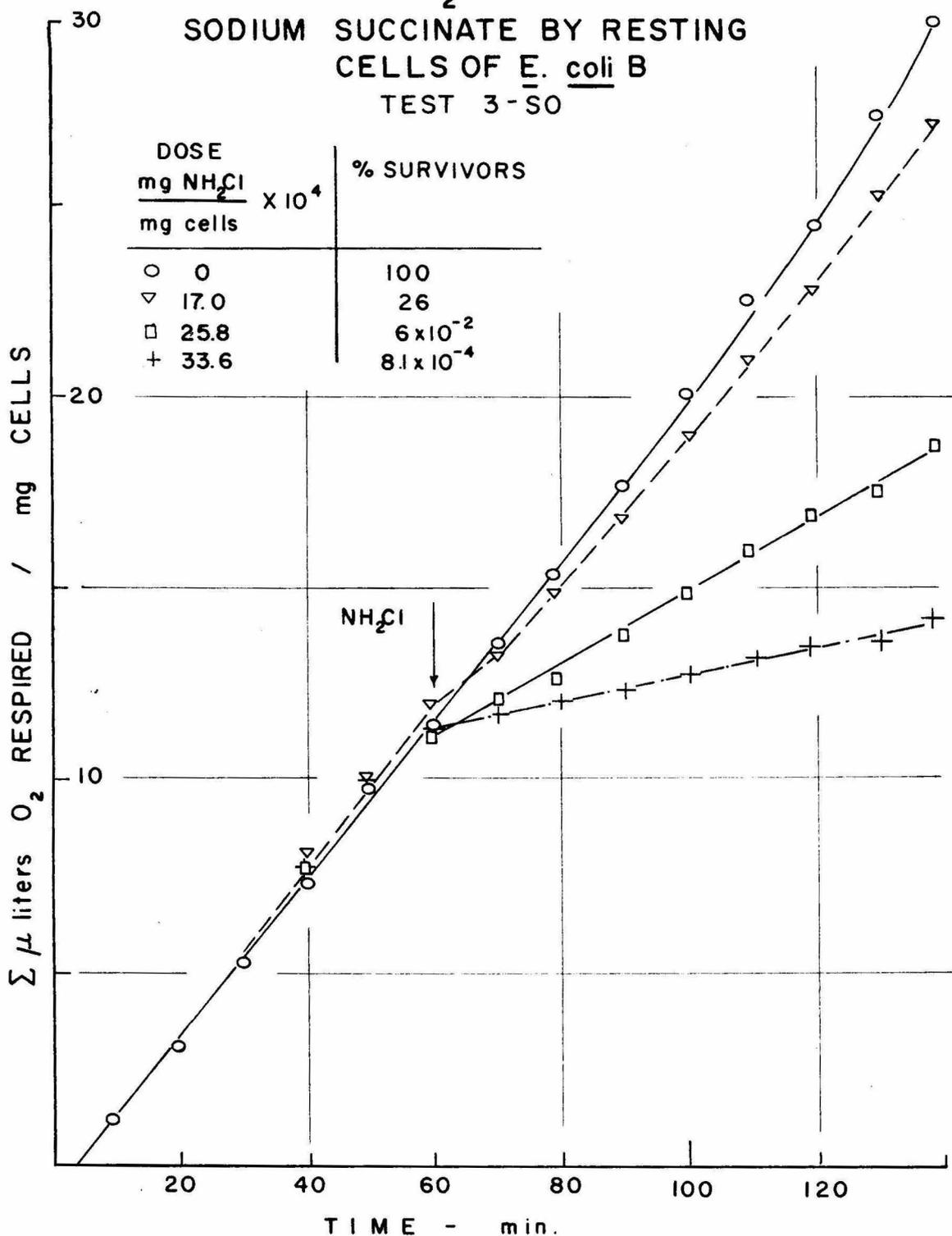


TABLE 6-7
EFFECTS OF NH₂Cl ON SUCCINATE
OXIDATION OF E. COLI B

Exp. No.	Treatment mg NH ₂ Cl mg cells × 10 ⁴	Q _{O₂} ul O ₂ /mg/hr	% Activity Remaining	% Survivors
1-5.0	0	16.0	-	-
	4.6	16.0	100	100
	14.0	16.0	100	65
	21.0	9.6	60	0.31
	32.0	2.4	15	9 × 10 ⁻³
2-5.0	0	12.0	-	-
	10.3	12.0	100	86
	19.4	8.4	70	0.11
	24.3	4.4	37	1.5 × 10 ⁻³
3-5.0	0	10.8	-	-
	17.0	8.9	83	26
	25.8	4.5	42	.061
	33.6	1.0	9	8.1 × 10 ⁻⁴
4-5.0 (sonic)	0	13.2	-	-
	9.2	13.2	100	-
	18.4	7.8	64	-
	27.6	3.4	26	-

FIGURE 6-20

EFFECT OF NH_2Cl ON
SUCCINATE OXIDATION BY
RESTING CELLS OF E. coli B
SUMMARY

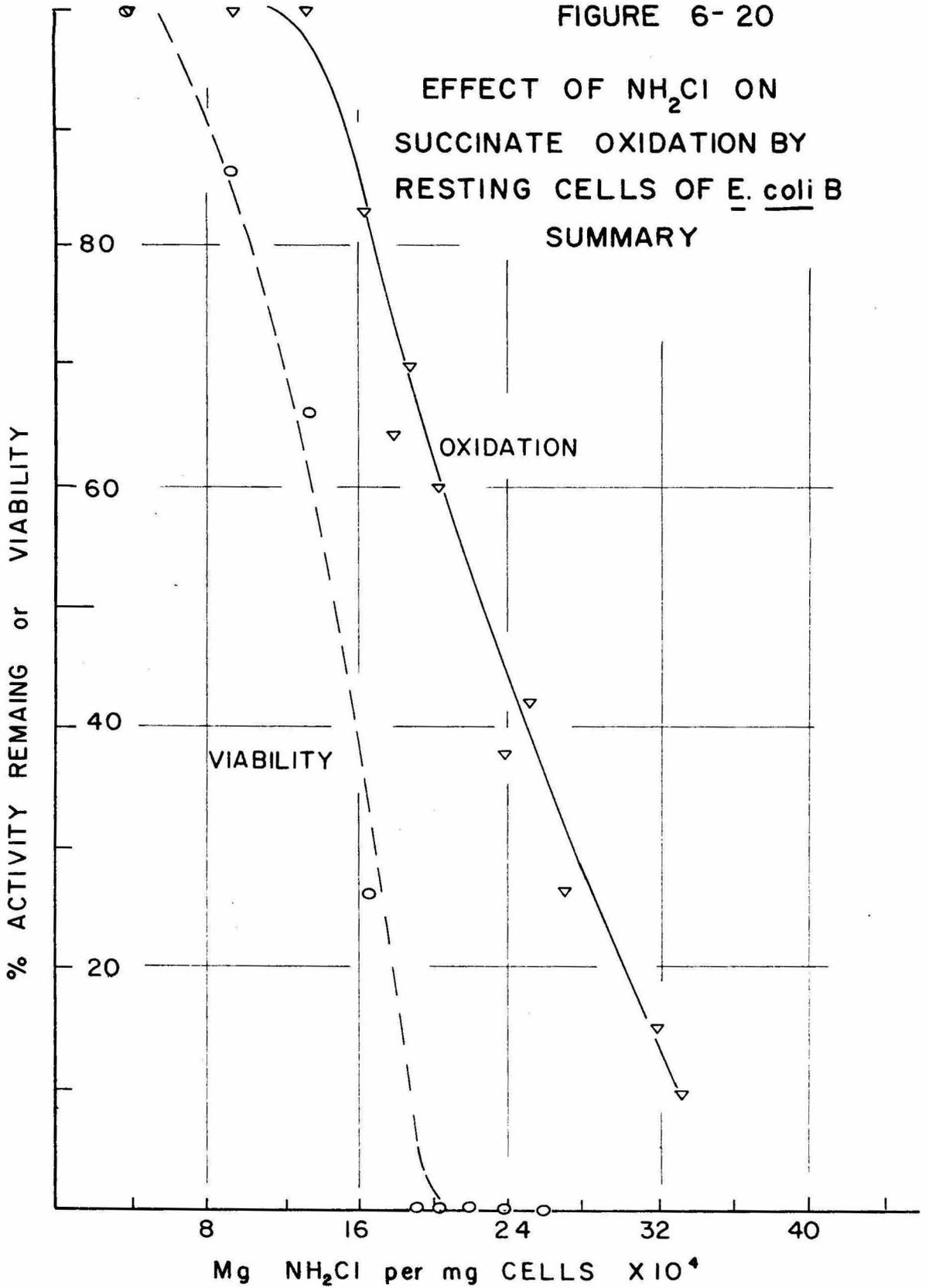


TABLE 6-6
EFFECTS OF NH₂Cl ON OXIDATION OF
SUCCINATE BY CELL FREE SYSTEM

Treatment Mg NH ₂ Cl Mg cells × 10 ⁴	Non-sonicated		Sonicated*	
	Q _{o2}	% Activity Remaining	Q _{o2}	% Activity Remaining
0	13.2	-	12.8	-
9.2	13.2	100	12.7	99
18.4	7.8	64	9.0	70
27.6	3.4	26	1.8	14

* Survivors - 4% of total cell population
Oxidation 97% of total oxidation

was only slightly lower than that obtained in the glucose oxidation experiments.

3. Acetate

One series of tests was made with 0.8% (0.098 M) sodium acetate as the substrate. Results of this test are graphically represented in figures 6-21 and 6-22. The control sample indicated that the oxidation rate was not initially linear. Approximate linearity was obtained after about 50 minutes, and the Q_{o2} value was computed from the portion of the curve between 50 and 100 minutes. All monochloramine doses effected decreases in activity from 13% to almost 100%. Flasks with lower doses of monochloramine showed some increase in activity 30 to 60 minutes after the monochloramine tip. As with glucose and succinate, viability was considerably more sensitive to monochloramine than was acetate oxidation.

FIGURE 6-21

EFFECT OF NH₄Cl ON OXIDATION OF SODIUM ACETATE BY RESTING CELLS OF E. coli B

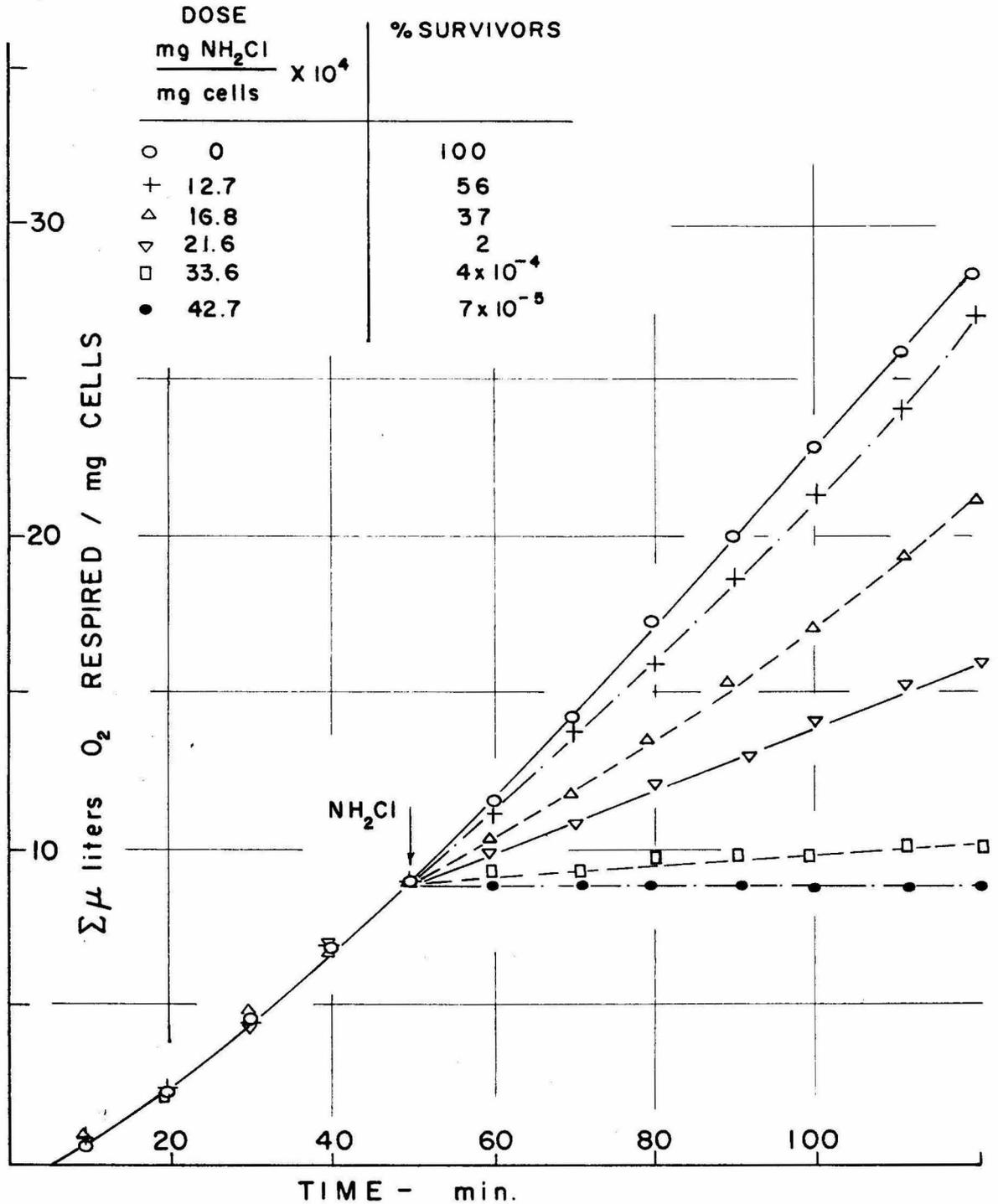
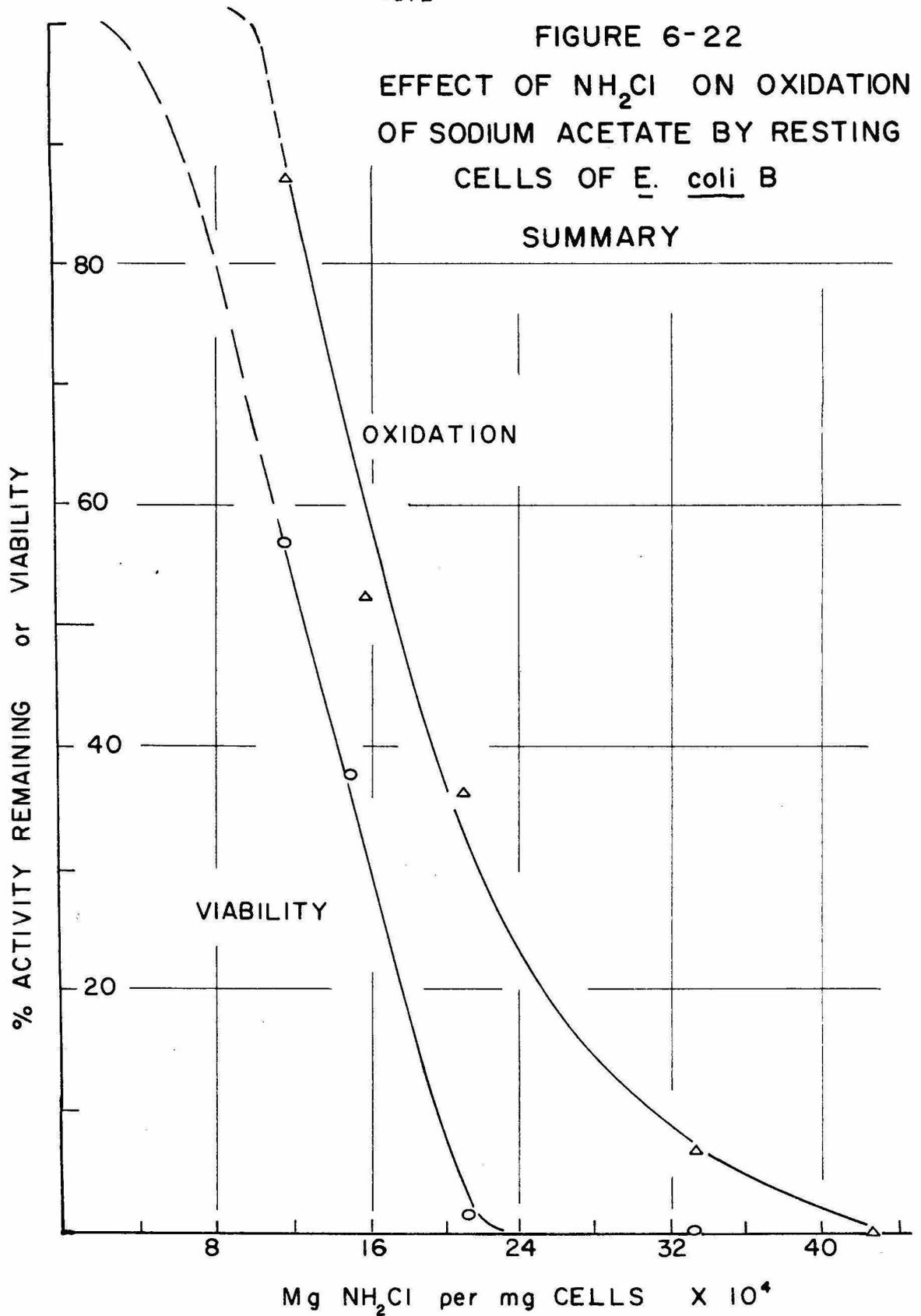


FIGURE 6-22
EFFECT OF NH_2Cl ON OXIDATION
OF SODIUM ACETATE BY RESTING
CELLS OF *E. coli* B

SUMMARY



4. Pyruvate

The effects of monochloramine on pyruvate oxidation (0.5% sodium pyruvate) of resting cells of *E. coli* pretreated for 15 minutes, washed and placed in Warburg vessels was studied. The oxidation rate of pyruvate was linear during the entire 125-minute test period, and the inhibiting effects of monochloramine on this oxidation were clearly demonstrated (figure 6-23). Only the sample with the lowest dose showed any revival activity over the testing period. A comparison of effects on viability and activity are illustrated in figure 6-24. Both viability and activity were lowered to comparable degrees at doses of less than 7×10^{-4} mg monochloramine per mg cells, but viability, as with other substrates tested, was more sensitive than pyruvate oxidation at the higher monochloramine levels.

B. RESULTS - FERMENTATION

1. Glucose

The effects of monochloramine on glucose fermentation were studied using pretreated, neutralized cell suspensions of *E. coli* B. The results of a single test series (figure 6-25) covered a range of effects of monochloramine. The fermentation rates were linear up to about 70 minutes, and the Q_{CO_2} for the control was 30.4. The sudden decrease in activity after about 90 minutes was found to result from the high concentration of acids produced during the fermentation. Brom thymol blue (0.2 ml 1:10,000) placed in several flasks, turned yellow within 80 minutes after the glucose tip. Computations of pH on the basis of acid CO_2 evolution indicate that the pH was

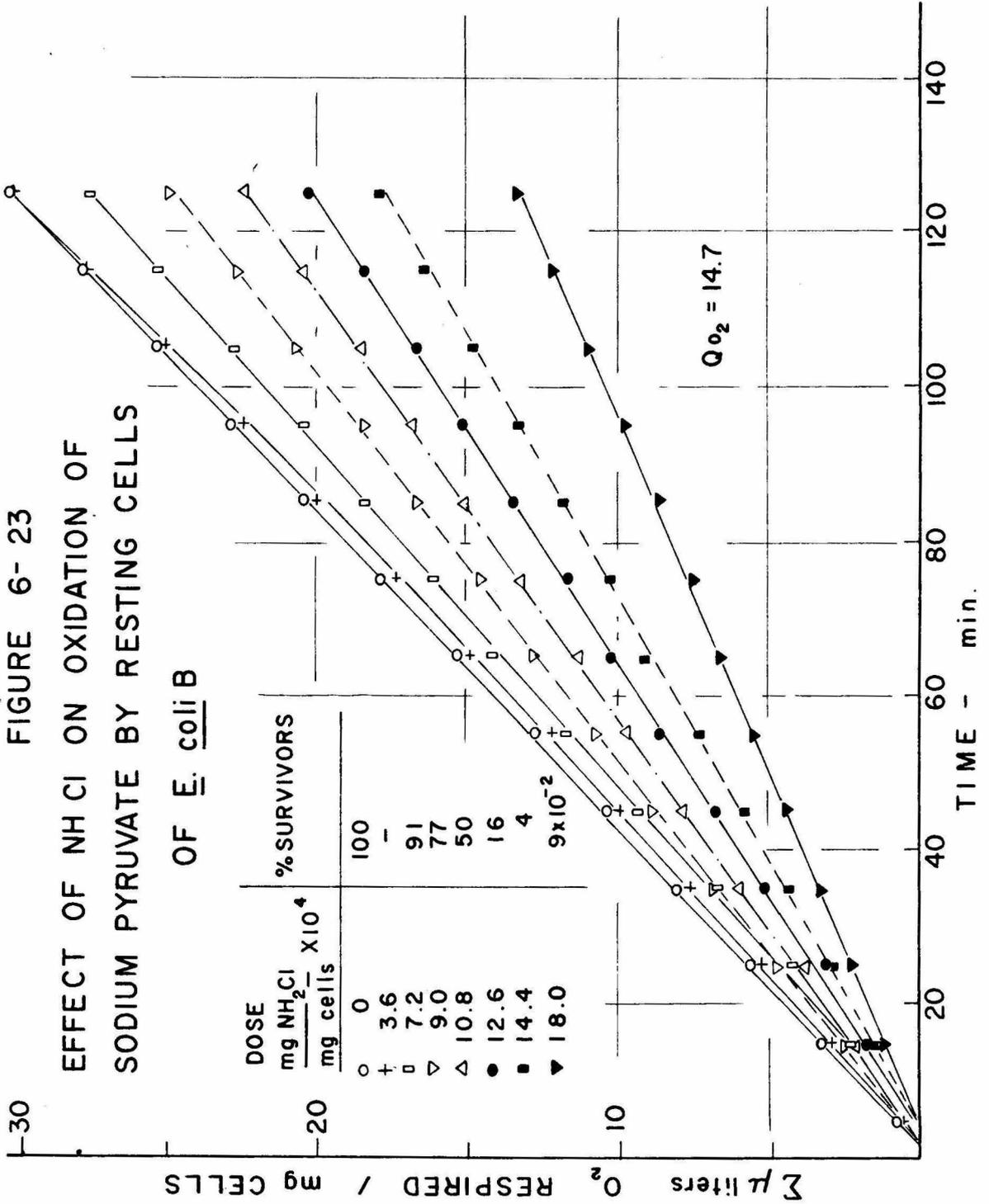
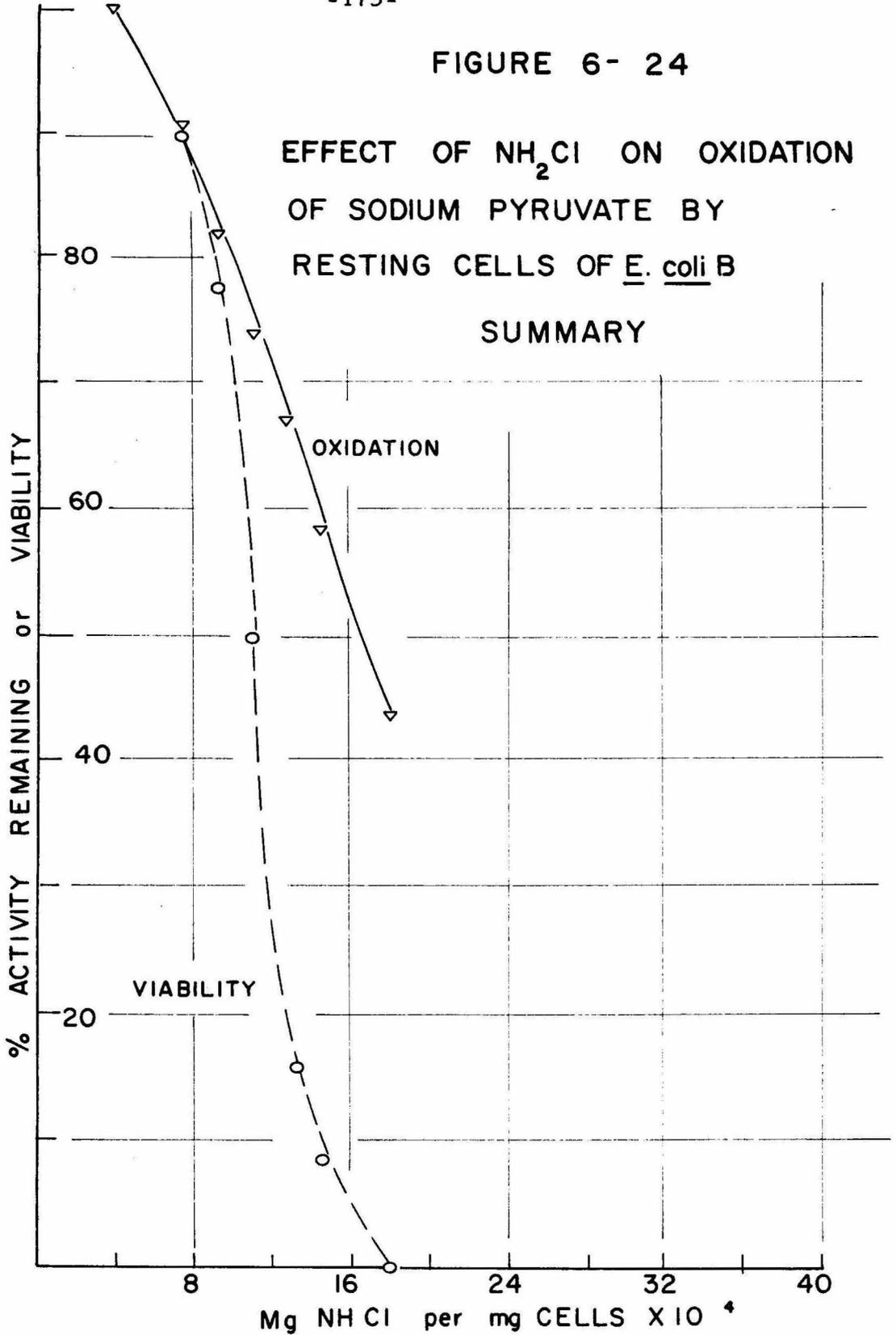
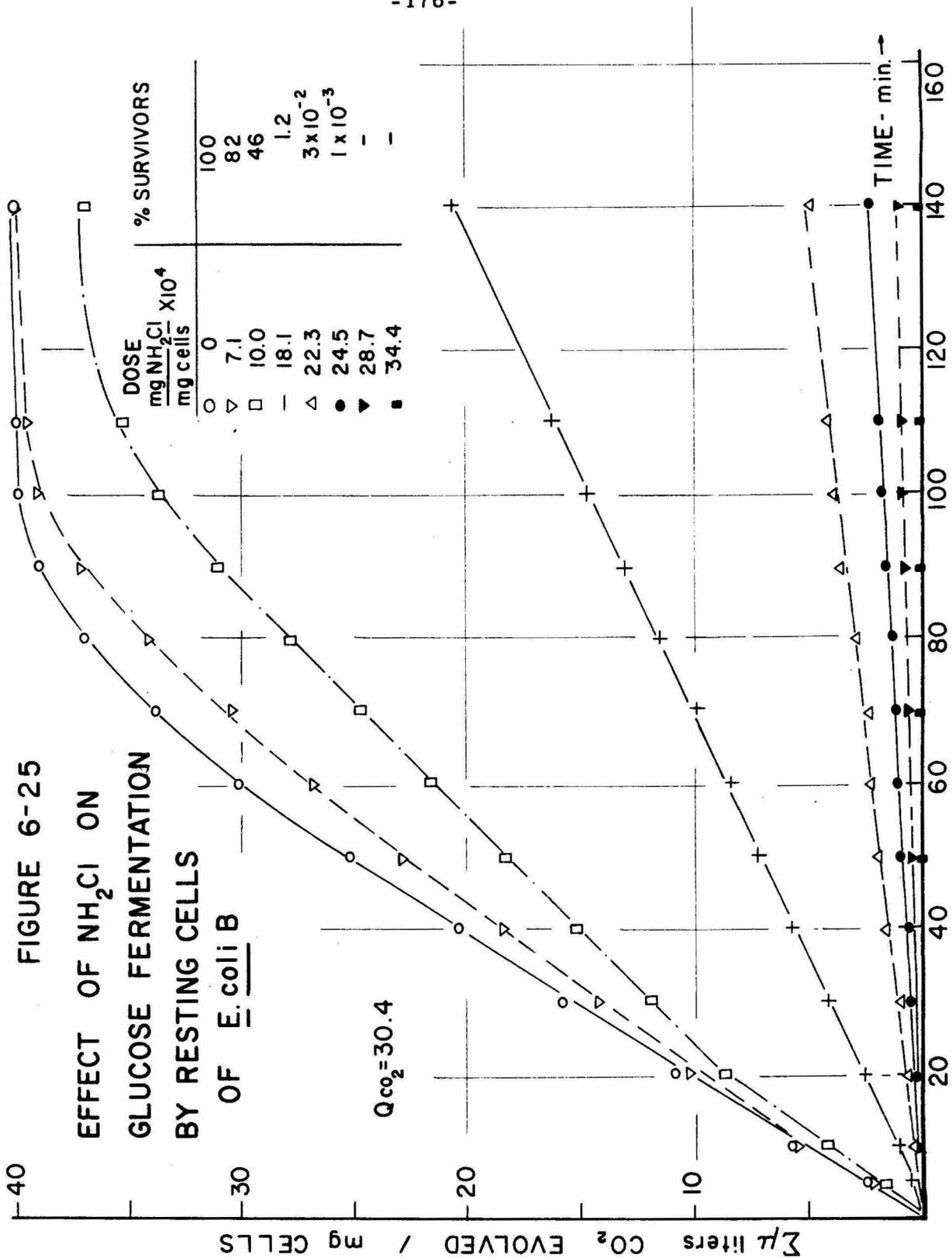


FIGURE 6- 24

EFFECT OF NH_2Cl ON OXIDATION
OF SODIUM PYRUVATE BY
RESTING CELLS OF E. coli B

SUMMARY





approximately 6.1. Note the break in the fermentation rate at both 10.0 and 7.14×10^{-4} mg NH_2Cl per mg cells, occurring between 10 and 20 minutes after the glucose tip.

It was of interest to determine what effect monochloramine might have on the ratio of the metabolic CO_2 to acid CO_2 produced.* The metabolic CO_2 evolved in control samples was 9.2% of the total CO_2 evolved. Treatment of cell suspensions with monochloramine appeared to decrease the proportion of metabolic CO_2 to acid CO_2 (table 6-8) to a point where no metabolic CO_2 was evolved.

Since the enzyme aldolase has been implicated as the site of the lethal biochemical lesion of chlorine (17), it was desirable to determine the effects of monochloramine on the fermentation of fructose-1,6-diphosphate. It is evident from this study (figure 6-26) that this substrate was fermented at the same rate (Qco_2 26.9) as glucose and was affected by monochloramine to the same degree. Note, again, the break in the fermentation rate curves for the suspensions done with the lower levels of monochloramine. The results of these tests are summarized in graphical form in figure 6-27. Cell viability was similar to that for the respiratory system.

The effects of monochloramine on the glucose fermentation by the nutritionally fastidious homofermentative organism, Streptococcus faecalis R, were also determined using pretreated, neutralized suspensions (figures 6-28 and 6-29). This organism utilized

* Metabolic CO_2 is that CO_2 evolved directly from the fermentative mechanisms, while acid CO_2 is that which evolves from the bicarbonate buffer as acidic end products are produced.

TABLE 6-8

EFFECTS OF NH_2Cl ON THE RATIO OF
METABOLIC CO_2 TO ACID CO_2 EVOLVED IN
GLUCOSE FERMENTATION BY E. COLI B

Treatment mg NH_2Cl /mg cells $\times 10^4$	% Metabolic CO_2	% Acid CO_2
Control	9.2%	90.8%
10.1	3.6%	94.4%
17.3	2.5%	97.5%
22.1	Nil	100 %

Obtained by difference from average of duplicate tests using 0.2 ml $3\text{NH}_2\text{SO}_4$ tip initially and 65 minutes after glucose tip.

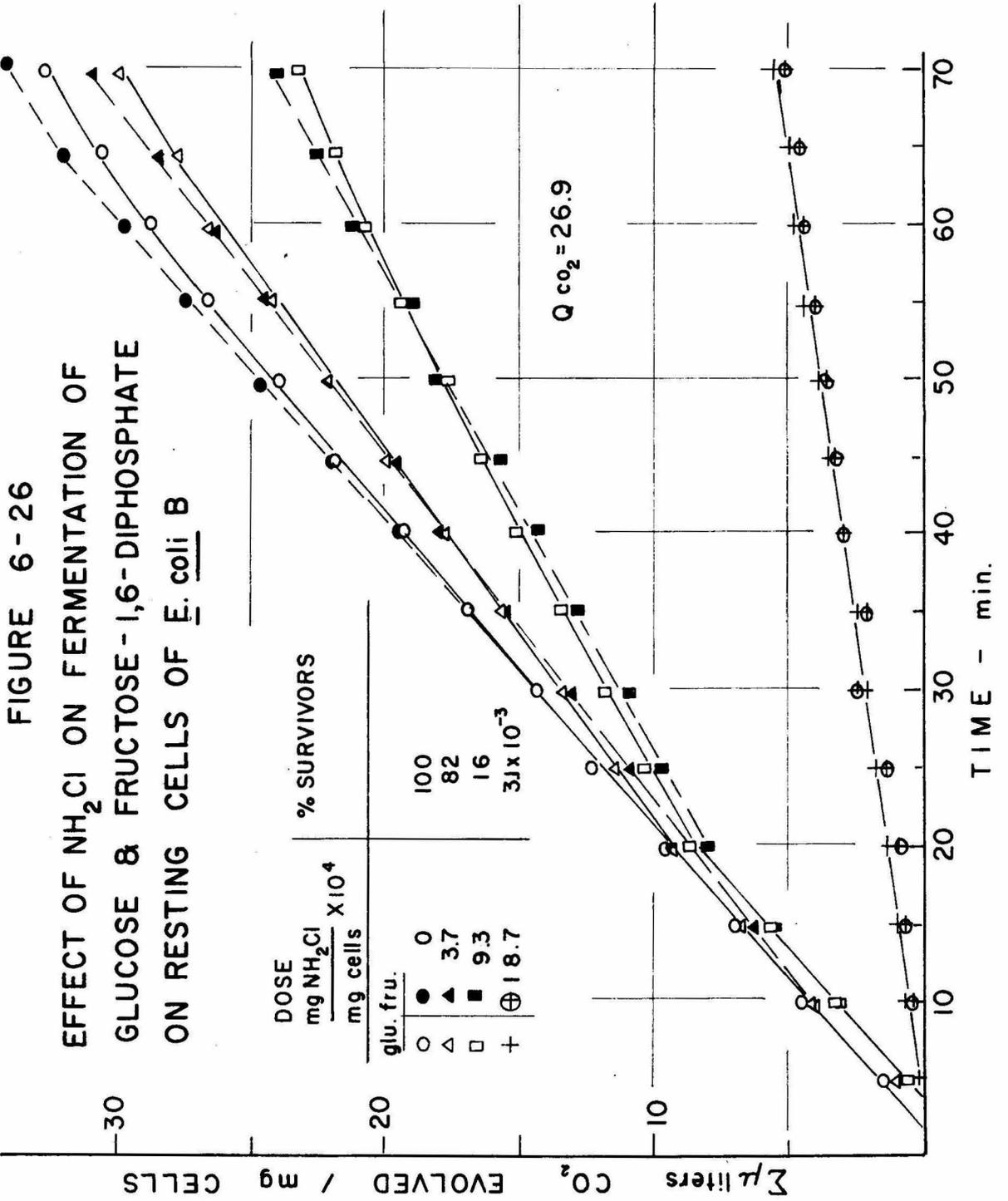


FIGURE 6-27
EFFECT OF NH_2Cl ON FERMENTATION
OF GLUCOSE BY RESTING CELLS OF
E. coli B

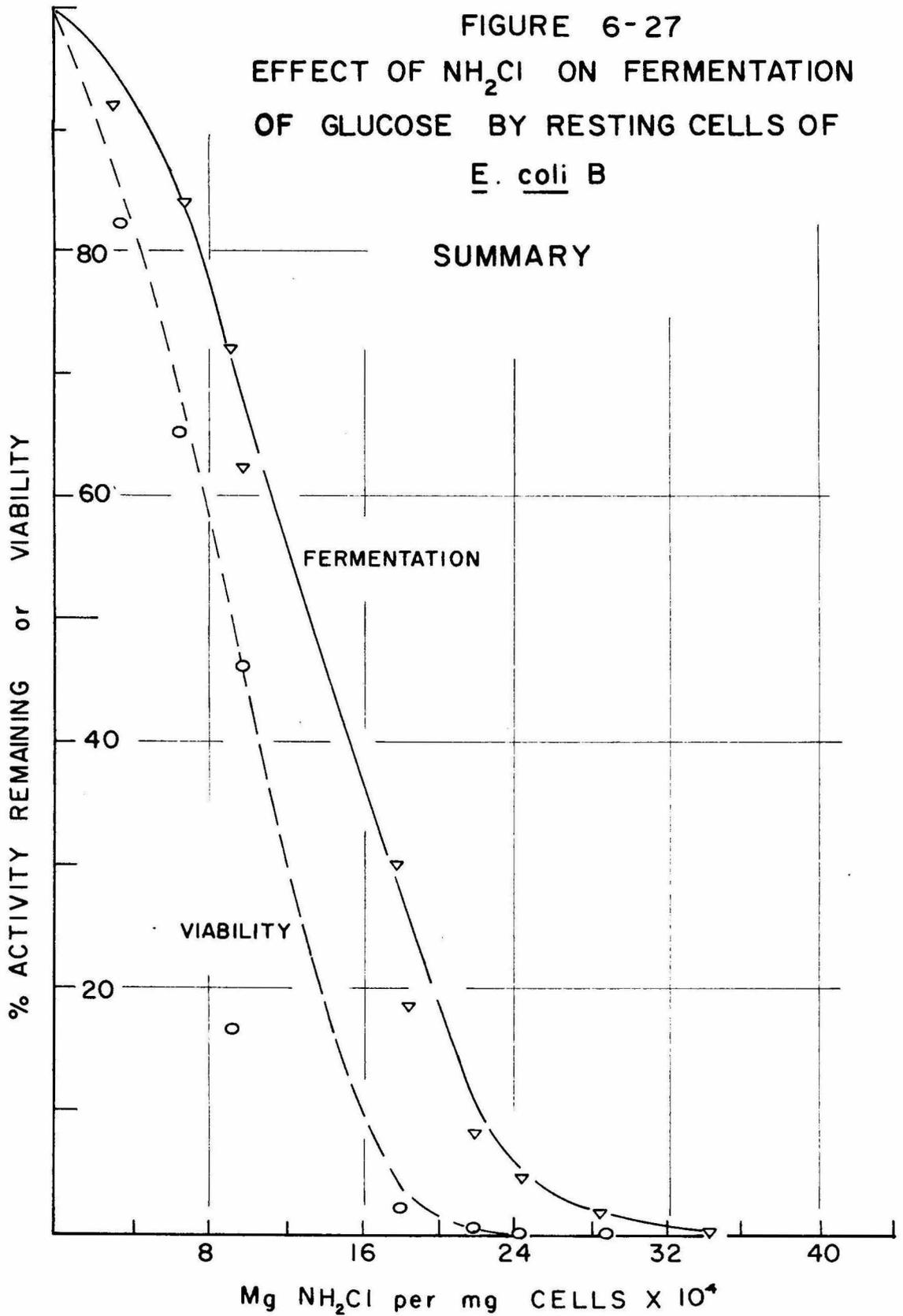


FIGURE 6-28

EFFECT OF NH_2Cl ON
FERMENTATION OF
GLUCOSE BY RESTING
CELLS OF *S. faecalis* R

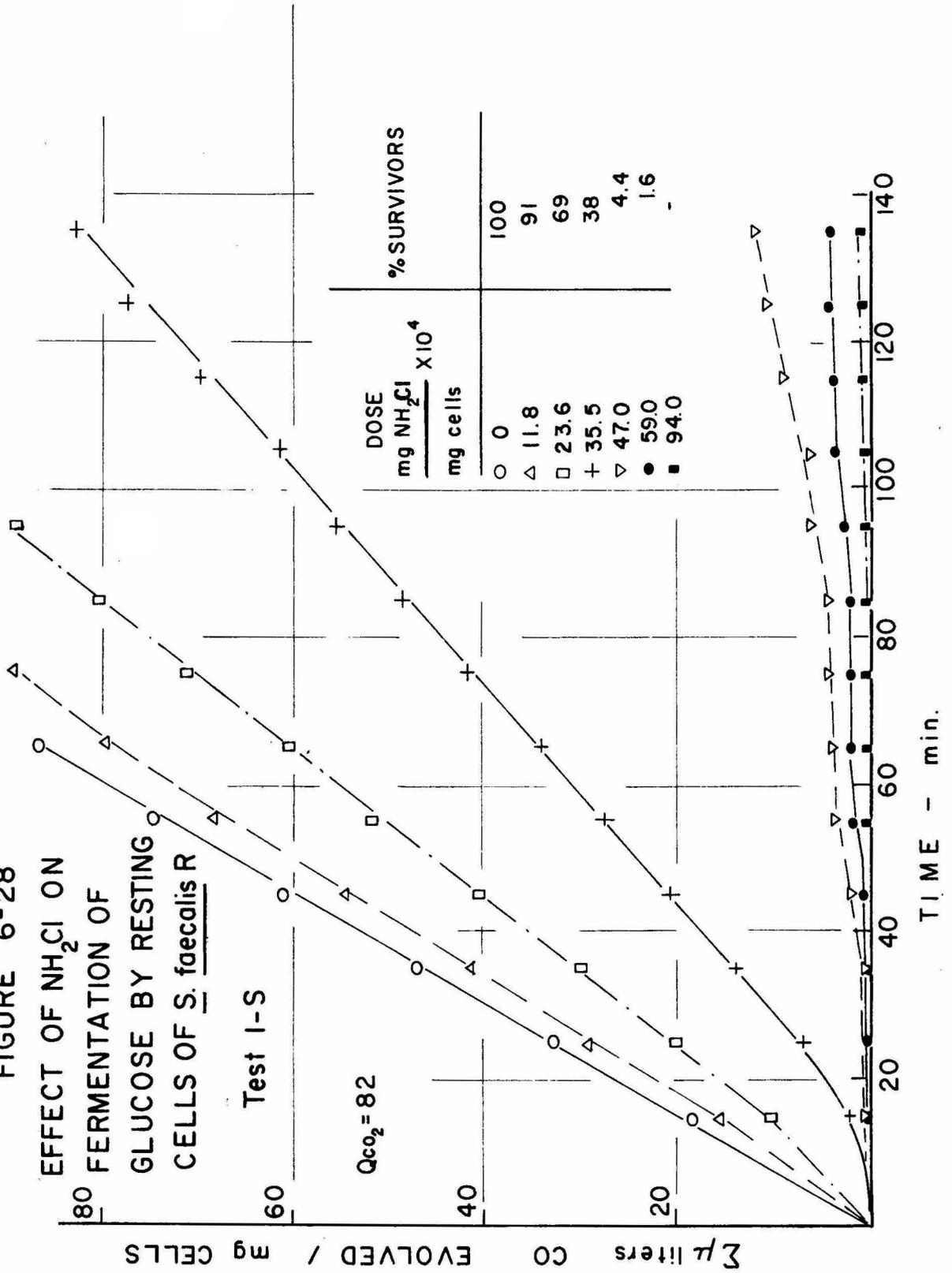
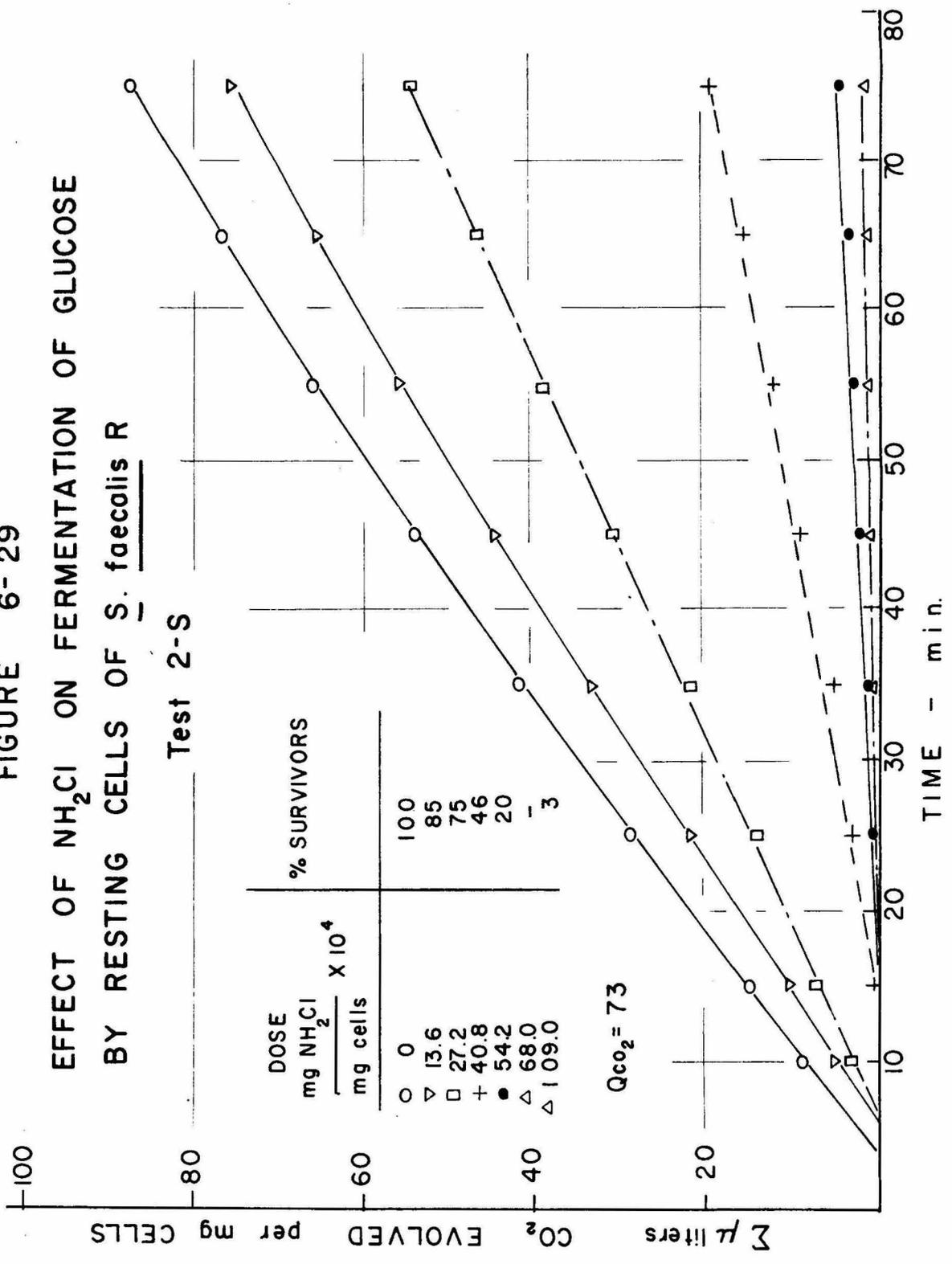


FIGURE 6-29
EFFECT OF NH₂Cl ON FERMENTATION OF GLUCOSE
BY RESTING CELLS OF S. faecalis R



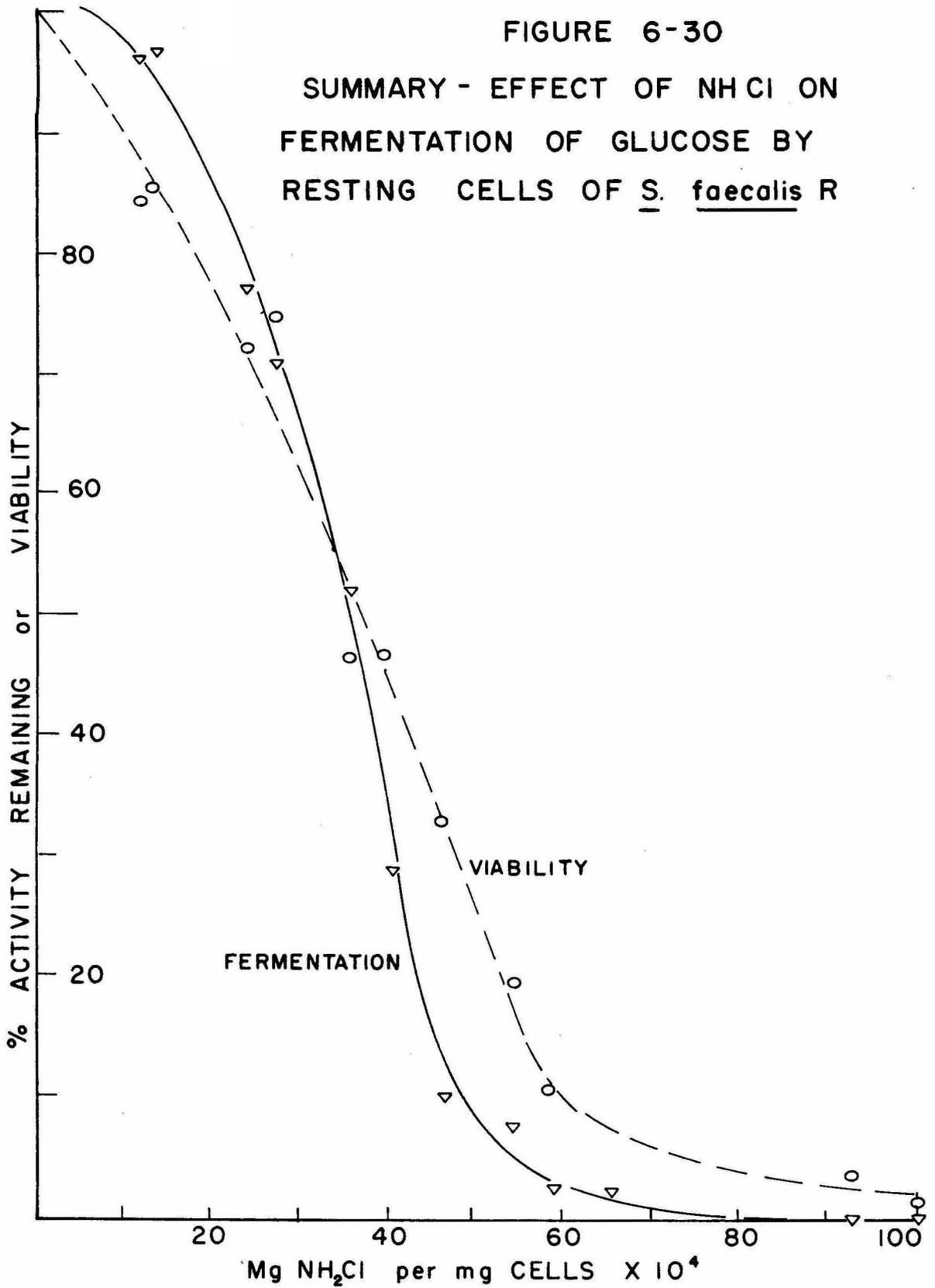
glucose at a much higher rate ($Q_{CO_2} = 82$ and 73), and the rates were linear from the start. The monochloramine dosages required to give comparable reductions in fermentative activity of S. faecalis were considerably higher than for E. coli. Thus, for a 50% reduction in fermentation, 38×10^{-4} mg NH_2Cl per mg cells was required, as opposed to 13×10^{-4} mg NH_2Cl per mg cells for E. coli. The summary of results from these two tests with S. faecalis appear in figure 6-30. It is significant that the sensitivity of glucose fermentation to monochloramine exceeded that of viability at all ratios of monochloramine to dry cells $> 30 \times 10^{-4}$. Under the conditions tested, S. faecalis R was considerably more resistant to monochloramination than E. coli B.

2. Pyruvate

A series of three tests were conducted to investigate the action of monochloramine on the rate of dissimilation of pyruvate by pretreated suspensions of E. coli B (figures 6-31, 6-32 and 6-33). The rate of metabolism of pyruvate was not linear, and Q_{CO_2} values were estimated on the basis of average slopes obtained between 100 and 120 minutes. The average Q_{CO_2} of the control cells for the three tests was 21.5. From the ratios of accumulated $\mu l CO_2$ for the treated suspension at a given time period to that for the control at the same time interval, the per cent reduction in activity effected by monochloramine could be calculated. These values are slightly lower at the lower monochloramine doses and higher at the higher doses than those computed by the slope-averaging method. The results of the three tests are summarized in figure 6-34.

FIGURE 6-30

SUMMARY - EFFECT OF NH₄Cl ON
FERMENTATION OF GLUCOSE BY
RESTING CELLS OF S. faecalis R



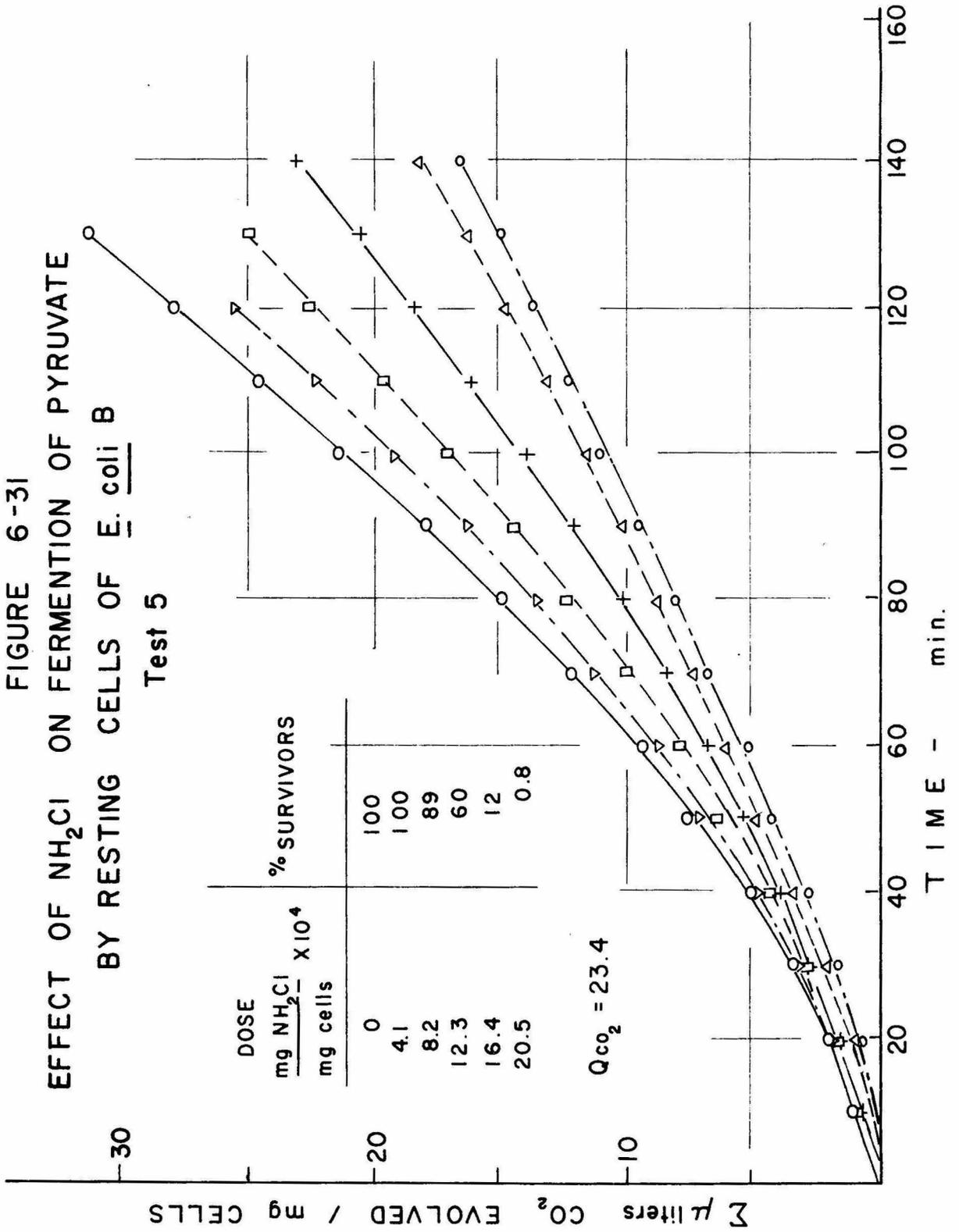


FIGURE 6-32
EFFECT OF NH₂Cl ON FERMENTATION OF PYRUVATE
BY RESTING CELLS OF E. coli B
Test 6

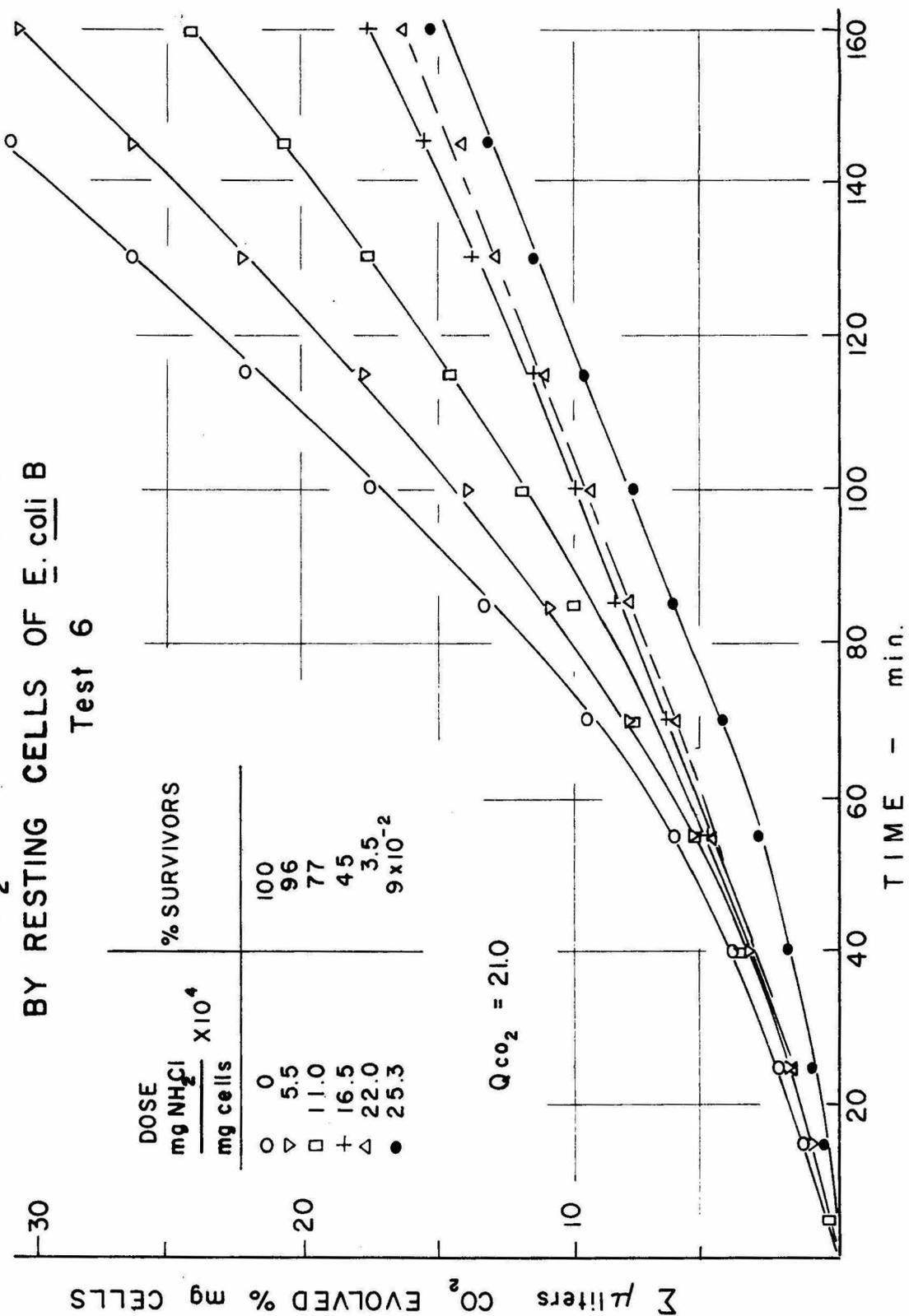


FIGURE 6-33

EFFECT OF NH_2Cl ON FERMENTATION OF
 PYRUVATE BY RESTING CELLS OF E. coli B
 TEST 7

CO₂ EVOLVED / mg CELLS

DOSE mg NH_2Cl mg cells	% SURVIVORS
0	100
4.5	93
13.5	53
18.0	4.4
22.5	0.3
45.0	—

Q ccg 20.6

TIME - min.

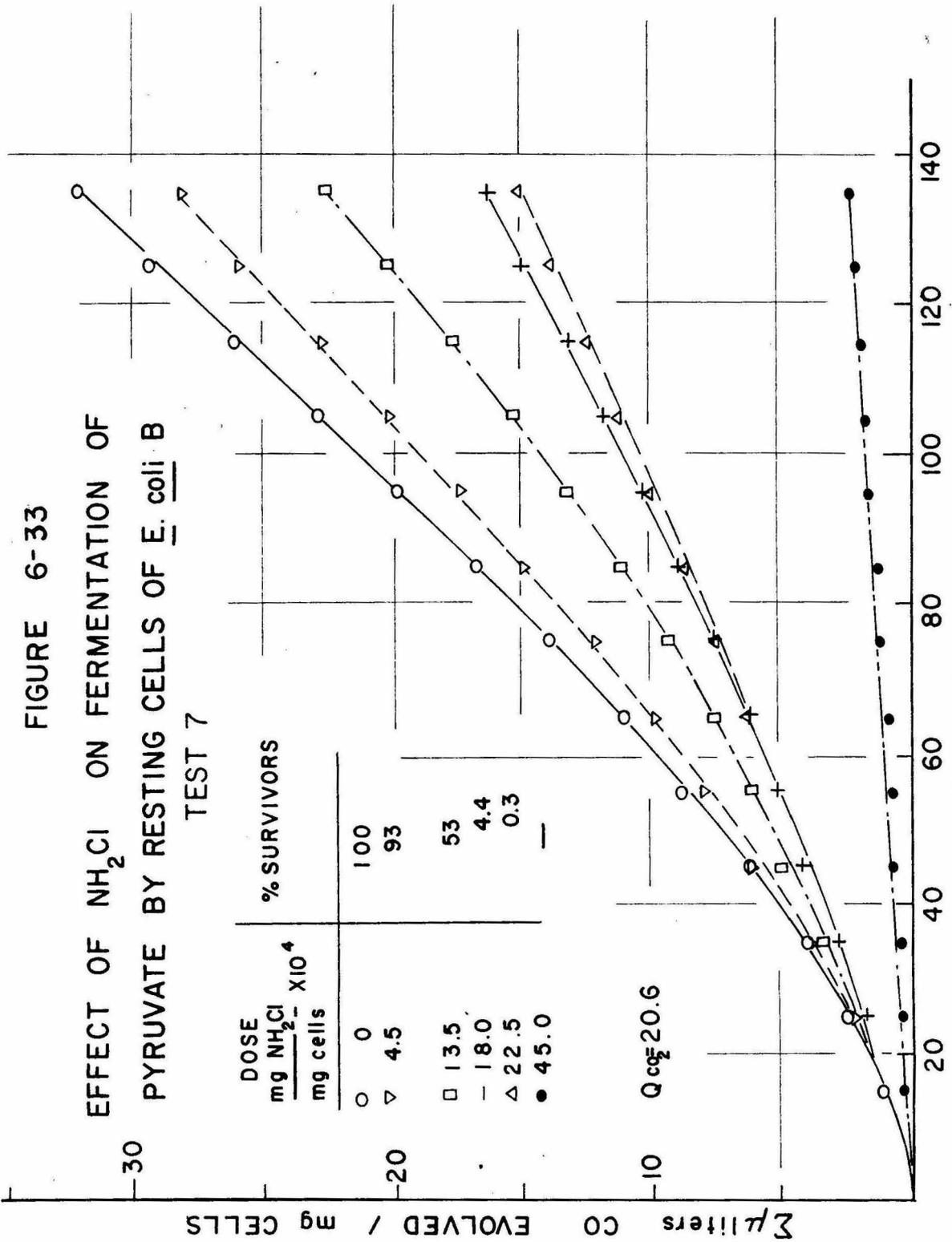
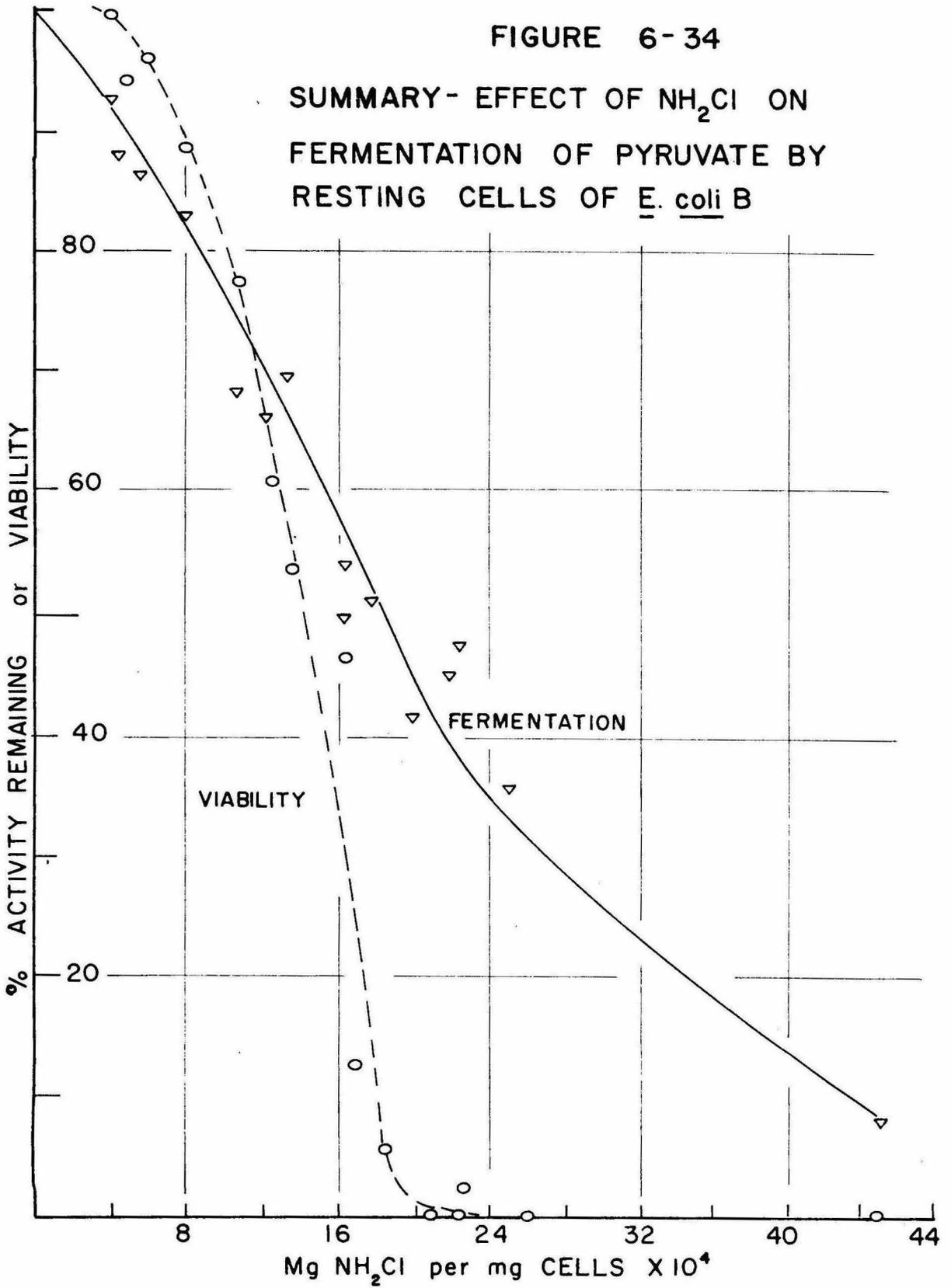


FIGURE 6-34

SUMMARY- EFFECT OF NH_2Cl ON
FERMENTATION OF PYRUVATE BY
RESTING CELLS OF E. coli B



There is considerably more scatter of data in these tests than in preceding tests. Note that reductions in pyruvate dissimilation were greater in viable cells at the higher monochloramine doses (10×10^{-4} mg NH_2Cl /mg dry cells). The effects of monochloramine on the ratio of metabolic CO_2 to acid CO_2 in pyruvate fermentation are tabulated in table 6-9. The per cent metabolic CO_2 evolved by control samples utilizing pyruvate was more than twice as high as that from glucose (21% vs. 9.2%), but monochloramine diminished output of metabolic CO_2 from either substrate.

C. RESULTS - DEHYDROGENASE

1. Succinic Dehydrogenase

The activity of succinic dehydrogenase in resting cells of E. coli B was determined by methylene blue reduction in evacuated Thunberg tubes. Yudkin (78, 79) and Quastel and Wooldridge (80) found that the rate of reduction of methylene blue by succinic dehydrogenase was not proportional to the enzyme concentration. Since succinic dehydrogenase is a ferro-flavoprotein, one might expect it to have some residual activity toward molecular oxygen and oxidation-reduction dyes, such as methylene blue. It has been shown that beef heart succinic dehydrogenase reacts with methylene blue under anaerobic conditions, probably reacting directly with the leucoflavoprotein formed in the reaction (81).

Cells were grown in the 1% sodium succinate-mineral salts medium, washed and suspended in buffer at densities which would reduce 90% of the methylene blue in less than 10 minutes. The

TABLE 6-9

EFFECTS OF NH_2Cl ON RATIO OF
METABOLIC CO_2 TO ACID CO_2 EVOLVED
IN PYRUVATE FERMENTATION BY E. COLI B

Treatment Mg NH_2Cl /mg cells $\times 10^4$	% Metabolic CO_2	% Acid CO_2
Control	21	79
4.2	18	82
8.6	13	87
12.1	15	85
16.3	9.5	90.5
21.2	7.8	92.2

Obtained from average of duplicate tests using 0.2 ml $3\text{NH}_2\text{SO}_4$
tip initially and 150 minutes after sodium pyruvate tip.

Thunberg tubes were purged with nitrogen for 3 minutes prior to evacuation in order to minimize the presence of residual molecular oxygen. The results of a typical test series are shown in figure 6-35. The 90% reduction time (T_{90}) was determined by measuring light transmittance of a 1:10 dilution of the stock methylene blue. The blank contained all the components of the system being studied, including the appropriate density of cells, except methylene blue. The results of the disproportionate decrease in succinic dehydrogenase with dilution are shown in figure 6-36. The experimental curve was obtained from assays of activities of several dilutions of the original cell suspension (e. g., a 2:3 dilution represented a 33% reduction in activity and the T_{90} was plotted accordingly). The theoretical curve was computed by multiplying the T_{90} for the original cell density by the reciprocal of the fraction of the activity remaining. Both the theoretical and experimental activity reductions have been estimated in these studies from the curves in figure 6-36. The results of two test series on succinate appear in table 6-10 and are graphically illustrated in figure 6-37. Note that cells were treated with monochloramine and counted prior to dehydrogenase assay.

One test series was designed to study the effect of monochloramine on toluene-treated cell suspensions. Toluened cells were treated with several doses of monochloramine for 10 minutes and analyzed along with non-toluened monochloraminated cells. The untreated toluened cells yielded higher succinic dehydrogenase activities (table 6-11) than did untreated non-toluened cell suspensions. The treated toluened cells were more sensitive to

FIGURE 6-35
EFFECT OF NH₂Cl ON SUCCINIC DEHYDROGENASE
ACTIVITY OF RESTING CELLS OF E. coli B

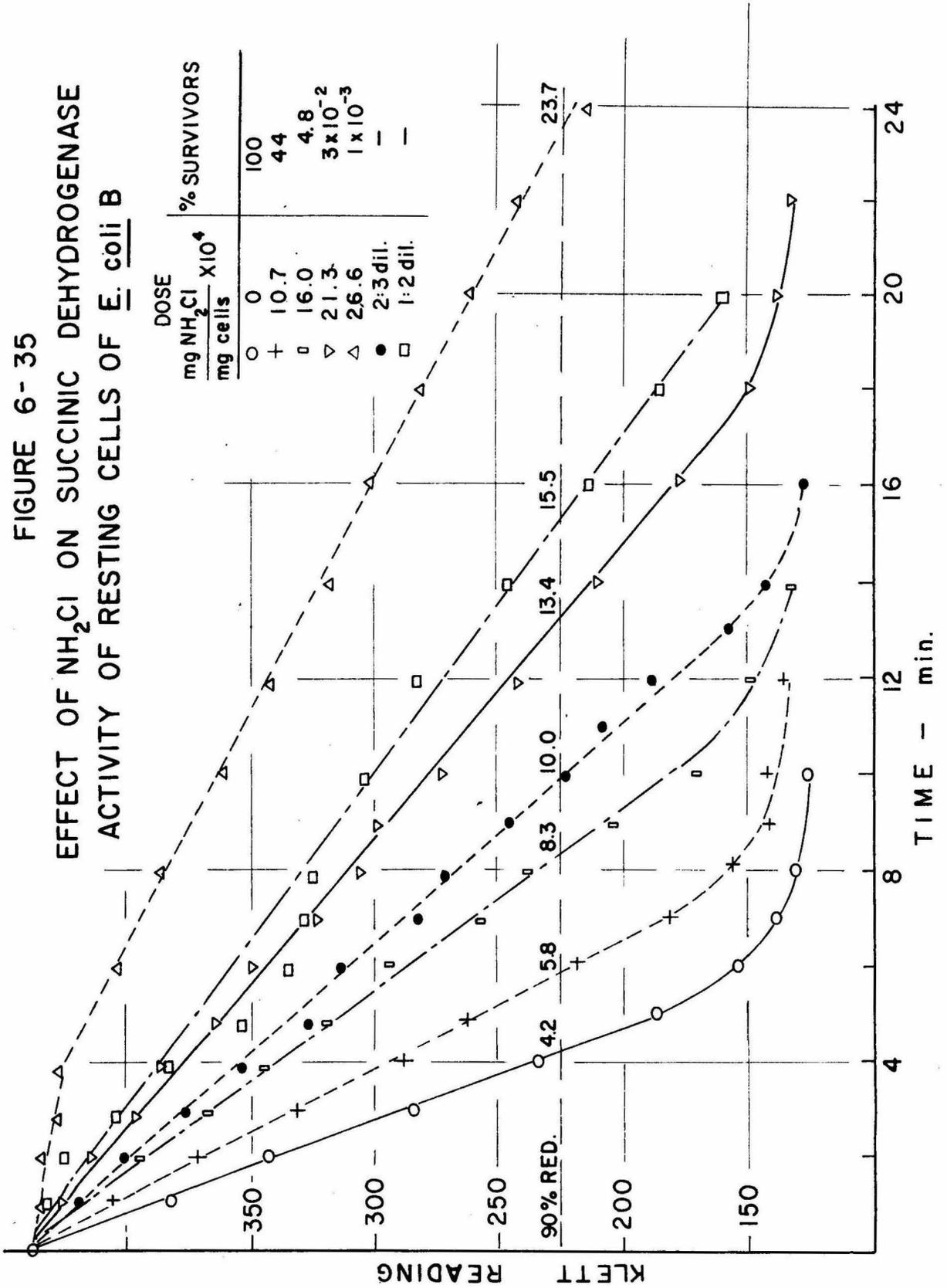


FIGURE 6-36

DISPROPORTIONATE DECREASE
IN SUCCINIC DEHYDROGENASE
ACTIVITY OF RESTING CELLS
OF E. coli B WITH DILUTION

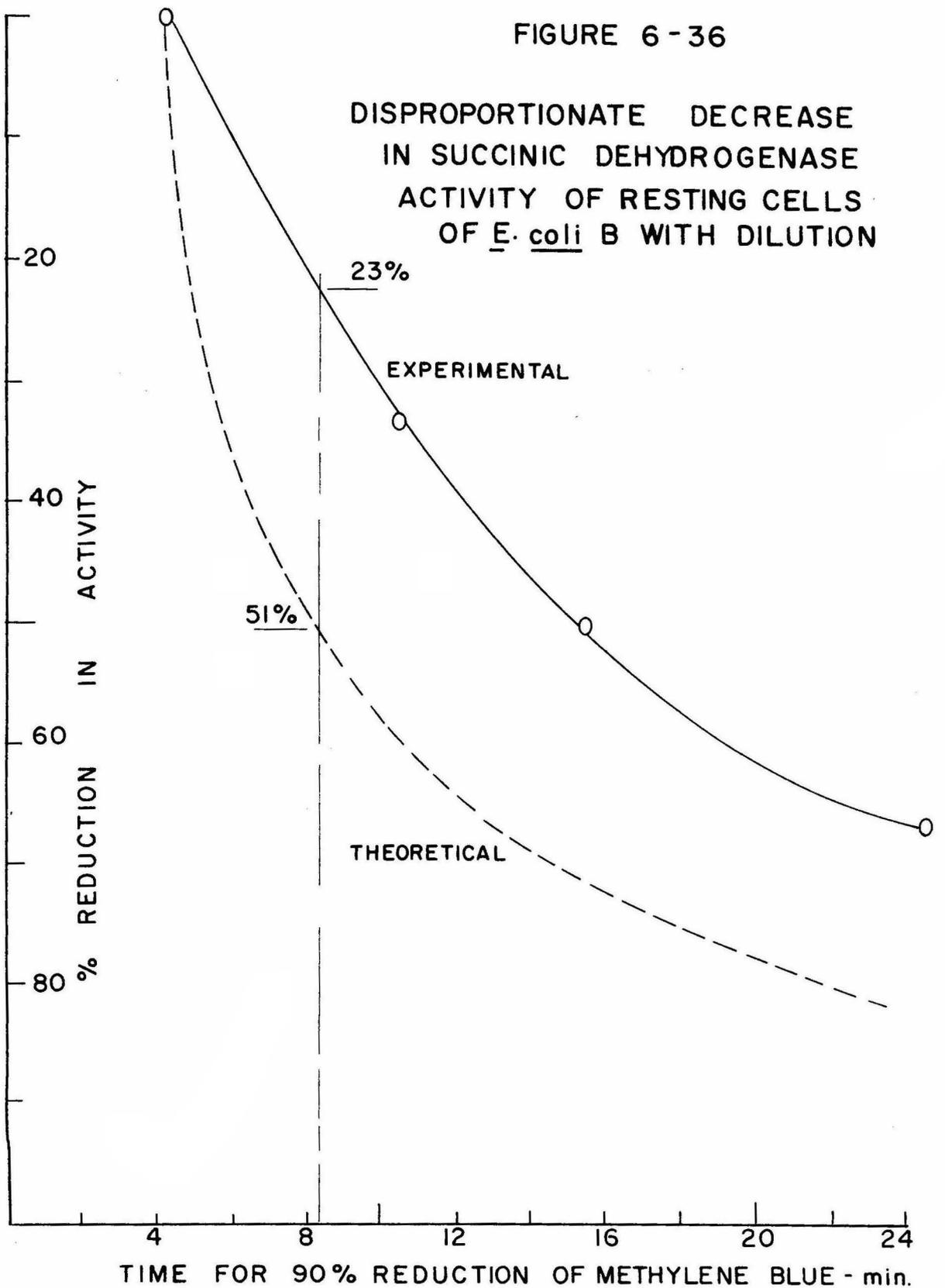


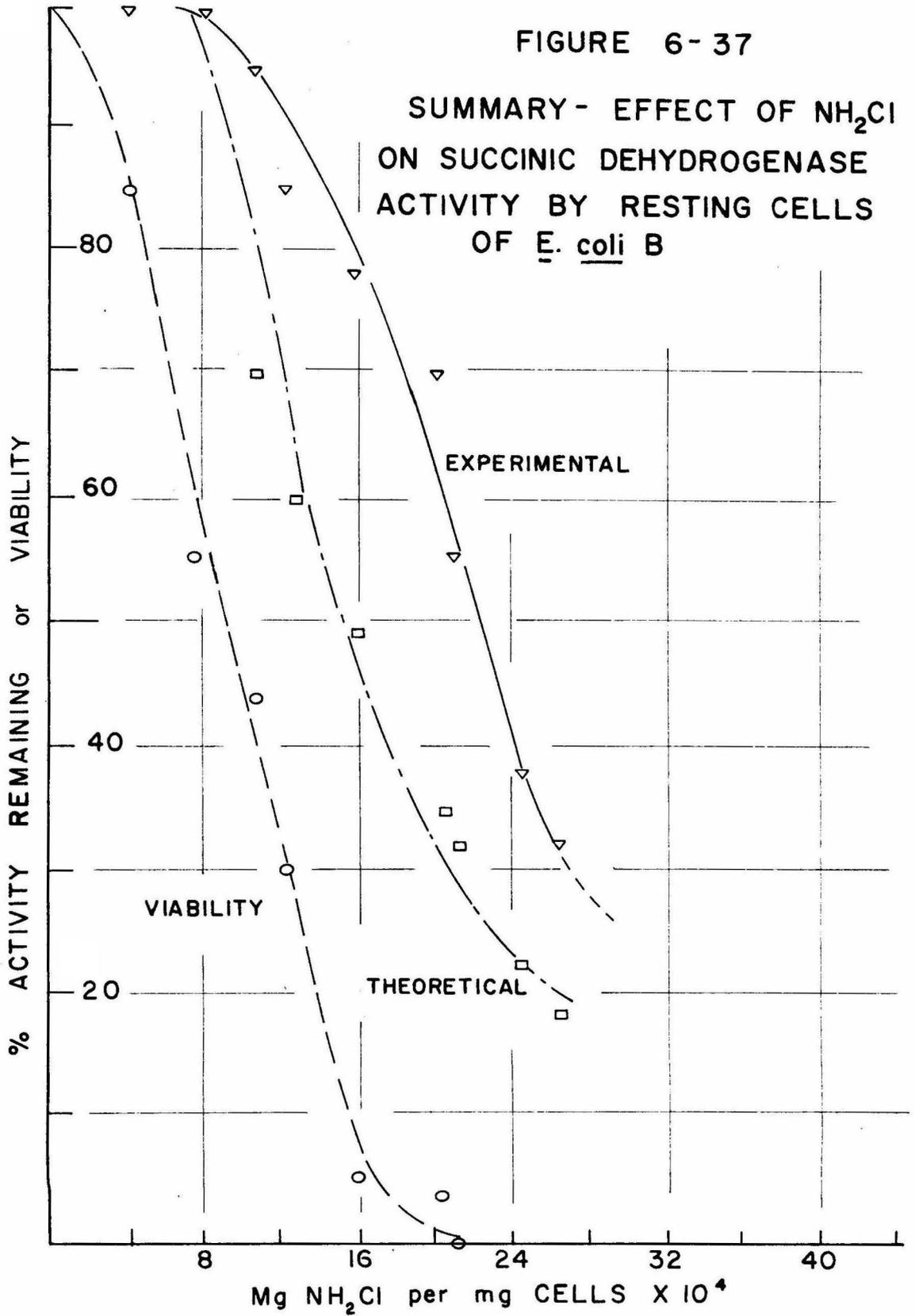
TABLE 6-10

EFFECT OF NH_2Cl ON SUCCINIC DEHYDROGENASE
ACTIVITY OF RESTING CELLS OF E. COLI B

Expt.	Treatment Mg NH_2Cl / Mg cells $\times 10^4$	T_{90} (min.)	Proportionate	Disproportionate (Experimental)	% Survivors
1	0	4.2	-	-	-
(3.8 mg cells)	10.7	5.8	70	95	44
	16.0	8.3	49	77	4.8
	21.3	13.4	32	55	.03
	26.6	23.7	18	32	<.001
2	0	5.4	-	-	-
(3.45 mg cells)	4.1	5.2	97	100	84
	8.2	5.4	100	100	54
	12.3	9.0	60	83	30
	20.5	16.8	32	70	4
	24.6	26.5	20	38	.003

FIGURE 6-37

SUMMARY - EFFECT OF NH_2Cl
ON SUCCINIC DEHYDROGENASE
ACTIVITY BY RESTING CELLS
OF E. coli B



monochloramine at the lower monochloramine doses than were non-toluenized samples.

TABLE 6-11

EFFECTS OF NH_2Cl ON SUCCINIC DEHYDROGENASE
ACTIVITY OF TOLUENIZED VS NON-TOLUENIZED E. COLI B

Treatment Mg NH_2Cl / Mg Cells $\times 10^4$	Toluenized % Activity Remaining	Non-Toluenized % Activity Remaining
0	100% $\cdot (T_{90} = 4.25 \text{ min.})$	100% $(T_{90} = 5.50 \text{ min.})$
9.8	87	100
19.6	50	70
24.5	45	45

Using 3.2 mg cells; computed by means of proportionate methods using respective controls.

2. Fructose-1,6-Diphosphate System

Since both aldolase and triose phosphate dehydrogenase had been implicated as the site of lethal lesion in bacterial chlorination (17), a study of the effects of monochloramine on fructose-1,6-diphosphate dehydrogenase would be of considerable interest. One simple method of studying both systems is to follow the reduction of fructose-1,6-diphosphate by resting cells. If either or both of these enzymes were inhibited, the reduction time of methylene blue would be increased proportionately, and, provided this reduction was extremely sensitive to monochloramine, more accurate methods could be employed subsequently to study either system separately. Iodoacetate is known to inhibit triose phosphate

dehydrogenase; therefore, this compound was employed in initial experiments. It was found that a 5×10^{-5} M iodoacetate inhibited the activity of the fructose-1,6-diphosphate system by 72%; 5×10^{-4} M reduced activity by 92%. (Plate counts were lowered 8% and 65% respectively.) These results encouraged the use of this method to assay the combined effects of monochloramine on aldolase and triose phosphate dehydrogenase.

Typical results obtained with a M/100 fructose-1, 6-diphosphate substrate are shown in figure 6-38. Escherichia coli B was grown in the basal medium supplemented with 1% glucose. The reduction of methylene blue was proportionate to cell concentrations; thus, reductions in activity were computed directly from T_{90} values, i. e., % activity remaining = T_{90} control + T_{90} NH_2Cl . A summary of the results of this and two other comparable trials appear in table 6-12 and figure 6-39. Since in no case did reductions in activity exceed reductions in viability, further studies of these enzymes were not pursued. Toluenization or sonication of cells prior to monochloramine treatment completely destroyed all activity of one or both of these enzymes, thereby making it impossible to study the role of permeability on the effects of monochloramine with this system.

3. Lactic Dehydrogenase

The results of a typical experiment showing the effects of monochloramine on the lactic dehydrogenase in resting cells of E. coli B recovered from the glucose-mineral salts medium appear in figure 6-40. This system was found to reduce methylene

FIGURE 6-38
 REDUCTION OF FRUCTOSE-1,6-DIPHOSPHATE BY NH₂CI
 TREATED RESTING CELLS OF E. coli B

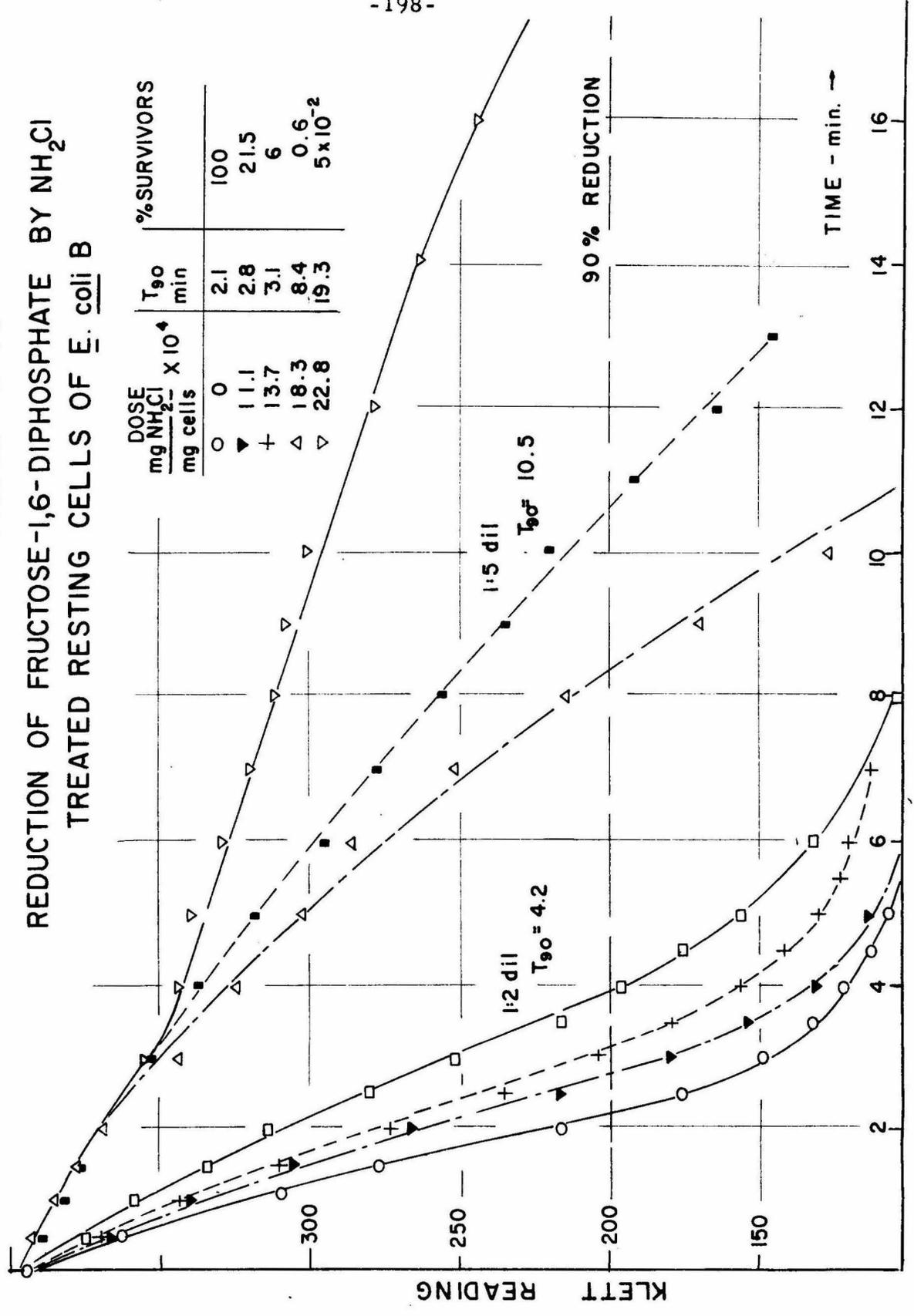


TABLE 6-12

EFFECTS OF NH_2Cl ON DECOLORIZATION OF
 METHYLENE BLUE WITH M/100 FRUCTOSE-1,
 6-DIPHOSPHATE SUBSTRATE BY RESTING
 CELLS OF E. COLI B

Expt.	Treatment (Mg NH_2Cl / Mg Cells $\times 10^4$)	T_{90} (min.)	% Activity* Remaining	% Survivors
1	0	2.1	-	-
(3.6 mg cells)	11.1	2.8	75	21.5
	13.7	3.1	68	6
	18.3	8.4	25	0.6
	22.8	19.3	11	0.05
2	0	3.2	-	-
(2.7 mg cells)	8.2	3.5	91	58
	10.1	3.8	84	30
3	0	2.3	-	-
(3.6 mg cells)	1.7	2.2	100	100
	3.4	2.3	100	84
	6.8	2.3	100	73
	10.2	3.2	72	38
	15.3	4.3	53	3
	22.0	27.2	8.5	.04

* Computed from ratio of T_{90} control to T_{90} treated.
 T = 37° C; pH 7.2.

FIGURE 6-39
SUMMARY - EFFECT OF NH_2Cl ON
ANAEROBIC METABOLISM OF
FRUCTOSE-1,6-DIPHOSPHATE BY
RESTING CELLS OF E. coli B

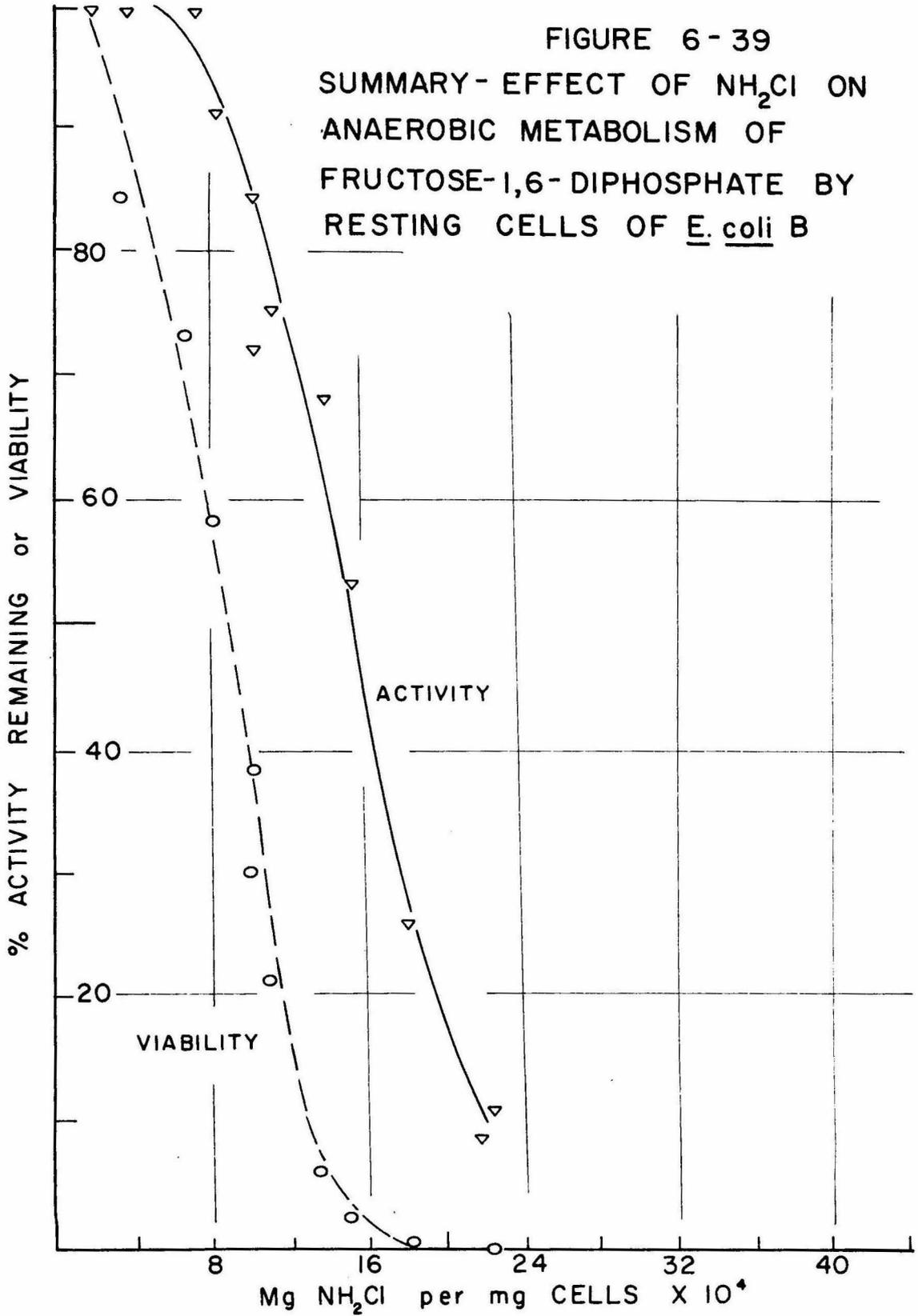
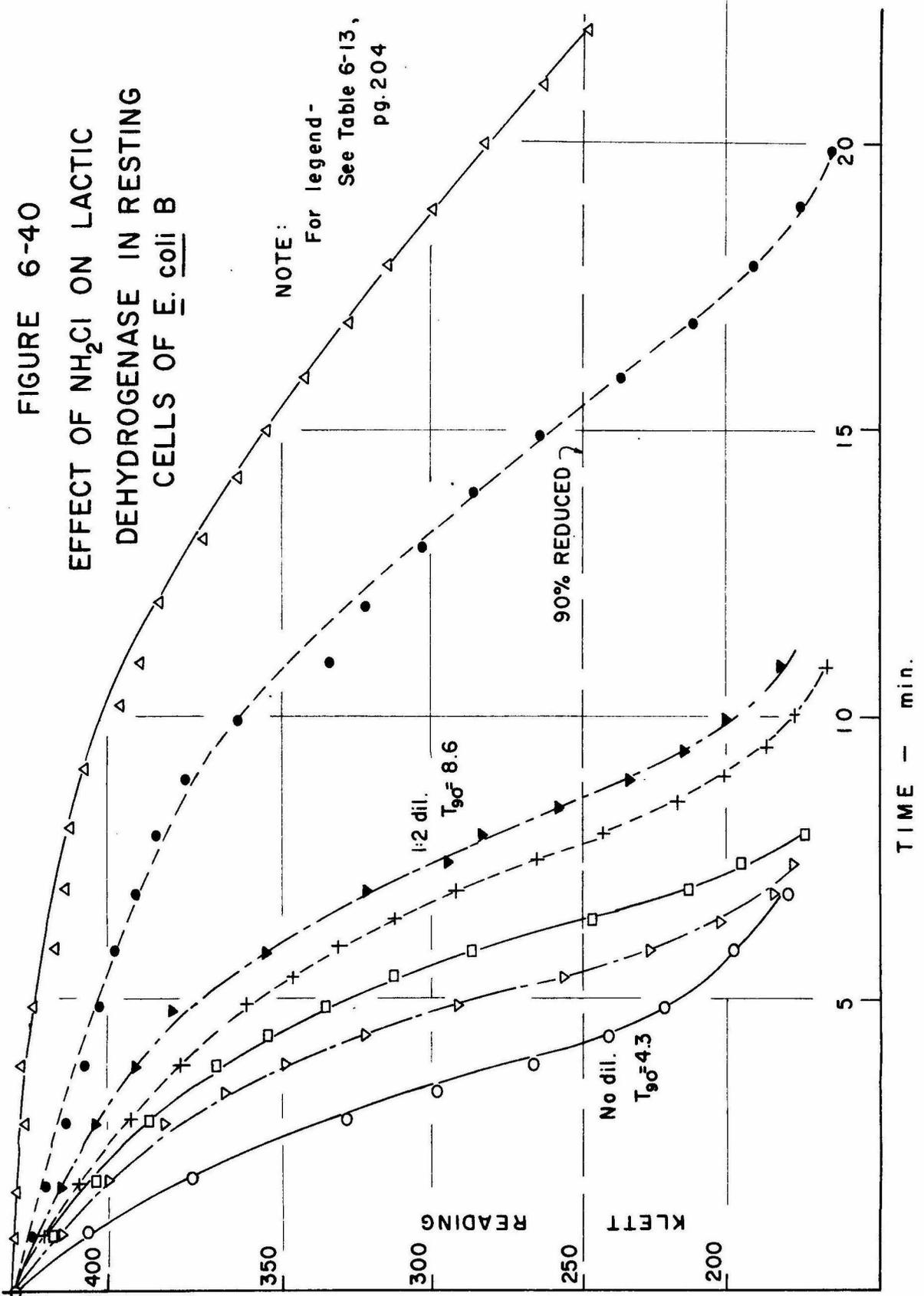


FIGURE 6-40
EFFECT OF NH₂Cl ON LACTIC
DEHYDROGENASE IN RESTING
CELLS OF E. coli B

NOTE:
For legend -
See Table 6-13,
pg. 204



blue at a rate proportional to enzyme concentration. Reductions in activity were computed by direct proportionality methods using the 90% reduction time as the measure of activity. As in preceding experiments, the density of control suspensions was selected so that T_{90} was under 10 minutes. Two test series were made with lactic acid, the results of which are tabulated (table 6-13). A graphical summarization of these tests appears in figure 6-41.

4. Formic Dehydrogenase

Initial studies of formic dehydrogenase activity were made with cells grown aerobically in the 1% glucose-mineral salts medium. The formic dehydrogenase activity for these cells was low, however (T_{90} for 2 mg cells = 35 minutes). Another cell crop which had been grown anaerobically in tryptose broth for 30 hours was much more active since the methylene blue reduction time, T_{90} , was 3.4 minutes/2 mg cells. At first one might assume that formic dehydrogenase activity was stimulated either by substances present in the richer second medium, or by anaerobic metabolism. However, knowledge of the formic acid enzymes of E. coli indicates that hydrogenlyase, an adaptive enzyme, is not produced in nutritionally minimal media under aerobic conditions (55, 56). Thus, rich media and anaerobic conditions provide conditions under which an enzyme, hydrogenlyase, can be induced to split formic acid into $H_2 + CO_2$. Furthermore, hydrogenase, also present in E. coli, will transfer the hydrogen to a carrier (methylene blue in the assay system employed). Therefore, these studies, conducted with tryptose grown cells under anaerobic

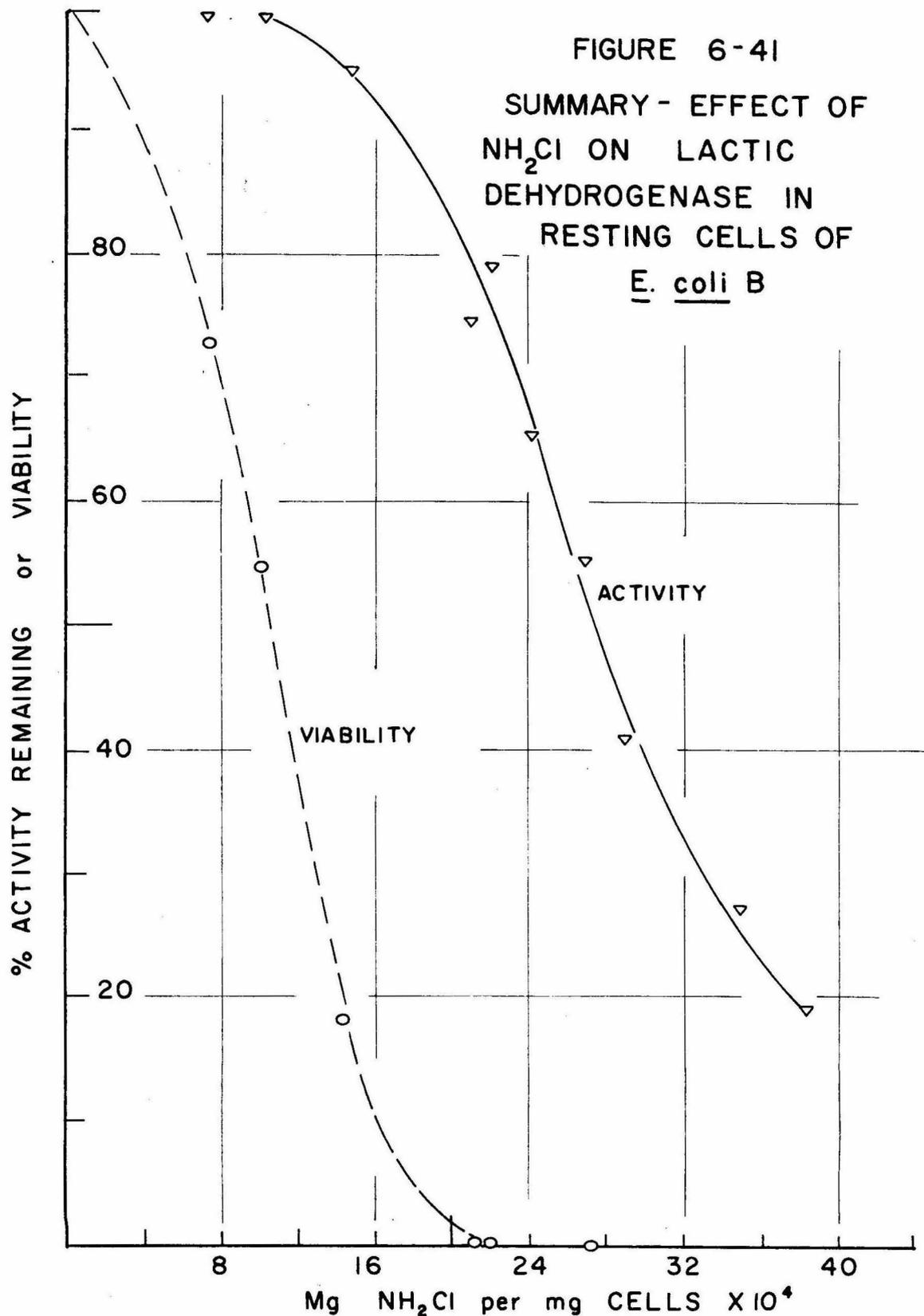


TABLE 6-13

EFFECTS OF NH_2Cl ON LACTIC DEHYDROGENASE
ACTIVITY IN RESTING CELLS OF E. COLI B

Expt.	Treatment Mg NH_2Cl / Mg cells $\times 10^4$	T_{90} (min.)	% Activity Remaining	% Survivors
1	○ 0	4.3	-	-
(2.1 mg cells)	○ 10.1	4.3	100	54
	▽ 21.0	5.7	75	.07
	□ 24.2	6.6	65	.003
	+ 27.0	7.9	55	-
	● 35.0	15.7	27	-
	△ 38.5	22.5	20	-
2	0	4.9	-	-
(2.7 mg cells)	7.3	4.8	100	73
	14.6	5.1	95	18
	21.9	6.0	80	.09
	29.2	11.4	42	-

Dilutions yielded proportional T_{90} increases, thus ratio of T_{90} treated : T_{90} control was used to determine % activity remaining.

conditions, measured the effects of monochloramine on all three enzymes: formic dehydrogenase, hydrogenlyase and hydrogenase.

The results of the single experiment made with this system are shown in figures 6-42 and 6-43. Note that these enzymes demonstrated proportionality between dilution and activity and were considerably insensitive to monochloramine.

D. RESULTS - INDUCTION OF β -D-GALACTOSIDASE

One relatively simple method for studying the effects of monochloramine on protein synthesis is to determine the ability of the organism to produce an inducible enzyme in the presence of a specific inducer. The inducible enzyme, β -D-galactosidase, was selected for this investigation, since it has been thoroughly studied and techniques for its assay have been perfected. The study was divided into four phases: induction in (a) resting cell preparations, (b) growing cell preparations, (c) toluenized resting cells and (d) toluenized growing cells. The toluenization of the cells should not be confused with the toluenization procedures used in the β -D-galactosidase assay (see Methods - Section III). Growing cell suspensions were prepared by harvesting cells grown for 19 hours at 37° C in a 1% succinate-mineral salts medium and inoculating the appropriate density of these cells into fresh medium of the same composition. Colorimetric readings were made at 1-hour intervals from this suspension which was aerobically incubated at 37° C. When logarithmic growth was fully attained, cells were monochloraminated, neutralized with thiosulfate and

FIGURE 6-42
EFFECT OF NH₂Cl ON FORMIC DEHYDROGENASE
OF RESTING CELLS OF E. coli B

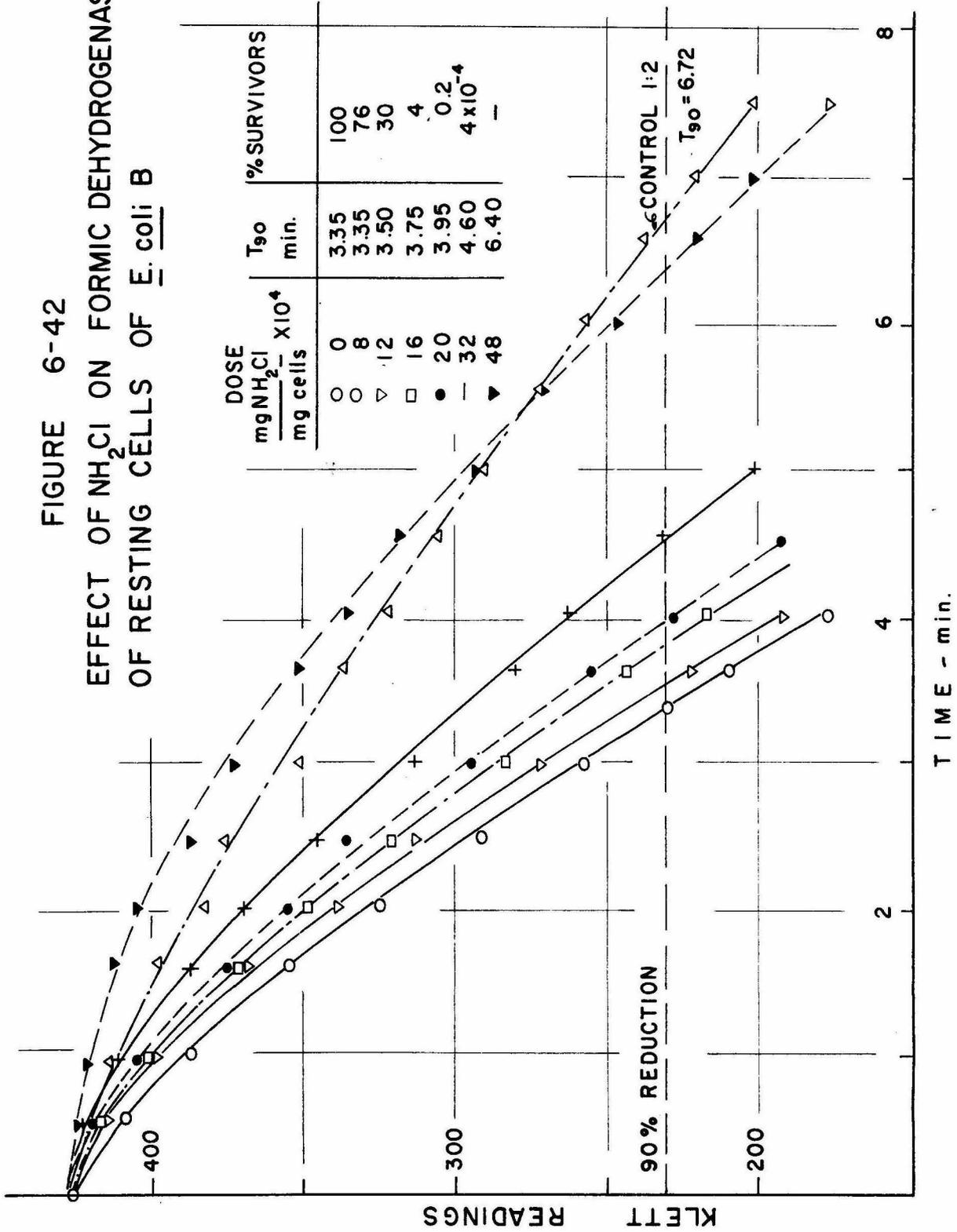
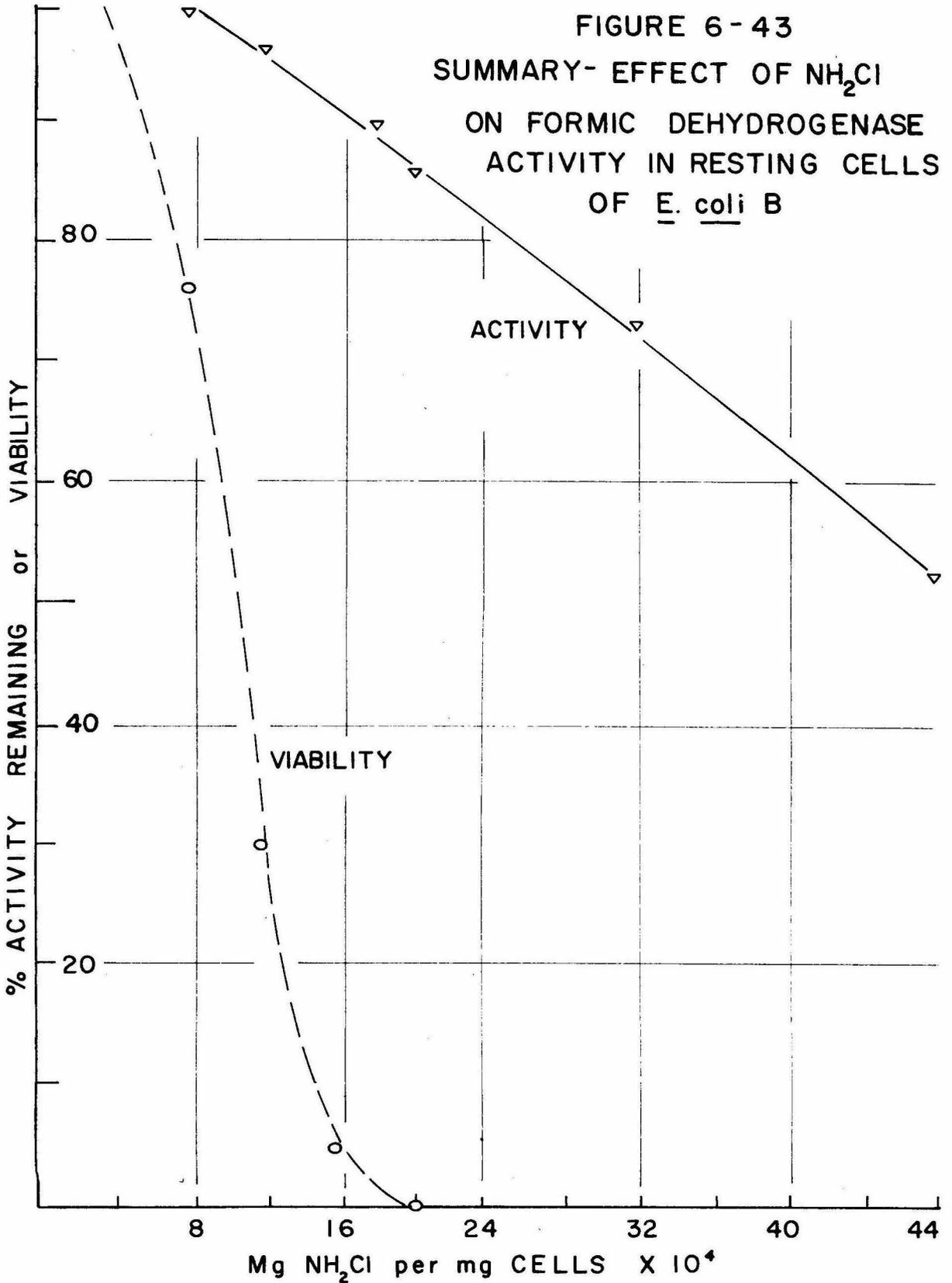


FIGURE 6-43
SUMMARY- EFFECT OF NH_2Cl
ON FORMIC DEHYDROGENASE
ACTIVITY IN RESTING CELLS
OF E. coli B



assayed for β -D-galactosidase activity. Toluened cells were prepared by adding 0.1 ml toluene and 10 μ g of sodium deoxycholate to 5 ml cells (0.7 mg/ml) and shaking the mixture for 15 minutes prior to treatment with monochloramine. The results of these experiments appear in tables 6-14, 6-15 and 6-16. Enzyme activities were computed using Beer's Law and expressed as activity units (m μ moles substrate transformed/mg cells/minute). The enzyme activity was linear with respect to cell concentrations, as is illustrated in figure 6-44: thus, reduction in activity could be calculated directly from absorbence. The effects of monochloramine, summarized in figure 6-45, show that actively growing cells were considerably less sensitive than resting cells to monochloramine, both with respect to viability and enzyme synthesis. Toluening of cells prior to monochloramine treatment made no appreciable difference. Another significant point illustrated from these results is that the effect of monochloramine on cell viability was considerably greater than it was in the respiration, fermentation and dehydrogenase studies. The only difference in procedure here is that the densities of cells treated with monochloramine were considerably lower than in the latter tests (0.7 mg/ml vs. 4 mg/ml).

E. DISCUSSION

Although studies of the effects of bactericidal reagents on pure enzyme preparations are valuable in determining sites of lethal action, the true test of these reagents must be proved on

TABLE 6-14

EFFECTS OF NH_2Cl ON INDUCTION OF
 β -D-GALACTOSIDASE BY RESTING E. COLI B

T = 37° C.

Treatment	Absorbance 2 cm cell	Activity (units)	% Activity Remaining	% Survivors
<u>Controls</u>				
No Dilution	.220	7.95		
3:5	.132	4.75		
1:2	.110	3.95		
1:5	.045	1.57		
1:10	.030	1.08		
<u>Samples</u>				
$\frac{\text{mg NH}_2\text{Cl}}{\text{mg cells}} \times 10^4$				
5.7	.220	7.95	100	100
11.4	.222	8.02	100	69
22.8	.210	7.60	93	30
34.2	.195	7.05	87	.72
45.6	.170	6.15	76	.067
57.0	.130	5.09	58	-
91.0	.058	2.10	26	-

$$\text{Activity} - C = \frac{A}{ab} = \frac{A}{4.4 \times 10^3 \times 2} \text{ moles/liter}$$

$$\therefore A_{\beta} = \frac{1}{8.8} \times 10^{-5} \times \frac{1}{45} \times \frac{1}{0.7} =$$

$$= 36.1 \times 10^{-9} \text{ A mols/mg-min}$$

+ = 45 min.

cells - 0.7 mg

volume - 10 cm

TABLE 6-15

EFFECTS OF NH_2Cl INDUCTION OF
 β -D-GALACTOSIDASE BY GROWING E. COLI B

T = 37° C.

<u>Treatment</u>	<u>Absorbance (2 cm cell)</u>	<u>Activity (units)</u>	<u>% Activity Remaining</u>	<u>% Survivors</u>
<u>Controls</u>				
No Dil.	.130	2.79		
1:2	.066	1.42		
2:5	.052	1.12		
1:5	.027	0.58		
<u>Samples</u>				
$\frac{\text{Mg NH}_2\text{Cl}}{\text{mg cells}} \times 10^4$				
5.7	.131	2.81	100	96
11.4	.130	2.79	100	87
22.8	.132	2.83	100	83
45.6	.123	2.64	95	8
68.4	.115	2.47	89	0.37
91.0	.106	2.29	82	-

+ = 75 min. cells - 0.7 mg $A_\beta = 21.5 \times A$
 v = 10 ml

TABLE 6-16

EFFECTS OF NH_2Cl ON INDUCTION OF
 β -D-GALACTOSIDASE BY E. COLI B AFTER TOLUENIZATION

		<u>Growing Cells</u>		T = 37° C	
<u>Treatment</u>	<u>Absorbance</u> (2cm. cell)	<u>Activity</u> (Units)	<u>% Activity</u> <u>Remaining</u>	<u>% Survivors</u>	
<u>Controls</u>					
No Dil.	.197	4.45			
1:2	.102	2.30			
1:5	.042	0.94			
<u>Samples</u>					
$\frac{\text{Mg NH}_2\text{Cl}}{\text{Mg cells}} \times 10^4$					
4.9	.197	4.45	100	100	
10.0	.197	4.45	100	91	
20.0	.195	4.40	99	83	
40.0	.195	4.40	99	16	
60.0	.185	4.16	94	5	
80.0	.170	3.82	86	0.12	

+ = 60 min. cells - 0.84 mg

v = 10 ml

$A_{\beta} = 22.6 \text{ A}$

		<u>Resting Cells</u>			
<u>Treatment</u>	<u>Absorbance</u>	<u>Activity</u> (Units)	<u>% Activity</u> <u>Remaining</u>	<u>% Survivors</u>	
<u>Controls</u>					
No Dil.	.310	7.55			
1:2	.146	3.56			

TABLE 6-16 (Cont'd)

<u>Treatment</u>	<u>Absorbance</u>	<u>Activity (Units)</u>	<u>% Activity Remaining</u>	<u>% Survivors</u>
2:5	.117	2.85		
1:5	.057	1.34		
<u>Samples</u> $\frac{\text{Mg NH}_2\text{Cl}}{\text{Mg cells}} \times 10^4$				
5.2	.310	7.55	100	100
10.4	.292	7.10	94	75
20.8	.287	7.00	92	41
41.6	.267	6.55	89	.16
62.4	.229	5.60	74	-
83.2	.165	4.02	53	-

FIGURE 6-44

SYNTHESIS OF β -D-GALACTOSIDASE AS
A FUNCTION OF INCREASE IN BACTERIAL
DENSITY

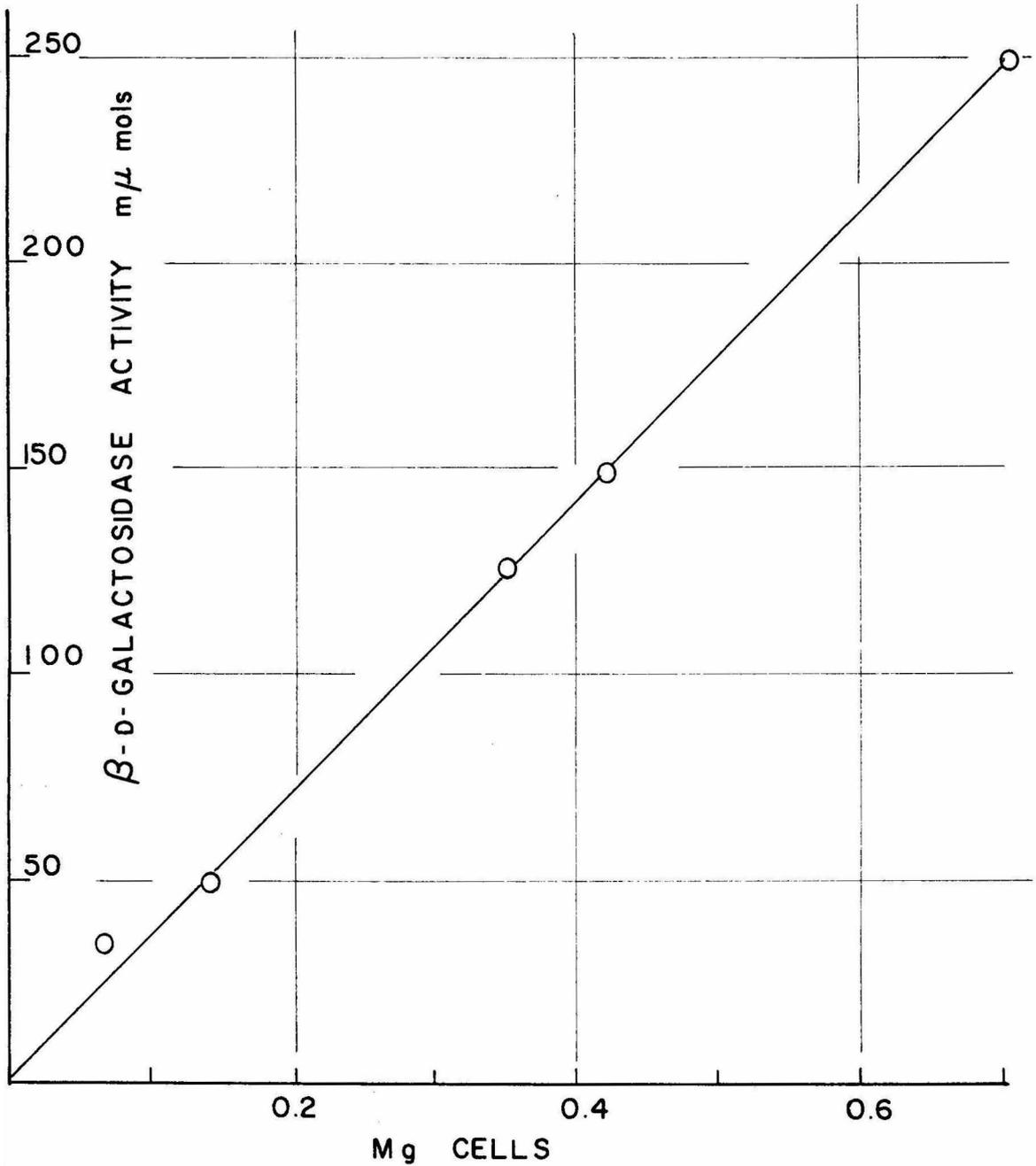
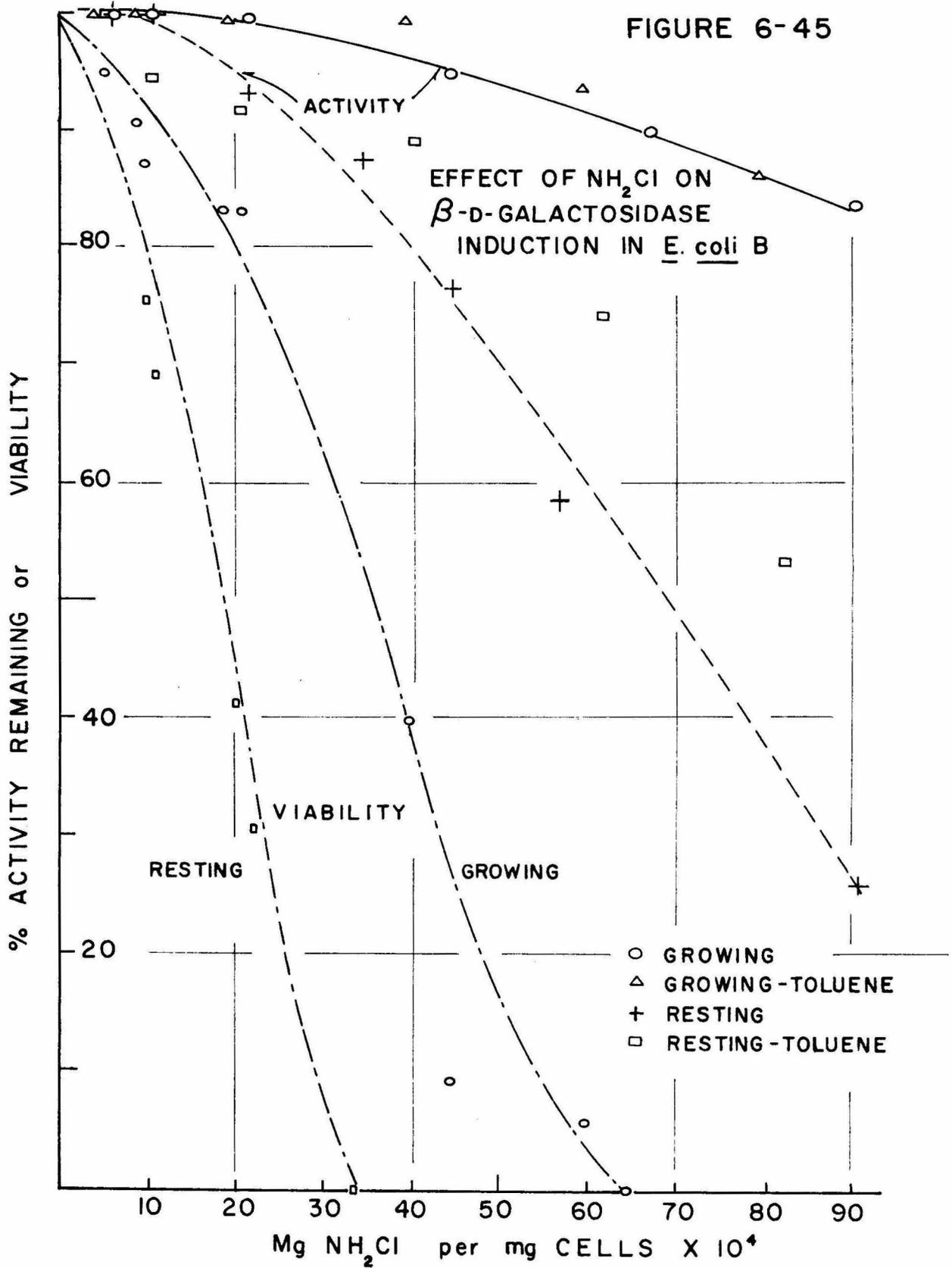


FIGURE 6-45



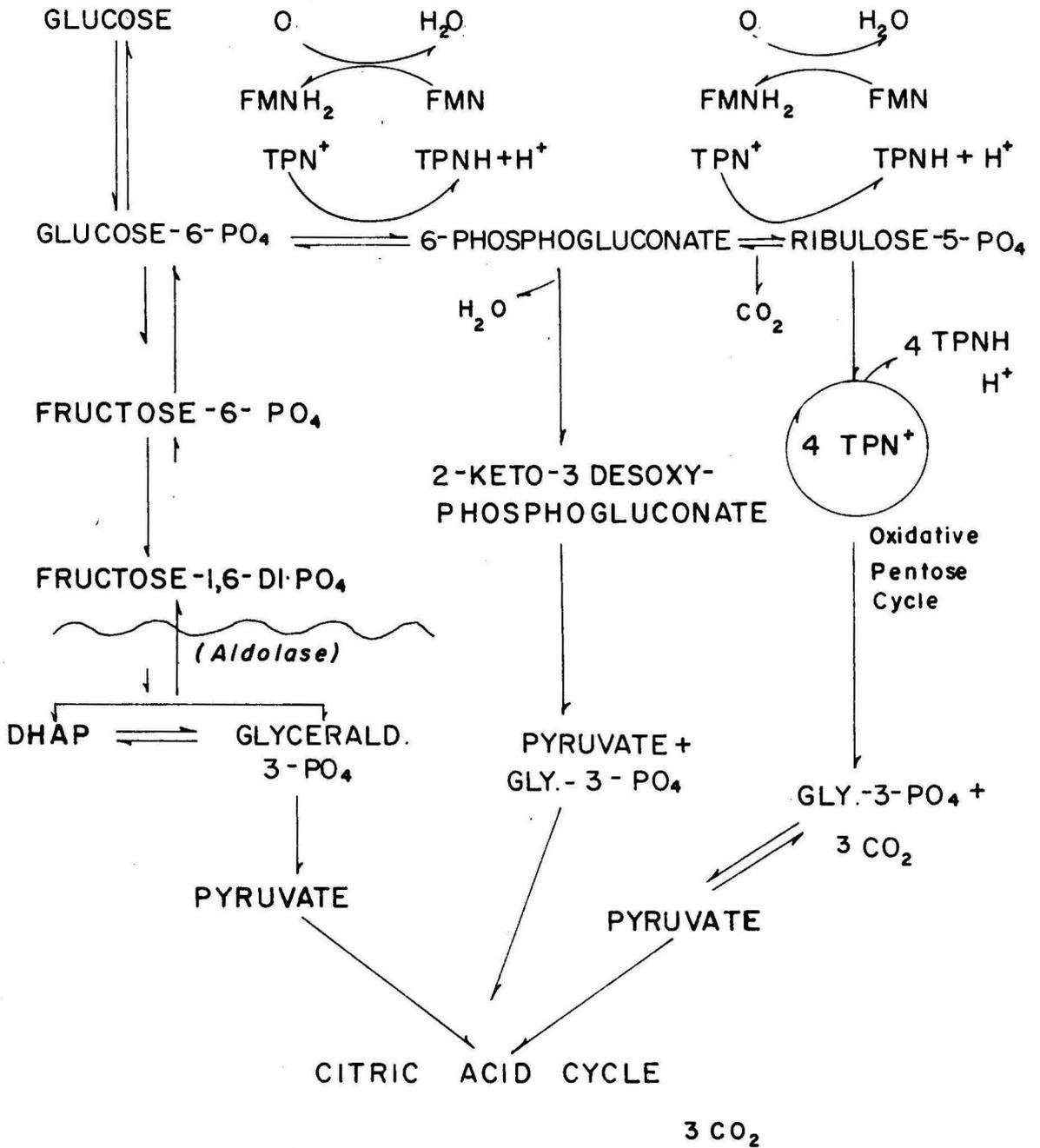
the intact biological system. Resting cell suspensions of E. coli B provided the intact biological system for investigating the site of lethal lesion of monochloramine. The primary criterion used to uncover clues to the biochemical lesion(s) was the relationship of cell viability to specific activities of certain enzymes in cells exposed to monochloramine. Logically, if a critical enzyme were truly impaired by a reagent, cell viability would suffer. It is important to point out, however, that bacterial death, as measured here, depended upon the cell's ability to multiply in a rich agar medium. Therefore, the criterion employed for detecting critical enzymic sites depended ultimately upon the reproductive ability of the affected organism. The plate count provided a sensitive tool for measuring bacterial viability, affording measurements in loss of viability as great as 10^{-4} per cent. The measurement of respiration was also accurate, but it would have been difficult to distinguish between 99 and 100% reduction of activity. In most of the experiments, respiration had to be reduced by monochloramine to a maximum of about 8% of the original value before nearly all cells were killed.

The effects of monochloramine on glucose oxidation indicate that "dead" cells respire. The argument that the more metabolically active cells were selected by such treatment is not valid, as it seems inconceivable that 10% of the cell population would account for over 80% of the respiratory activity (figure 6-11). Glucose oxidation involves a great number of enzymes and several alternate pathways. It is conceivable that a blockage of one enzyme

might not completely inhibit glucose oxidation but simply shift its usual pathway of metabolism. One important example of this would be a shift to the hexose monophosphate shunt (oxidative pentose pathway - figure 6-1). For example, if aldolase were completely inhibited, fructose-1,6-diphosphate instead of accumulating could be converted to fructose-6-phosphate by fructose-1,6-diphosphatase. The equilibrium between fructose-6-phosphate and glucose-6-phosphate is in the direction of the latter (70 to 30%) which would result in an excess of glucose-6-phosphate (82). Escherichia coli contains glucose-6-phosphate dehydrogenase, an enzyme which oxidizes glucose-6-phosphate to 6-phosphogluconate with the reduction of triphosphopyridine nucleotide (TPN) (83). The 6-phosphogluconate may be converted to ribulose-5-phosphate which enters into the oxidative pentose cycle, or it may be dehydrated to yield a 2-keto-3-deoxyphosphogluconic acid which is cleaved into pyruvic acid and glyceraldehyde-3-phosphate (84) (figure 6-46). Thus, aldolase can be bypassed, and both the citric acid cycle and oxidative pentose cycle may be utilized. One important result of this shunt involves the respiratory quotient (R.Q.). If only the oxidative pentose cycle and citric acid cycle were utilized, the moles of CO_2 produced would balance the moles of O_2 utilized; therefore, the R.Q. would equal 1. If, however, the dehydration of 6-phosphogluconate were a major pathway, no CO_2 would be evolved, and the R.Q. value would fall below 1. Although these systems are operative in E. coli, it is difficult to conclude with the data at hand whether or not this alternate pathway is used. Monochloramine

FIGURE 6-46

POSTULATED EFFECT OF BLOCKAGE OF ALDOLASE UPON THE OXIDATION OF GLUCOSE



did decrease the R.Q. to some extent, indicating that the $\text{CO}_2:\text{O}_2$ balance was disrupted.

Another important outcome from these studies is the lack of effect of excess thiosulfate and L-cysteine on respiring, monochloraminated cells. Presumably if monochloramine reversibly affected sulfhydryl sites on enzymes, sodium thiosulfate and/or L-cysteine would have permitted partial or complete recovery of respiratory rates. This finding substantiates the chemical studies in which monochloramine was shown to oxidize L-cysteine to stable sulfur. It is apparent that if thiols or sodium thiosulfate do reverse the action of monochloramine on enzymes or other organic constituents of the cell, the catabolism of glucose is not involved.

The occasional stimulatory effects of monochloramine may have been the result of some "growth" occurring in the Warburg vessels. Increases in population during the manometric experiments seemed to parallel the late rise in activity of monochloraminated cells. Growth could have been promoted by leakage of cell contents, which, together with sulfate (monochloramine was prepared by chlorination of ammonium sulfate solutions), ammonia from decomposition of the chloramine, phosphate (buffer), glucose and trace elements, would conceivably provide a growth menstruum for surviving cells. It is possible that a revival instead of true growth occurred. In any event, the "growth" which may have taken place would not have affected the comparisons of viability

and activity reductions, since plate counts and enzyme studies were made in the initial stages of the test.

The pretreatment of cells (monochloramination, washing and placing in Warburg cups) resulted in about the same degree of suppression of glucose oxidation as did treatment in the Warburg vessels. The reductions in cell viability were somewhat lower, however. This suggests that monochloramine's effects on cell multiplication and glucose oxidation are independent to some degree.

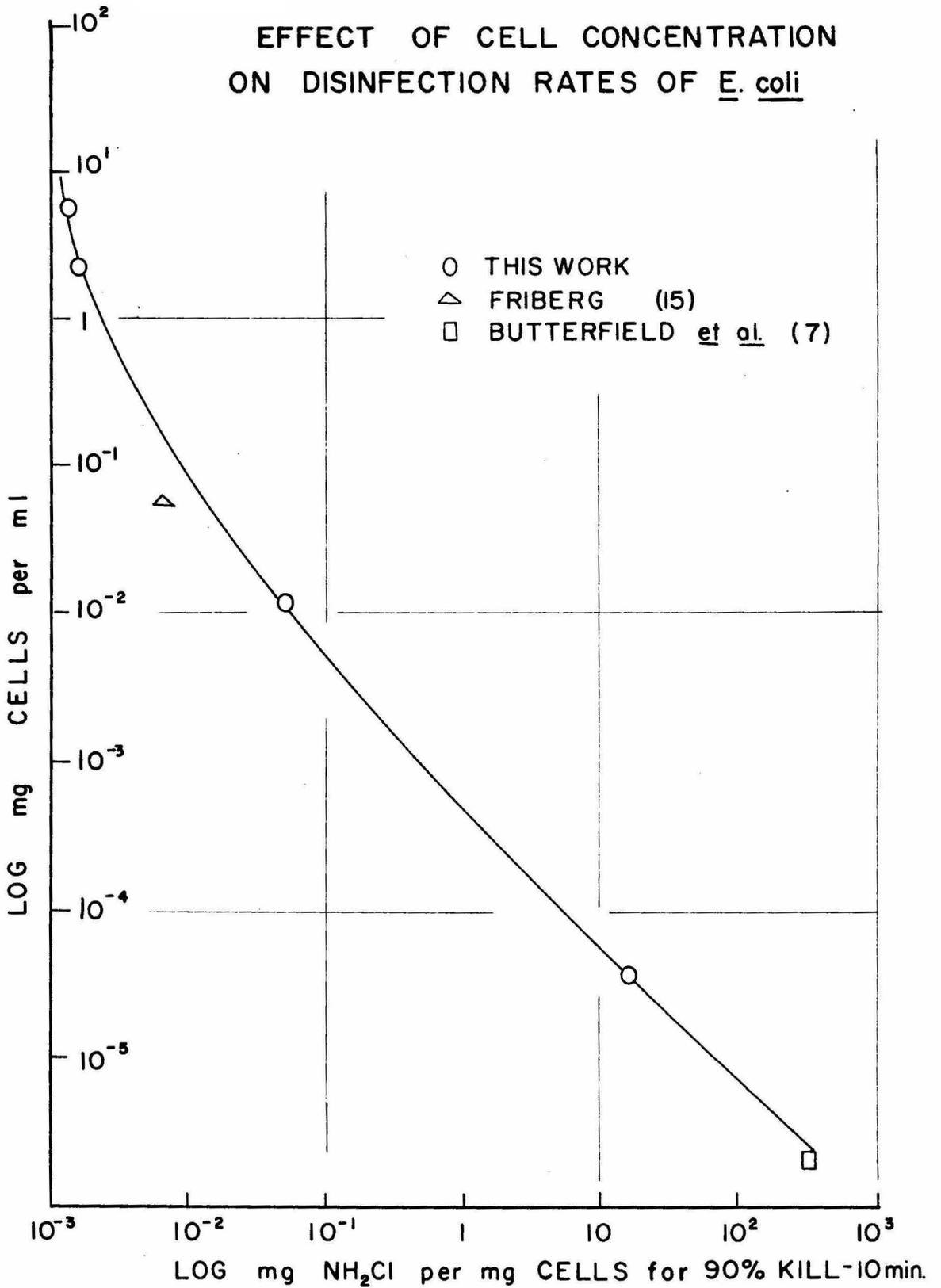
One interesting aspect of this study was the response of cell-free systems to monochloramine. Fair et al. (11) suggested that the antimicrobial efficacy of chlorine and its compounds is partly due to their small size, thus enabling them to penetrate the cell wall and membrane with little effort. The oxidative activities of monochloraminated, sonicated, cell-free suspensions toward glucose (table 6-5) and succinate (table 6-6) indicate that permeability is not a problem in monochloramine action. Similarly, toluene, which is known to destroy cell walls and thereby to increase cell permeability (60), had no effect on the sensitivity of either succinic dehydrogenase activity (table 6-11) or β -D-galactosidase induction (table 6-16) to monochloramine. These tests were not used to explore the effect of time, except insofar as both cellular and cell-free systems were exposed to monochloramine for the same time interval. The effects of this disinfectant appear to be nearly instantaneous at the temperature and pH used in these studies. Enzyme activities were immediately

affected after the monochloramine tip, and in very few instances did this activity change within the next 40 to 60 minutes. Therefore, these analyses were merely observations of the sensitivity to monochloramine of cellular enzymes, with and without a functional cytoplasmic membrane. Since the enzyme is equally sensitive in both intact and disrupted cells, the cell wall and membrane appear to offer no protection through non-specific side reactions or physical blockage of the chloramine molecules.

Upon careful analysis of data obtained from treating various cell densities with monochloramine, it was discovered that increases in cell density did not require proportional increases in monochloramine to effect a predetermined per cent kill in a constant time interval. A plot of the concentration of cells (mg dry weight/ml buffer) treated versus the ratio of monochloramine to unit weight of dry cells (mg monochloramine/mg cells) for a 90% kill in 10 minutes reveals that as cell density increased, the amount of monochloramine per cell required for a given per cent kill decreased, approaching asymptotic limits above 5 mg cells per ml. Although a degree of variability undoubtedly exists with respect to methods of treatment of cells, data from other sources (7, 15) agree with these figures (figure 6-47). Thus, near the asymptotic limits (5 mg cells/ml), 1.7×10^7 molecules of monochloramine per cell were required to kill 90% of the cells, whereas at the other end of the scale (2.8×10^{-6} mg cells/ml) 4.7×10^{12} molecules of monochloramine per cell were required for the same

FIGURE 6-47

EFFECT OF CELL CONCENTRATION
ON DISINFECTION RATES OF E. coli



relative kill. Physically, this is to be expected, for it is easy to visualize that as the number of particles in a given volume are increased, the probability of a collision increases. This is not a simple linear function, however, even for simple neutral particles, for the probability of collision would increase more rapidly as the number of particles increased. An analysis of the monochloramine-cell system would be extremely complex, for there is no way to define a lethal "hit," and particle velocity distribution would be difficult to analyze. Qualitatively, however, these results strongly indicate that cell density is an important determinant and is interdependent with concentration in the bactericidal action of monochloramine. The results of this phenomenon are clearly demonstrated in glucose oxidation (figures 6-13 and 6-14) and in the β -D-galactosidase tests (tables 5-14, 5-15 and 5-16). It was essential, therefore, to hold cell densities constant within a certain range in order to compare the effects of monochloramine on the enzymes studied.

The investigations of succinic acid oxidation were designed to study the effects of monochloramine within the citric acid cycle. The enzyme, succinic dehydrogenase, which catalyzes the oxidation of succinate to fumarate, is an iron-flavoprotein. The iron, which is in the ferric state, is bound to the protein through sulfhydryl groups; thus, the enzyme is completely blocked by sulfhydryl inhibitors such as heavy metals (85). Blockage of this enzyme by monochloramine would result in the accumulation of succinate which might be converted to succinyl CoA via the "P"

enzyme of E. coli (figure 6-2). This enzyme strongly favors the formation of succinate. However, there appears to be no way for E. coli to oxidize succinate, and thus blockage of the dehydrogenase would undoubtedly result in a cessation of oxidative activity. The results from these tests indicate that monochloramine does not completely inhibit this enzyme or the associated cytochrome system, since reductions in activity are considerably less than viability reductions.

The oxidation of acetate was studied in order to determine effects of monochloramine on the incorporation of this substrate into the citric acid cycle. Acetate probably enters the citric acid cycle via an ATP-CoA-acetate transferase, forming acetyl CoA (86). From there the dissimilation would proceed through the citric acid cycle pathway (figure 6-2). It may be significant that Coenzyme A contains a sulfhydryl group which is directly involved in the activation of acetate (figure 6-3). The effects of monochloramine on acetate oxidation were similar to those on the succinic acid system. This may imply that succinic dehydrogenase is the more critical enzyme in this cycle (with respect to monochloramination), or simply that the CoA sulfhydryl, as well as other important sulfhydryl groups in enzymes of the citric acid cycle, are equally sensitive to this reagent.

The final oxidation study was made upon sodium pyruvate. Monochloramine might interfere with the conversion of pyruvate to active acetate (figure 6-3). Other pathways of pyruvate oxidation in E. coli have been elucidated, including a "clastic"

cleavage and a "noncoupled" acetate-generating pyruvic oxidase, usually termed a "Proteus" type (87). The former cleavage involves formate as an electron acceptor yielding acetylphosphate coupled to ATP via acetokinase. The mechanisms involved are still obscure, but biotin and folic acid have been implicated in these reactions (87). The "Proteus" mechanism, an aerobic system involving flavoprotein and cytochrome b, probably is induced upon accumulation of pyruvate in the final phases of growth (87). The pathways for oxidation of pyruvate, therefore, are loaded with sulfhydryl-containing enzymes and cofactors, and the effects of monochloramine on this system provided interesting results. Figure 6-24 shows excellent agreement at the lower monochloramine doses between pyruvate oxidation and viability reductions. However, the correlation persisted only up to the point of approximately a 10% diminution in activity and viability; thereafter, viability became predominantly affected. There are several explanations for this phenomenon. The inhibition of one or a part of an enzyme may be independent of cell viability. Thus, blockage of pyruvate oxidation may not be in itself lethal to cell function. There exists in some bacteria a "malic enzyme" which catalyzes the reaction of pyruvate, CO_2 and TPNH to yield malic acid and TPN (88). Thus, the conversion of pyruvate to active acetate may be bypassed without impairing normal function of the citric acid cycle.

The aerobic breakdown of carbohydrates by E. coli involve many alternative routes, each of which may provide the cell with

the necessary energy and chemical materials for synthesis and growth. The fermentation of carbohydrates is considerably less versatile, and the efficiency of energy production is lower than in true respiration. Information on the effect of monochloramine on glucose and pyruvate fermentation might give some insight into the importance of alternative pathways. Not surprisingly, both systems were considerably more sensitive to monochloramine than the respiratory systems. Thus, approximately 1.4 times as much monochloramine per cell was required to reduce the activity of glucose oxidation to the same extent as fermentative activity.

Under anaerobic conditions, the oxidative pentose pathway is unavailable to glucose dissimilation. The cofactor, TPN, which is reduced in the conversion of glucose-6-phosphate to 6-phosphogluconate, is oxidized through the cytochromes, hence leading to oxygen as the ultimate hydrogen and electron acceptor. In addition, under anaerobic conditions reduced DPNH must be reoxidized via lactic acid dehydrogenase (Pyruvate \rightarrow lactic acid) or alcohol dehydrogenase (acetaldehyde \rightarrow ethanol), rather than through molecular oxygen via the flavin-cytochrome system.

The greater sensitivity of fermentative mechanisms (relative to aerobic metabolism) to monochloramine suggests that alternative pathways in the aerobic dissimilation of carbohydrates may compensate for enzyme blockage along the normal aerobic pathway. The anaerobic systems were, however, less sensitive

to monochloramine than cell viability, with certain exceptions. Anaerobic dissimilation of pyruvate was more sensitive than viability to low doses of monochloramine. Study of two enzymes, lactic acid and formic dehydrogenase, associated with pyruvate fermentation indicate that enzymes or cofactors involved in the conversion of pyruvate to acetyl CoA may be highly sensitive to monochloramine. That is, both lactic and formic dehydrogenases were very insensitive to monochloramine, thereby implicating monochloramine in the conversion of pyruvate to acetate. The relative insensitivity of acetate oxidation to monochloramine implies that the sulfhydryl containing cofactor, CoA, may not be the most sensitive component in the pyruvate conversion scheme. This suggests that an enzyme, or the cofactors, lipoic acid amide and/or thiamine pyrophosphate, could be the sensitive site(s). It is important to note, however, that the sensitivity of pyruvate metabolism was critical only for low doses of monochloramine. This means that the cell either bypasses this mechanism, or that this effect is completely unrelated to the cell survival. It is difficult to conceive that blockage of pyruvic acid metabolism alone would account for death of cells directly, since no energy can be derived from pyruvate under anaerobic conditions.

The response of metabolic CO_2 to monochloramine by fermenting cells offers another area of speculation. Carbon dioxide is released via the cleavage of formic acid by hydrogenlyase and by the decarboxylation of pyruvate to yield acetaldehyde. The

proportions of formic acid and CO₂ which are produced by formic hydrogenlyase are sensitive to pH and initial growth conditions of E. coli (89, 90). Thus, above pH 7.8, CO₂ formation is retarded or non existent, whereas at lower pH values little formic acid is formed and CO₂ evolution is very high. Aerobically grown cells yield little CO₂ in glucose fermentation (90). The induced enzyme, formic hydrogenlyase, probably is an important determinant in these findings. The experiments performed in this investigation showed that metabolic CO₂ constituted between 15 and 20% of the total CO₂ evolved by resting E. coli while fermenting glucose. This value decreased with increasing monochloramine doses (tables 6-8 and 6-9). Since hydrogenlyase was probably not present in these suspensions, such information suggests that either (a) the cleavage of pyruvate to acetaldehyde via oxidative decarboxylation and thiamine pyrophosphate (TPP), or (b) the "clastic" cleavage of pyruvate to acetyl phosphate and formate may here have been affected. The reaction sequence, mentioned earlier as the "clastic" cleavage of pyruvate, probably involves biotin, folic acid, and CoA (87), whereas the decarboxylation of pyruvate involves the sulfhydryl cofactor, TPP (86).

A final note regarding the fermentation studies concerns the effects of monochloramine on glucose fermentation by Streptococcus faecalis. Streptococcus faecalis is homofermentative, producing mostly lactic acid as the end product of glucose fermentation. There is evidence that ethanol and volatile acids besides lactic are produced by S. faecalis (91). Moreover, this organism

cannot aerobically oxidize glucose or other organic substrates. Its nutritional requirements are complex (92), requiring an array of B vitamins, amino acids, purines and pyrimidines for growth. In contrast, E. coli is heterofermentative, possesses a cytochrome system and a citric acid cycle, and has relatively simple nutritional requirements. The latter was evidenced by the excellent growth E. coli attained in a mineral salts-glucose medium. It is not surprising, therefore, that S. faecalis responded differently to monochloramine than E. coli. In fact, glucose fermentation and viability of S. faecalis were considerably less sensitive to monochloramine than they were in E. coli. There was close correlation between viability reduction and fermentative reduction throughout the entire range of monochloramine doses. Since S. faecalis can be presumed to have fewer enzymes than E. coli, thus being less versatile in its metabolism, inhibition of an enzyme in S. faecalis may be more closely related to viability than in E. coli. Caution must be used in concluding that the "death" of S. faecalis resulted from inhibition of enzyme function in the glycolytic cycle. The reduction in fermentative activity may have been the result of secondary effects of monochloramine, i. e., interference with the synthesis of protein or nucleic acids would affect the cell's ability to ferment glucose. This leads to the question of the specificity of action of monochloramine on enzyme systems. It is difficult to separate specific from non-specific effects of monochloramine on the enzyme systems studied. Since inhibition of enzymic activities was rarely greater than the proportion of

cells killed by monochloramine (with the exception of pyruvate fermentation using low doses of monochloramine), one might say that monochloramine acted non-specifically upon these systems. Since we were dealing with populations of differing physiological states rather than individuals, the net effect of impairment of many enzymes in a cell could of course be death. In measuring the general result of such inhibition, i. e., reduction in glucose fermentation activity, succinate dehydrogenase activity, etc., we have no way of knowing what proportion of the cells were "killed" by enzyme inhibition in that system and what proportion of the cells were already "killed" through involvement of other critical sites.

One point of sheer speculation is concerned with the existence of iso-enzymes (93, 94). Since the effects of monochloramine on the fermentation of glucose by S. faecalis are less than for E. coli, and since both organisms have a glycolytic pathway, it is conceivable that the enzymes in one organism are not exactly identical to those effecting the same catalysis in the other. Thus, triose phosphate dehydrogenase of E. coli may be somewhat more sensitive to monochloramine than the isologous enzyme in S. faecalis. This may be directly related to differences in the positioning of the -SH group in the active site of the enzymes.

The dehydrogenases were studied to check certain assumptions and to determine the variation of effects of monochloramine on other specific enzyme functions. The reliability of the results obtained by the Thunberg method depends upon the efficiency of removal of oxygen, the effect of methylene blue on the enzyme

studied and on the characteristics of the enzymes themselves. As was mentioned earlier, the nitrogen purge and careful setting of joints insured reasonably air tight systems. The only enzyme studied that gave non-linear responses was succinic dehydrogenase. This enzyme has an affinity for methylene blue (79, 80, 81), thereby yielding reductions disproportionately high as the cells are diluted. The question arises, however, as to the analysis of enzyme activity reduction resulting from monochloramine action. Since the quantity of enzyme, i. e., cell density, was the same in all tubes, it would seem most logical to use direct proportionality to calculate reduction in activity. If, however, monochloramine interferes directly with the leucoflavoprotein so that methylene blue cannot react with it, activity reductions should be calculated on the basis of an intermediate disproportionality which would be difficult to determine. A comparison of reduction of succinic oxidation activity (figure 6-20) by chloramine with that of succinic dehydrogenase activity (figure 6-37) indicates that the apparent reduction in activity was greater when calculated by the proportionality method than the disproportionality method. The latter gave reductions much the same as those found in the succinate oxidation studies. Since both the succinic oxidation and dehydrogenase act via the same enzyme complex, it is difficult to understand why there should be any degree of disagreement in these two methods of analysis unless, as was suggested above, monochloramine prevents the side reaction of methylene blue with this enzyme complex.

Because of the postulated involvement of aldolase and

triose phosphate dehydrogenase in the biological action of chlorine (17), results of the action of monochloramine against the fermentation of fructose-1,6-diphosphate were especially interesting. This enzyme complex was found to be less sensitive to monochloramine at low doses than glucose fermentation, and about the same at higher doses. It was virtually as sensitive as succinic dehydrogenase to monochloramine, as computed by the proportionality method. Thus, there is doubt that this enzyme complex is the sole site of lethal lesion in E. coli B.

Both lactic and formic dehydrogenase were less sensitive to monochloramine than the other systems studied. These two enzymes have been reported to be less sensitive to iodine and bromine than succinate and glucose dehydrogenases (95).

In comparing the relative sensitivities of the various dehydrogenases studied, it is clear that monochloramine is effective against sulfhydryl enzymes. Both triose phosphate dehydrogenase and succinic dehydrogenase contain sensitive sulfhydryl groups in the active site of the enzyme (54, 85). These enzymes are equally affected by monochloramine in vivo. It is significant to point out that lactic dehydrogenase also contains sulfhydryl groups in the active site (96), yet it is considerably less sensitive to monochloramine. Velick (97) has found that sulfhydryl groups of different enzymes behave in different ways, lactic acid dehydrogenase sulfhydryls being more sluggish than those of triose phosphate dehydrogenase.

There is no doubt that the synthesis of β -D-galactosidase is insensitive to monochloramine in the critical range of concentrations tested. It is significant that the synthesis mechanism, at least for this enzyme, is very resistant to the oxidations of monochloramine. The synthesis of β -D-galactosidase is known to occur de novo from the amino acid pool rather than from a simple change in form of a preformed protein (76). Thus, induction of the enzyme involves the entire process of amino acid activation, transfer via transfer RNA to messenger RNA, and release into the bacterial ribosome. Evidence derived from chemical reactivity of the purines and pyrimidines with monochloramine indicated that these nucleic acid building blocks were rather insensitive to monochloramine oxidation. The results with β -D-galactosidase further substantiated this. It has been shown that chloramphenicol inhibits enzyme induction due to effects on RNA (98). Monochloramine cannot be classified in this category. The fact that this single synthesis was not affected by monochloramine does not imply that monochloramine might, in fact, inflict its lethal lesion in the synthesis of some other essential protein. It is, however, important to find that the RNA transferring mechanism is relatively insensitive to monochloramine. The experiments dealt directly with the induction of β -D-galactosidase, involvement of a permease being eliminated by toluene treatment of the cells (58).

VII. A PRELIMINARY ATTEMPT TO ISOLATE
MONOCHLORAMINE-RESISTANT MUTANTS AND
AUXOTROPHS INDUCED BY MONOCHLORAMINE

The preceding chapter centered around two basic physiological systems, namely, carbohydrate metabolism and protein synthesis. These systems represent two of the most important components controlling cell integrity, i. e., energy and growth. There are, of course, many other vital physiological functions. Underlying all cellular activities, however, is the regulatory mechanism residing in the genome. While amazingly stable, the genetic material is susceptible to minor changes, permitting organisms to adapt themselves to new environments. These changes, mutations, may occur naturally, or be induced more rapidly by a number of physical or chemical agents. A large number of chemicals are mutagenic, but chlorine or chloramines have not been reported to induce genetic change. It is conceivable, however, that monochloramine could cause chemical changes in DNA, thereby causing genetic mutations. Such mutations, not at all uncommon in E. coli, could express themselves in a variety of ways, e. g., altered resistance to inhibitory agents, increased nutritional requirements (auxotrophs), altered colonial morphology, inability to reproduce (lethal mutations), etc.,. Methods have been devised for detecting the presence of auxotrophs and determining the rate at which they are induced by a mutagen (49, 62). It is impossible, however, to determine whether or not an agent has induced any lethal mutations.

Resistance to inhibitory agents (disinfectants, antibiotics) is not uncommon in bacteria. It has been argued that the inhibitor acts as a mutagenic agent, giving rise to a mutant population that is resistant to that agent. Demerec (99) and others (100, 101) have shown that bacterial resistance to antibiotics is not induced by these compounds, but that it originates through the process of selection by the antibiotic of spontaneous mutants endowed with resistance to the drug. In previous sections of this dissertation it was noted that E. coli exposed to monochloramine could be recovered. Whether this is evidence of the selective action by monochloramine of monochloramine-resistant cells is not known.

The research along the aforementioned lines was very preliminary because of time limitations.

A. RESULTS

1. Monochloramine Resistance in Escherichia coli

Monochloramine resistance was studied by several techniques. Initially, various doses of monochloramine were incorporated in nutrient agar which was poured into Petri plates.

Escherichia coli was spread on the surface of these plates and incubated at 37° C for 24 to 48 hours. Distinct colonies were then picked and streaked on monochloraminated agar. These survivors were tested against control organisms for monochloramine resistance. In no case were these survivors found to have any greater resistance to monochloramine than the parent population. In another series of tests, E. coli was inoculated into a minimal

broth containing 1% glucose, and incubated at 37° C for 18 hours. Twenty milliliters of this culture was inoculated in 180 ml of fresh medium and treated with approximately 1.5 mg monochloramine per liter. The treated suspension was incubated 18 hours and, again, 20-ml of this sample was added to fresh medium containing monochloramine (2 mg/l). These serial transfers were continued several times, increasing the dose of monochloramine gradually to 5 mg per liter, and the final suspensions were tested for resistance to monochloramine. As before, however, it was not possible to select a population of monochloramine-resistant mutants.

In the course of the enzyme experiments, it was often noted that plate counts increased markedly in a second 24-hour incubation period. Typical results appear in table 7-1. Note that

TABLE 7-1

Treatment (mg NH ₂ Cl/mg cells × 10 ⁴)	Average plate count		% Increase
	24 hour	48 hour	
0	337 × 10 ⁷	337 × 10 ⁷	0
7.2	265 × 10 ⁷	306 × 10 ⁷	15
10.8	128 × 10 ⁷	169 × 10 ⁷	32
14.4	84 × 10 ⁶	123 × 10 ⁶	47
18.0	100 × 10 ⁴	301 × 10 ⁴	301

the per cent increase during the second incubation period rose with the monochloramine dose. In all enzyme studies reported earlier, the plate counts were always higher after 48 hours of incubation

than after 24 hours. These data suggested that monochloramine might act as a mutagenic agent. Such an agent might induce a spectrum of auxotrophs, including a number of blocked mutants which required an initial period of adjustment prior to multiplication in the nutrient agar (49).

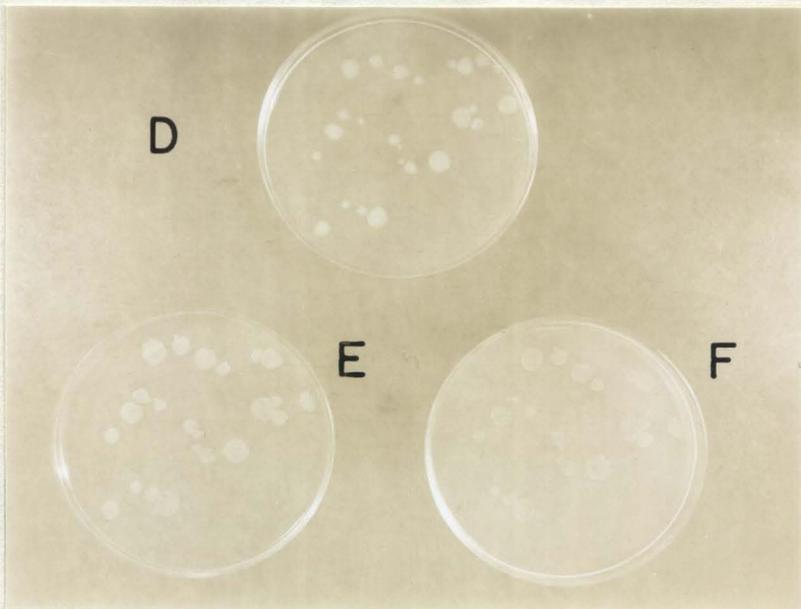
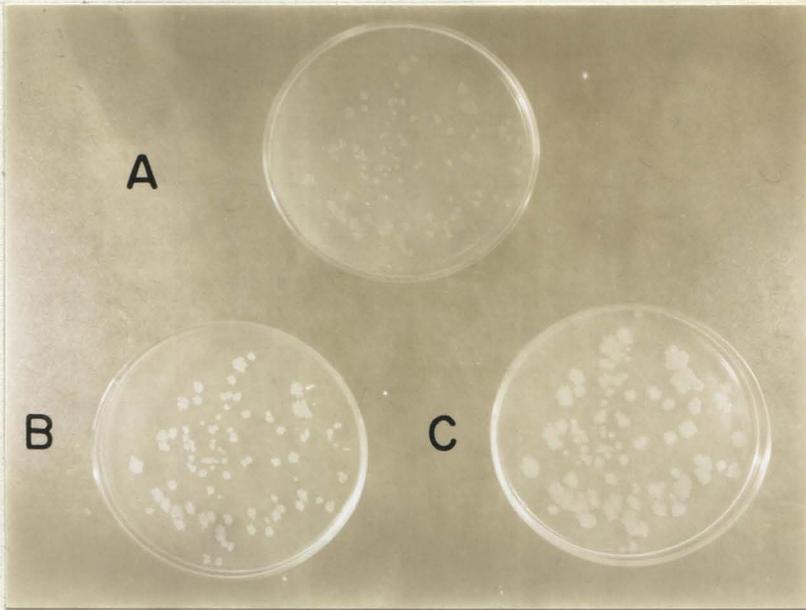
2. Induction of Auxotrophs of *Escherichia coli*

The replica plate technique was used in an attempt to isolate and characterize auxotrophic mutants that may have been induced by monochloramine. Cells were treated with monochloramine sufficient to kill from 10 to 80% of the organisms. The treated suspensions were evenly inoculated on the surface of 8 parallel nutrient agar plates and the plates incubated at 37° C for 18 hours. Replica plates in duplicate were made from these templates by sterile velveteen pads, and these plates were incubated for 24 hours. The results of a typical test are illustrated in figure 7-1. Plates A and D were the original templates (two monochloramine doses), and plates B, C, E, and F were duplicate glucose-minimal agar plates. The original cell density was 13×10^8 cells/ml, and per cent kills were 58% and 15% for samples A and D, respectively. It was apparent from this test and from four others like it, that auxotrophic mutants were not induced by monochloramine in the range of the doses applied. A computation of mutation rate would, of course, be impossible, but one might estimate the lower limits by using the equation of Luria and Delbruck (102):

$$a = \frac{-\ln 2 \ln p}{N}$$

FIGURE 7-1

REPLICA PLATES OF NH_2Cl TREATED E. coli B



where a is the mutation rate per bacterium per generation, p is the fraction of plates (in parallel) containing no mutants and N is the number of survivors appearing on the plates. Since $p = 1$ in these experiments, " a " would equal zero. If, however, we say that $p = 7/8$, i. e., if one plate gave mutants, then we can compute " a ". For a typical test, as cited above, $N = 75 \times 10^7$, and " a " would equal about 10^{-9} mutations per bacterium per generation. Thus, we may say that the mutation rate induced by 12×10^{-4} mg NH_2Cl per mg dry weight of cells is less than 10^{-9} per bacterium per generation. This rate is extremely low compared to natural mutation rates of E. coli (103).

Another procedure, the penicillin technique, was used in a further attempt to isolate auxotrophs induced by monochloramine. Cells were treated with monochloramine to give a considerably greater range of cell reduction than before (50 to 0.03% survivors). The results of two typical tests appear in table 7-2. The colonies appearing within 24 hours after the addition of penicillinase are classified as penicillin-resistant mutants, whereas those appearing in 48 hours were presumed to be a mixture of penicillin-resistant and blocked mutants. Any colonies appearing after 24- to 48-hours' incubation with nutrient agar would be classified as auxotrophs. It is again very clear that auxotrophy is not expressed through any effects of monochloramine. Some 48-hour colonies were picked and tested for auxotrophy on minimal and rich agar with no success. Of some interest is the high percentage of penicillin-resistant colonies appearing in the monochloramine treated samples.

TABLE 7-2

PENICILLIN TECHNIQUE FOR MONOCHLORAMINATED CELLS

Plate Counts*

Test A	Control		Treated Sample	
	<u>Dilution</u>	<u>Colonies</u>	<u>Dilution</u>	<u>Colonies</u>
Initial	1:10 ⁷	200	1:10 ⁴	74
Penase** 24 hr.	1:10 ³	39		
	1:10 ⁴	1	1:100	117
Penase** 48 hr.	1:10 ³	125	1:10	TNTC
	1:10 ⁴	7	1:100	166
Total 48 hr.	1:10 ³	164	1:100	283
	1:10 ⁴	8		
Nutrient Agar*** 48 hr.	1:10 ³	0	1:100	0
	1:10 ⁴	0		

Test B	Control		Sample (1)		Sample (2)	
	<u>Dilution</u>	<u>Colonies</u>	<u>Dilution</u>	<u>Colonies</u>	<u>Dilution</u>	<u>Col.</u>
Initial	1:10 ⁷	364	1:10 ⁷	94	1:10 ³	60
Penase** 24 hrs.	1:10 ³	53	1:10 ³	170	1:10	50
	1:10 ⁴	8				
Penase** 48 hrs.	1:10 ³	90	1:10 ³	34	1:10	20
	1:10 ⁴	9				
Total 48 hrs.	1:10 ³	143	1:10 ³	204	1:10	70
	1:10 ⁴	17				
Nutrient Agar*** 48 hr	1:10 ³	0	1:10 ³	0	1:10	0
	1:10 ⁴	0				

* Average from 3 plates

** Hours after addition of penicillinase-minimal agar

*** Hours after addition of nutrient agar

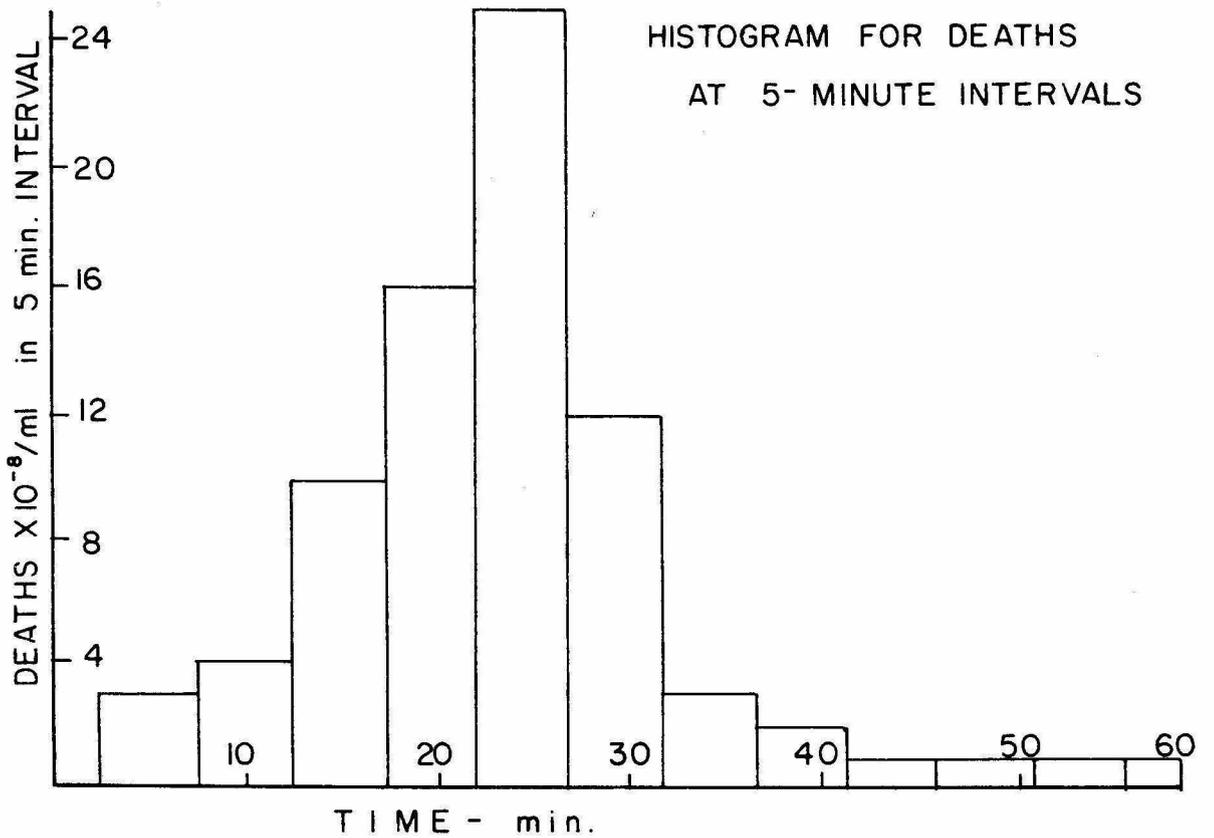
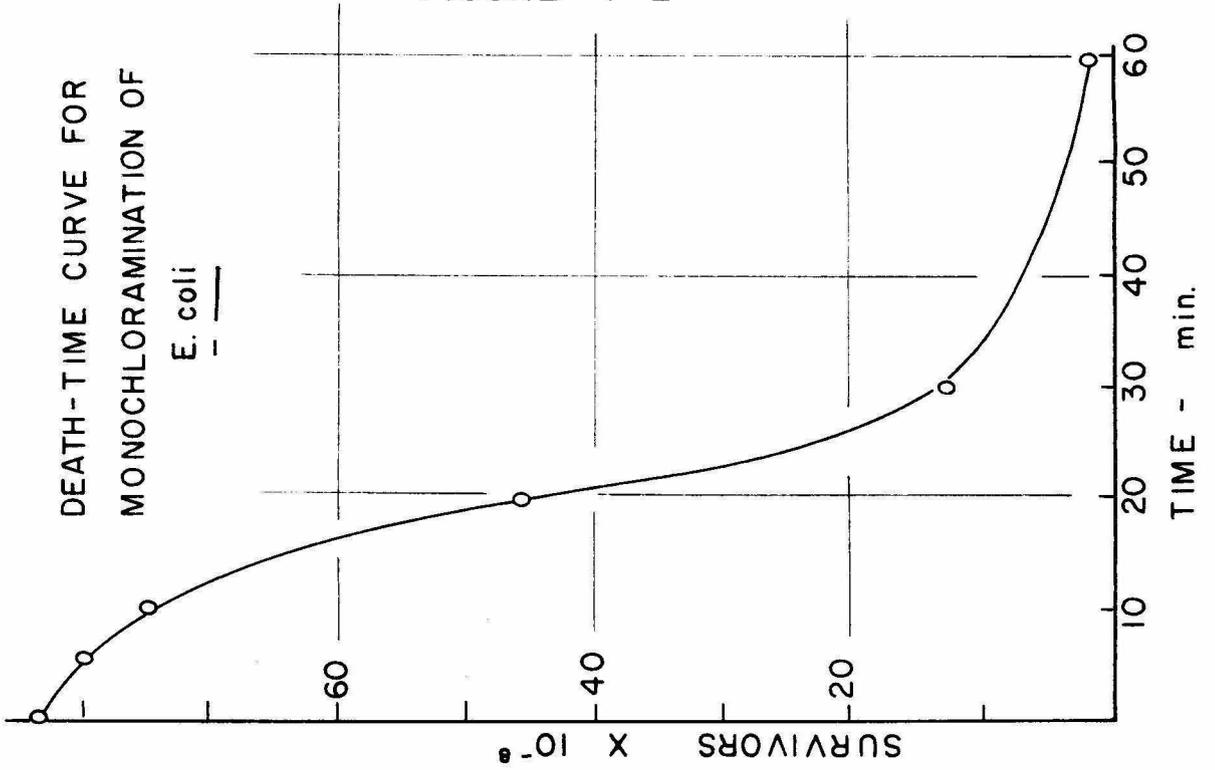
B. DISCUSSION

It is not surprising that monochloramine mutants could not be found. In general, mutants to non-specific agents such as chlorine are ordinarily organisms that have devised some mechanism of keeping the agent from getting into the cell (104). There is no information in the literature regarding monochloramine mutants, although there was mention of such a mutant isolated at the Fort Detrick Laboratories (105). Wyss (106) found that radiation-resistant and peroxide-resistant organisms are also somewhat more resistant to chlorine.

The methods employed to find monochloramine-resistant mutants may have been too crude to detect them. Mutants to phenol, a non-specific disinfectant, can be isolated only by very careful procedures, growing them on extra rich media (106). These mutants generally form extremely small colonies, presumably because they are diverting a great portion of their normal metabolism to produce materials essential for resistance. Similarly, one might expect any monochloramine-resistant mutants to be extremely weak, having poor survival value.

Since repeated monochloramination of survivors yielded a population with essentially the same sensitivity to monochloramine as the original population, it can be concluded that the organisms which were not killed early escaped by chance. The death curve for monochloramination follows the S-shaped logistics curve (figure 7-2). Although the exponential death curve suggests that the chemical reagent reacts with a "hard to find" spot in the organism (107),

FIGURE 7-2



such reasoning can be seriously questioned. One may cite several purely physical phenomena which involve exponential removal, yet it would hardly be sensible to suggest that the removing agent actually seeks out a scarce particle for removal. If one plots a histogram of bacteria killed versus time, one would note that a normal curve results (figure 7-2). This suggests that individual deaths in the population are randomly distributed. It is important to realize, however, that such analyses have shortcomings insofar as the complexity of the cell is concerned. One must use care in drawing simple conclusions from a very complex system involving cells in many physiological states.

The auxotrophic mutant studies also met with little success. The rates of induced mutation must be relatively low, at least for the formation of any auxotrophs detectable by the methods tried. Adelberg (49) found that the penicillin technique selected auxotrophs equal to 2 or 3% of the initial population exposed to ultraviolet radiation (about 100 colonies from 3.6×10^3 ultraviolet survivors). The apparent low rate of monochloramine induced mutations in E. coli B could have resulted from non-specificity of this "inducer". Thus, many sites on the gene may be affected resulting in a high lethal mutation rate with few, if any, auxotrophs. Also these investigations specifically sought out auxotrophs, whereas monochloramine could have induced a large population of drug resistant mutants, as well as mutants dependent on specific nutrients or nutrilites. Finally, strain B of E. coli may have been a poor choice for this study. The notable highly

mutagenic coliform strain, K-12, might have responded differently.

The results in table 7-2 indicate one interesting aspect to this study. For example, in test A only 0.03% survival was found in the sample treated with monochloramine. Yet, the 24-hour penicillinase plate counts indicate that the survivors on treated plates made up 12/39 or 31% of the control survivors. Similarly, after 48 hours this value was 6.4%. These values are considerably higher than the original survival rates, indicating that monochloramine selected out a population of penicillin-resistant mutants. Thus, 39 out of 2×10^6 control cells were penicillin-resistant, whereas 117 out of 7.4×10^3 surviving treated cells were penicillin-resistant. Similar results are found in test B. The trend from the three treated samples indicates that the higher the monochloramine dose, the greater the percentage of penicillin-resistant cells in the surviving populations. It is difficult to advance any mechanism for this apparent selection. While monochloramine may have induced a penicillin-resistant mutant, it is more likely that a relationship exists between penicillin resistance and monochloramine "resistance." For example, since penicillin is known to interfere with cell division, it could be suggested that monochloramine interferes with cell division mechanisms. More work in this area is indicated.

The question of blocked mutants and 48-hour monochloramine survivors is difficult to answer. The 48-hour survivors are not auxotrophs, per se, but may be physiologically impaired cells.

Unfortunately the nature of these cells was not investigated.

In conclusion the work of Milbauer and Grossowicz (37) may be relevant to our study. They found incubation of neutralized chlorine treated *E. coli* B/r⁴ on minimal agar (1% glucose) to yield considerably fewer colonies than similar suspensions plated on nutrient agar. Parallel suspensions incubated in minimal agar plus 2% yeast extract, 1% peptone or 0.3% "Dovril" resulted in intermediate recovery. The authors attributed these results to reactivation of treated cell suspensions through the provision of metabolites essential for recovery from injuries caused by chlorine. However, no mention was made of the possibility that auxotrophic mutations may have been induced by chlorine. True auxotrophs, if present, would have been unable to grow on minimal media, but would have flourished on nutrient agar. These investigators also found that additions of ammonium sulfate to the bacterial suspension prior to treatment with chlorine greatly encouraged "recovery," suggesting involvement of monochloramine. Examination of these data in the light of genetic effects leaves to speculation the question of the mutagenicity of chlorine and its derivatives. In our investigations with strain B, no differences were noted between parallel plates of minimal agar and nutrient agar inoculated with treated cell suspensions; nor did replica plating or penicillin selection techniques reveal any auxotrophy. If Milbauer and Grossowicz were observing a true mutagenic effect, strain differences and variation in techniques may explain our lack of agreement with them.

VIII. GENERAL DISCUSSION

The preceding investigations of monochloramine and its effects on bacteria have only partially answered many of the questions about which they were designed; in fact, they have begged more questions. As the study progressed it became increasingly apparent that the specific effects on bacteria of monochloramine, like other non-specific disinfectants, was very difficult to characterize. The chemical investigations indicated monochloramine to react, preferentially, with sulfur groups. The effects of monochloramine on the various enzymes tested implicates the sulfhydryl group, since the more vulnerable sulfhydryl containing enzymes were the most sensitive. Thus, one might say that monochloramine is specific toward compounds which it most readily oxidizes. This is not to say, however, that its reaction with disulfide bonds or other sulfur groupings is not important. There is also a possibility that the N-chloro substitution of essential heterocyclic nitrogen rings, or of amino groups which they possess, may cause considerable damage to cell function. Thus, the purines and pyrimidines, making up the structure of DNA and RNA, may be substituted to cause genetic anomalies. Such anomalies, if they exist, may be drastic enough to be expressed as lethal mutations. Since no auxotrophs were isolated in these studies, it is difficult to conceive of monochloramine behaving as a mutagenic agent.

One may further speculate that chlorinated metabolites within the cell might act as analogues or antimetabolites, thereby preventing normal biosynthesis and growth. The chemical studies

with amino acids indicated that the N-chloro derivatives were not stable, however, hydrolyzing to aldehydic fragments, ammonia, carbon dioxide and chlorides. It is questionable whether such unstable analogues could interfere significantly with normal cell function.

In order to facilitate comparisons of the effects of monochloramine on enzyme systems, the weight ratio of monochloramine to cells required to effect 10, 50 and 90% inhibition of each enzyme or enzyme system studied has been tabulated (table 8-1). It is obvious from this table that there is no precise order of sensitivity of these enzyme systems to monochloramine. For example, pyruvate fermentation was the most sensitive system at the level of 10% reduction in activity (5.0×10^{-4} mg NH_2Cl /mg cells), but at the 90% level of inhibition, it was one of the least sensitive (47.0×10^{-4} mg NH_2Cl /mg cells). However, one may generalize and note that pyruvate fermentation was, in general, the most sensitive system at low levels of monochloramine, whereas glucose fermentation and fructose-1,6-diphosphate "dehydrogenase" were most sensitive at levels above 8 mg NH_2Cl /mg cells. The relative sensitivity of all of these systems to monochloramine was similar, with the exception of β -D-galactosidase and formic dehydrogenase. This again suggests that this reagent is highly reactive and non-specific in its action. It is also important to note that these comparisons do not take into account viability effects. If per cent reductions in activity at a constant degree of loss in viability are tabulated (table 8-2), considerably more scatter appears in the data. Viability seems to be the most sensitive

TABLE 8-1

A COMPARISON OF ENZYME SENSITIVITIES TO MONO-
CHLORAMINE AT SELECTED ENZYME ACTIVITY REDUCTIONS

Enzyme or Enzyme System*	Dose (mg NH ₂ Cl/mg cells × 10 ⁴) for given reduction in enzyme activity		
	10%	50%	90%
Glucose Oxidation Warburg Treated (6-11)	11	18	31
Glucose Oxidation Pretreated (6-16)	10	20	30
Succinate Oxidation (6-20)	16	23	33
Acetate Oxidation (6-22)	12	18	31
Pyruvate Oxidation (6-24)	7	16	-
Glucose Fermentation <u>E. coli</u> B (6-27)	5	13	22
<u>S. faecalis</u> R (6-30)	11	36	47
Pyruvate Fermentation (6-34)	5	19.2	45.3
Succinic Dehydrogenase Theoretical (6-37)	10	15	-
Experimental (6-37)	13	22	-
Fructose-1,6-Diphosphate "Dehydrogenase" (6-39)	9	15	22
Lactic Dehydrogenase (6-41)	17	28	> 48
Formic Dehydrogenase (6-43)	17	48	-
β-D-Galactosidase Growing (6-45)	68	-	-
Resting (6-45)	28	70	-

* Numbers in parentheses - figure number.

TABLE 8-2

A COMPARISON OF ENZYME SENSITIVITIES TO MONOCHLOR-
AMINE AT SELECTED VIABILITY REDUCTIONS

Enzyme or Enzyme Sensitivity*	Percent Enzyme Activity Remaining When Percent Viability Remaining is:		
	90%	50%	10%
Glucose Oxidation Warburg Treated (6-11)	100	94	35
Glucose Oxidation Pretreatment (6-16)	99	79	18
Succinate Oxidation (6-20)	100	89	59
Acetate Oxidation (6-22)	100	82	30
Pyruvate Oxidation (6-27)	90	74	43
Glucose Fermentation <u>E. coli</u> B (6-27)	97	68	10
<u>S. faecalis</u> R (6-30)	82	41	<0.1
Pyruvate Fermentation (6-34)	84	62	38
Succinic Dehydrogenase Theoretical (6-37)	100	92	30
Experimental (6-37)	100	98	58
Fructose-1,6-Diphosphate "Dehydrogenase" (6-39)	98	90	32
Lactic Dehydrogenase (6-41)	100	100	80
Formic Dehydrogenase (6-43)	100	96	85
β -D-Galactosidase Growing (6-45)	100	97	91
Resting (6-45)	100	96	85

* Numbers in parentheses - figure numbers.

parameter to the effects of monochloramine. Slight variations in technique, dose, cell density or temperature have great effects on the lethality of monochloramine. This is emphasized most profoundly by observing the comparative sensitivities of glucose oxidation with pretreated versus cell suspensions treated in Warburg flasks (tables 8-1 and 8-2). Thus, at a given dose of monochloramine per cell, the reduction in glucose oxidation is approximately the same for both methods of cell treatment, but a comparison on the basis of per cent viability indicates that the pretreated suspensions were considerably more sensitive. Such results indicate that cell death and glucose oxidation are not directly related to monochloramine action. The data in both tables, however, substantiate the theory that enzymes containing sulfhydryl groups at the active site are most sensitive to monochloramine.

Whether the death of bacteria from monochloramination is caused by the denaturation of enzymes or to other changes in the cell cannot be determined from this study, although the evidence implicates the total physiology of the cell. Thus, effects of monochloramine on glycolysis in Streptococcus faecalis R were equal to or greater than reductions in viability, whereas enzymic sensitivity in Escherichia coli was considerably less than decreases in viability. The question then arises: can one attribute the loss in viability of S. faecalis to the inhibition of one or more of the respiratory enzymes? Since the oxidation of glucose, or other organic carbon compounds, is essential for growth and multiplication, and since

the death of these cells parallels the inhibition of glucose oxidation, one would be inclined to answer, yes. It is conceivable that the cell may have been killed via another mechanism resulting in the cessation of glucose metabolism. There are, however, many examples in the literature of bacteria which can still oxidize glucose after being killed by such agents as acetone, silver ions and ultrasonics (108, 109, 110, 111).

As for E. coli, it is apparent that monochloramine's effect on respiratory mechanisms does not entirely account for death. In other words, lethality of monochloramine was expressed more readily than inhibition of anaerobic or aerobic metabolism. This does not mean that adverse effects on respiration had no effect on cell viability. It does imply, however, that: (a) the site of lethal lesion is not within the respiratory mechanisms; and/or (b) the reagent is non-specific, acting at multiple sites and possibly affecting individual cells in different ways. Because of the interdependency of metabolic pathways, only one or several critical sites need be affected for an inhibitor to express a lethal effect. Presumably, if one looks long enough and hard enough, the key site may be uncovered. However, it seems logical to assume that a highly reactive reagent like monochloramine would interact with many sites instantaneously, thereby causing death via many different lesions. The instantaneous monochloramine demand of bacterial cells supports this assumption, since, as was pointed out in Section VI, this demand is probably exerted almost entirely by

sulfur compounds. Because many of the essential enzymes and cofactors in the cell contain sulfur, it would be difficult to implicate a single site. We have seen that the sensitive sulfhydryl enzyme, triose phosphate dehydrogenase, is not affected to as great an extent as is viability, yet Knox et al. (17) believe this enzyme to be critical to cell integrity. It seems more likely, however, that the death of E. coli B by the action of monochloramine, like phenol and other non-specific reagents, results from cumulative (or synergistic) effects on several essential systems in the cell, none of which by itself is the biochemical lesion.

One important facet of this study, the reversibility of the action of monochloramine, was alluded to throughout these investigations. The increase in cell population observed following neutralization of treated cells with excess thiosulfate (Section IV) cannot be explained on the basis of reversibility of the monochloramine reaction. There was no evidence in this investigation to support the theory of reversal of monochloramine action via reductions of disulfide to the sulfhydryl state (i. e., activation of sulfhydryl groups in functional sites of enzymes). Furthermore, all attempts to revive the activity of enzymes in monochloraminated cells by the addition of excess thiols and thiosulfate or by dilution in phosphate buffer were unsuccessful. The increased cell populations described in the reactivation studies (Section IV) could result from: (a) resuscitation of treated organisms through some

mechanism of chemical reversibility; (b) proliferation of surviving organisms utilizing trace nutrients both from the cells and from compounds added (monochloramine and sodium thiosulfate) during the testing procedure; or (c) proliferation of a mutant strain of bacterium, resistant to monochloramine and able to grow in the presence of very low amounts of nutrients. The first two possibilities have been discussed at some length, and neither completely applies to the situation. The third explanation seems as plausible as any. It has been reported in some recent literature (37, 39, 112), that the few survivors in populations exposed to doses of different physical and chemical disinfecting agents, under some circumstances, recover by virtue of exudates from dead cells, and thereby grow and produce progeny which approach, numerically, the original population. Evidently such resistant cells possess an unusual degree of adaptability and may be thought of as abnormal, or as mutants. Unfortunately, in our study, no controls were included to take into account the induction of a mutant which might flourish under very low concentrations of nutrients.

The terms bactericidal and bacteriostatic are often confused in studying the disinfection process. For example, compounds of mercury in high concentrations are bactericidal, but are bacteriostatic at low concentrations. Rahn (9) has attributed the bacteriostatic and bactericidal action of several disinfectants to different chemical reactions. Thus, Rahn suggested that lags in death curves with dilute concentrations of phenol and formaldehyde

were due to reversible chemical reactions, whereas at high concentrations these disinfectants directly affected essential enzymes in an irreversible manner. By analogy, therefore, low doses of monochloramine may be bacteriostatic, acting on enzymes in a reversible manner, whereas higher doses may affect other cell functions irreversibly. The experimental results obtained in this study do not completely support such an idea, since all attempts to reverse enzyme function were unsuccessful, even at very low monochloramine doses.

The experiments with β -D-galactosidase indicate very strikingly that the complexities of the synthesis of this protein are not significantly affected by monochloramine. The implications of these results transcend protein synthesis, considering the involvement of nucleic acids in protein formation. Thus, genetic direction of the synthesis of cellular enzymes apparently was not seriously deterred by monochloramine. It is important to note, however, that such reasoning is based only upon analysis of a single complex system and requires more rigorous investigation.

The brief genetic studies with E. coli B suggest that monochloramine does not predominantly affect genetic functions of the cell. It should be re-emphasized, however, that the results obtained with one strain and under very limited conditions are not sufficient to permit definite conclusions. The significance of the apparent selection by monochloramine of penicillin-resistant mutants is unknown.

The entire study presented here has only scratched the surface of the research that could be undertaken to understand better the biological activities of monochloramine and other chlorine derivatives. The paucity of definitive publications on this topic infers the great difficulties involved in conducting fundamental studies on these highly reactive compounds. The practical applications gleaned from this study are few. Water and waste treatment practices will not change radically because it was found that: (a) monochloramine is bacteriostatic under some conditions; (b) enzyme function is more refractory than cell viability to monochloramine; or (c) monochloramine is not a good mutagen. The information gained from these studies, however, does contribute much in support of the notion that monochloramine is rather non-specific in the mode(s) by which it kills cells. This investigation also can serve as a point of departure to more elegant chemical studies of monochloramine. Several fruitful approaches along these lines suggest themselves, e.g., (a) the mechanisms and kinetics of the reaction of monochloramine with organic compounds of sulfur, amino acids, nucleic acids, phenols and other organic compounds, especially those of importance in water and wastes, and (b) the importance of pH on some of the chemical reactions and biological functions affected by monochloramine. It is obvious from the results of this investigation that the interaction of monochloramine with enzymes, both isolated and in the intact cell, is obscure and

requires elucidation. Further efforts to resolve the question of the means by which bacteria recover from the effects of monochloramine treatment are also needed, particularly insofar as monochloramine-resistant mutants and genetic effects are concerned.

IX. SUMMARY AND CONCLUSIONS

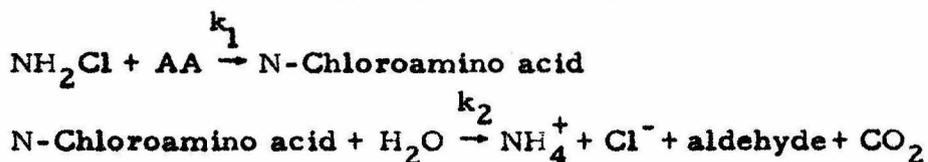
A sequence of studies was initiated in an effort to obtain a better understanding of the mode by which monochloramine exerts lethal effects against bacteria, principally Escherichia coli. The several major facets of these investigations dealt with: (a) the recovery of monochloramine-treated cells in the presence of excess thiol compounds or thiosulfate; (b) the relative reactivities between monochloramine and selected organic compounds; and (c) the effects of monochloramine on the enzymes of resting cells of E. coli and Streptococcus faecalis. Also, a preliminary attempt was made to determine (a) whether monochloramine-resistant mutants are selected or induced by monochloramine treatment of E. coli, and (b) whether monochloramine is mutagenic as expressed by auxotrophy in E. coli. A number of conclusions which can be drawn from the findings are itemized and briefly discussed in the following paragraphs.

1. Estimates of the coliform bacteria population in monochloraminated sewage were appreciably higher as determined by the dilution to extinction technique (most probable number - MPN) than by the membrane filter (MF) procedure. Similar, though less marked, effects were noted in suspensions of E. coli treated with monochloramine. The differentials observed varied with the strain and the nature of the environment. Several theories were proposed to explain these differentials in estimating numbers of coliforms.

2. Resting cell suspensions of E. coli B in phosphate buffer, treated with monochloramine and neutralized with excess sodium thiosulfate, hydrogen sulfide or cysteine, appeared to "recover" from the disinfecting action of monochloramine. The evidence indicates that this recovery was not due to the chemical reversibility of oxidized sulfhydryl groups, and probably did not occur as a result of revival of respiratory mechanisms. Nor was the recovery a result of proliferation of survivors; however, there was no assurance that monochloramine-resistant strains (or mutants) might not have been capable of growth in the buffered suspensions.

3. No evidence could be obtained to support the theory that the reaction of monochloramine with sulfhydryl groups can be reversed by thiosulfate or thiols. Chemical investigations indicated that sulfhydryl and disulfide groups are oxidized to elemental sulfur. The activity of monochloramine-treated enzymes (glucose oxidation) of resting cells could not be restored with mercaptans or thiosulfate.

4. Results from studies of the reaction between monochloramine and alanine suggest a general mechanism for the reaction of amino acids with monochloramine in water:



A brief study of the reaction constants using analog computer methods indicated the k_1 to be of the order of magnitude of 10 liter moles⁻¹, min⁻¹ and k_2 of 0.01, min⁻¹. The value

for the second-order rate constants of monochloramine and amino acids was found to be approximately one-billionth that of chlorine and amino acids. The reactions with sulfur containing amino acids were too rapid to permit determination of rate constants, a finding which supports the notion that monochloramine exerts its lethal effects on organisms via the oxidation of essential sulfur groups in enzymes, cofactors or metabolites of the cell.

5. The decreasing order of reactivity of the selected organic compounds tested with monochloramine were as follows: cysteine, cystine, and methionine > arginine > histidine, tryptophan, and alanine > adenine and guanine > cytosine, uracil, and thymine >> glucose, ribose, lactose. The rapidity of the monochloramine "demand" of E. coli cell suspensions indicated that the monochloramine reacted principally with sulfur containing compounds of the cell.

6. Monochloramine had a greater effect on the activity of enzymes with active functional sulfhydryl groups than those with sluggish sulfhydryl moieties. With only one exception (pyruvate metabolism), however, loss in activity did not exceed loss in viability of E. coli.

7. The fact that individual enzymes in resting cells of E. coli were less sensitive to monochloramine than viability supports the argument that this agent exerts its lethal effects through the simultaneous blockage or destruction of more than one enzyme or cofactor in more than one metabolic system. However, some evidence supports the hypothesis that monochloramine may exert

its lethal lesion within the glycolytic pathway of S. faecalis R.

8. Except at very high levels, monochloramine did not appreciably affect the synthesis of β -D-galactosidase in E. coli. This suggests that monochloramine may not exert a major detrimental influence on protein synthesis.

9. Monochloramine-resistant mutants, if they exist, were extremely difficult to isolate and culture. Monochloramine-induced auxotrophs could not be isolated by either the replica plate or penicillin techniques. Such studies indicated that if mutants were induced, the rates of mutation were very low. There was some evidence that monochloramine treatment selected penicillin-resistant cells.

10. Since enzymes in cell-free and resting cell preparations of E. coli were equally sensitive to monochloramine, it can be assumed that cell permeability is not a major consideration of the kinetics of the disinfection process with this agent.

11. Cell concentration was found to be an important variable in death rate studies with chlorination. An increase in cell concentration did not require a proportional increase in monochloramine for a given per cent kill under controlled conditions.

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