FINAL REPORT

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Study of Environmental Effects on Cellular Autoxidation

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SUMMARY

Working on the hypothesis that oxygen toxicity is a result of increased lipid peroxidation, three fields of effort have been developed: first, *in vivo* peroxidation effects on cell membranes have been measured using rats exposed to 100% oxygen at 260, 400, 600, and 760 mm Hg.; second, an *in vitro* method of studying membrane oxidation has been developed using isolated rat kidney lysosomes; third, a new reaction involving the products of lipid oxidation, malonaldehyde and other thiobarbituric acid reactive substances, has been discovered and investigated. Rats exposed to 100% oxygen grew less than those exposed to air. Oxygen-exposed rats showed changes in their lysosomal enzymes in lung and brain tissue. Lung showed the most dramatic changes, while inhibition of brain lysosomal enzymes was noted. Liver lysosomes showed latent changes in the lysosomal membrane indicating that very subtle effects from oxygen exposure may hitherto have gone unnoticed. The effects of oxygen-exposure on liver lysosomes was, however, potentially harmful since the lysosomes were more susceptible to physical damage. Brain lysosomes were found to be too fragile for use in preparative studies, while kidney lysosomes were isolated free of major mitochondrial contamination. An *in vitro* system for the study of the mechanism of lipid peroxidation and protective agents has been developed using kidney lysosomes. The effects of salts, pH, and osmolarity of the medium have been studied. The *in vitro* assay is being applied also to kidney lysosomes of oxygen-exposed rats. Aldehydes produced during lipid oxidation have, for the first time, been demonstrated to react with proteins and amino acids. Polymerization of ribonuclease, a lysosomal enzyme, results from reaction with aldyhydes and the polymers are less enzymatically active than the native enzyme. The relationship of the aldehyde-protein reaction product to oxidized lipid-protein polymers is discussed as well as the role of aldehyde-polymers as "age pigments". Preliminary studies indicate that a further outgrowth

of this study will be evidence supporting the hypothesis that vitamin A rather than vitamin E is the limiting factor in the antioxidant deficiency syndrome. Electron microscopic studies of the lysosomal and mitochondrial membrane of oxygen-exposed animals is in progress.

In summary, the above results support the original hypothesis that exposure of increased oxygen pressure and to 100% oxygen results in an increased rate of lipid peroxidation.

INTRODUCTION

The basic hypothesis of this work is that the toxic effects of increased oxygen partial pressures and of 100% oxygen are related to an increase in the rate of oxidation of the lipid of subcellular membranes. Since it is not possible to measure the rate of oxidation of the subcellular membranes directly *in vivo*, we have chosen to measure the changes in the permeability of isolated subcellular particles as an index of lipid oxidation. Lysosomes are suited for such measurements since the lysosome is a single membrane limited structure containing a well known mixture of acidic hydrolytic enzymes (1). A change in the permeability of the lysosome will result in the release of its enzyme content. Enzyme release is then a function of membrane damage. While the normal function of lysosomes appears to be the disruption of dead or injured cells (1) and the resorption of useless appendages (2), they have also been strongly implicated in the pathology of muscular dystrophy due to dietary antioxidant deficiency (3) and may also be involved in oxygen toxicity.

Effects of lipid peroxidation have not been limited to the lysosomal membrane. Packer (4) has reviewed the general aspects of lipid peroxidation-caused degeneration of subcellular particles and has pointed out that the disruption is common to such diverse structures as lysosomes, mitochondria, microsomes, and chloroplasts. The latter are unique in that Packer and co-workers have demonstrated a light-dependent peroxidation reaction.

Our work has dealt with three aspects of lipid peroxidation of biological structures: 1) *in vivo* exposure to 100% oxygen and subsequent examination of membrane damage through changes in lysosome stability; 2) *in vitro* peroxidation of isolated lysosomes and the development of model systems for the study of protective compounds, and 3) studies of the reactivity of the products of lipid peroxidation.

RESULTS

I. Exposure of Rats to 100% Oxygen. Through collaboration with Dr. G. A. Brooksby of Ames Research Center, N. A. S. A., we have exposed a number of animals to controlled pressures of 100% oxygen ranging from 260 to 760 mm Hg. The results are summarized in Appendix I, which is a copy of a manuscript currently in press in the Journal of Aerospace Medicine.

The enzymes contained within the intact lysosome are not available to external substrates. A membrane-disrupting agent, such as detergents, sonication, lipases, or proteases, must first act upon the lysosomal membrane before the latent enzyme activity can be revealed. A change in the permeability of the lysosome will result in either the release or availability of its enzyme content. Thus free or available lysosomal enzymes are an indication of the damage to the tissue, while the total enzyme content is an indication of the total lysosome content. Tissues were homogenized and lysosomal fractions were isolated in isotonic sucrose solutions to maintain intact lysosomes. The lysosomal enzymes, acid phosphatases, arylsulfatase and β-glucuronidase, were assayed by the use of automated procedures. Arylsulfatase was found to be the most satisfactory enzyme for detailed study since the enzyme assay was the most reproducible and since the enzyme was quite stable.

Three tissues were examined by this technique: brain, liver, and lung. The lungs of oxygen-exposed animals showed the most dramatic changes. There was an immediate increase in the total enzyme level in the oxygen-exposed animals at 4 days, increasing at 14 days, but not significantly different from the control, and again a significantly increased level at 28 days at 600 mm Hg. Increasing the oxygen pressure to 760 mm Hg. did not significantly increase the effect. The free arylsulfatase (presumably released by lysosomal damage) was also higher at 4 days at 600 mm Hg. and at 28 days and 760 mm Hg. The time sequence follows that found by Brooksby on histological examination of the lung. The brain showed the same

pattern as the lung, but the results were reversed; e.g., there was a significant decrease in both total and free enzymes. The decreased enzyme activity is probably related to inactivation of the enzyme by interaction with either the oxidizing lipid or the products of lipid oxidation. Enzyme inactivation by malonaldehyde, one of the products of lipid oxidation, will be dealt with in a later section.

While these results were found in brain and lung, liver lysosomal enzyme assays showed no significant differences. Exposure of liver lysosomal fractions to osmotic pressures other than that isotonic for the lysosome will result in rupture or lysis of the lysosome (Figures 3 and 4, Appendix I). The pH of the medium also affects lysosome stability. From these parameters it was possible to determine two conditions for assessing the ability of the isolated lysosomes to withstand osmotic stress. Exposure of liver lysosomal fractions to osmotic shock as shown as Figure 5 (Appendix I) revealed the presence of latent damage to the lysosome membrane. The lysosomal fractions were exposed to either isotonic (0.7M sucrose) or hypotonic (0.3M sucrose) solutions at 37°C. Lysosomal fractions from rats exposed to oxygen were more labile than those from air-exposed animals.

Two agents are thought to be important in determining the stability of cellular membranes: vitamin A and α-tocopherol (vitamin E). Preliminary experiments with tocopherol-sufficient and deficient rats exposed to 400 mm Hg. failed to show any significant differences in the free, total, or bound lysosomal enzymes, arylsulfatase, acid phosphatase, or β-glucuronidase. A trend was, however, present indicating that tocopherol-deficient animals might have a greater release of the liver lysosomes. The experiment is being repeated with one modification. Since the addition of excess tocopherol also did not decrease the lysosomal effect, tocopherol alone might not be the agent damaged by increased oxygen tensions; e.g., antioxidant depletion is not the toxic lesion. Vitamin A, both in deficiency

and excess, has been shown to affect the stability of lysosomes more so than tocopherol deficiency (5-10). This combined effect can be understood by assuming that vitamin A levels in the membrane must be maintained at a critical level in order for the maximum membrane stability to be affected. During tocopherol deficiency vitamin A might be oxidized and thus lost from the membrane. Excess vitamin A might be solubilized within the membrane and likewise causes disruption (perhaps by acting as a pro-oxidant). Tocopherol functions in membrane stability thus only as a general anti-oxidant and not as an unique structural part of the membrane. Methylene blue and santaquin, antioxidants of similar solubility properties to that of tocopherol, will reverse the effects of tocopherol deficiency. The study in progress tests the questions raised as to tocopherol and vitamin A function in lipid oxidation by subjecting rats to a defined regimen deficient in tocopherol, vitamin A, or the combination and comparing these animals with those receiving sufficient vitamin A and tocopherol or excessive vitamin A and tocopherol. Because of the weight differences resulting from the growth depression of oxygen-exposure, pair-fed controls have been utilized (Figures 1 and 2, Appendix I). The animals will be examined for changes in the stability of the kidney lysosomal fractions and for morphological and histological changes of the liver at the electron microscope level.

II. <u>In Vitro Peroxidation of Isolated Subcellular Particles</u>. From the results of the study of the <u>in vivo</u> effects of oxygen-exposure on isolated lysosomes and especially the latent effects, a study of the mechanisms of lipid oxidation of the lysosomal membrane and the effects of lipid oxidation on lysosomal enzymes was undertaken. In addition, by isolating the lysosome for detailed study in this manner, we hope to short-cut the delays of <u>in vivo</u> experiments which are extremely time-consuming and expensive. Further, the use of an isolated model system allows

better control of the phenomenon and may lead to mechanisms which may suggest protective agents. Trials of potential protective agents can then be made in the intact rat.

Lysosomes, while containing essentially all of the hydrolytic or destructive enzymes of the cell, constitute only about 10-20% of the total protein of the cell. Isolation procedures are difficult and wasteful of the starting materials. Yields of purified lysosomes are of the order of 2-3% of the starting material. Brain, liver, lung, and kidney were examined as sources for preparative yields of lysosomes. Brain was of particular interest since little is known about the brain lysosome composition. The convulsions associated with hyperbaric oxygen exposure are often suggested as indicating a direct effect of oxygen on higher nervous centers.

The results of a comparative study of brain vs liver lysosomes are presented in Appendix II (prepared for submission to Life Sciences). Liver lysosomal preparations are most commonly prepared and are considered the "standard" preparation. It is clear from the results that brain lysosomes are less stable than those of liver. The pH and osmotic stability range of brain and liver lysosomes were, however, similar. The fragility of brain lysosomes, especially in higher sucrose concentrations (1.2M) makes this tissue undesirable as a source for preparative isolation of lysosomes. Sucrose density gradient centrifugation is the method of choice for the preparative isolation of subcellular fractions. Concentrations of sucrose of 1.2M or greater are often necessary in order to isolate lysosomes from mitochondria which are of similar density.

Shibko and Tappel (11) have reported the isolation of a highly purified rat kidney lysosome fraction. The scheme is simple and the yield relatively high. For this reason rat kidney lysosomes were chosen for preparative study. The rat

kidney lysosome population, unlike the liver, is composed of two groups. The majority are large, about 1 micron in cross-section, while the remainder is small, about 0.4 micron. The latter are of about the same size as those of the liver. By sucrose density gradient centrifugation (11) it is possible to separate the large kidney lysosomes free of all but 5-10% mitochondria. The lysosome pellet is a distinct dark brown layer lying below the mitochondria, which are pinkish in color. An electron micrograph of the intact kidney illustrating the large lysosomes is shown in Figure 1A. Isolated kidney lysosomes are shown in Figure 1B.

Since the lysosomal preparations from kidney are almost free of mitochondria, enzyme assays are not necessary to determine changes in the permeability of their membranes. Light scattering can be used to measure the swelling of the lysosomes following peroxidative or other damage to their membranes. As the particles swell, the non-specific light absorbance at 520 mμ decreases. Shibko and Tappel (11) have shown that enzyme release follows changes in the light absorbance. Figure 2 illustrates typical swelling curves for kidney mitochondria and lysosomes caused by lipid peroxidation. Peroxidation was initiated by the addition of ferrous ion (10 μmoles $FeCl_2$). The absorbance at zero time has been taken as 100%. Mitochondria and lysosomes swell at about the same initial rate. The change in the light scattering and hence the size of the particles is greater for lysosomes than for mitochondria. Since mitochondria are more complex, containing internal structures, it is not surprising that the light scattering is greater for the lysosome. Wills and Wilkenson (17) reported that lysosomes also peroxidize to a greater extent than mitochondria. The similarity in the initial rate of swelling indicates that the maximal rate of lipid oxidation is taking place in both preparations. A similar rate for both preparations is expected, since the rate of

oxidation involves the unsaturated fatty acids of the membrane. It is unlikely that the mitochondrial and lysosomal membranes would differ significantly in their lipid composition.

The rate of swelling depends upon the medium used to suspend the lysosomes. In sucrose solutions lysosomes swell spontaneously below 0.6M. Addition of ferrous ion did not appreciably affect the rate of swelling. Similar results were observed in mannitol solutions. Considerably more spontaneous swelling occurred in mannitol solutions than in sucrose solutions of equal osmolarity. Typical peroxidation initiated swelling was observed in 0.25M mannitol (Figure 3), which also was the most stable mannitol medium (higher spontaneous rates were observed in 0.6, 0.45, and 0.3M solutions). The lack of differences in the rate of swelling between the spontaneous and peroxidation initiated preparations is probably due to a lack of sensitivity of the method and not indicative of a protective effect of sucrose or mannitol. The spontaneous rate of swelling was so rapid as to make differentiation difficult. In 0.25M mannitol, where the spontaneous rate was reasonable, peroxidation initiated swelling was observed.

Ionic media appeared to be more promising. Further, a convenient method for measuring the rate of lipid oxidation in the preparation is to determine the formation of colored products on treatment with 2-thiobarbituric acid. This method of determining lipid oxidation is known as the TBA reaction and measures mainly the amount of malonaldehyde formed on oxidative cleavage of polyunsaturated acids. The TBA reaction is, however, interfered with by reducing sugars, thus adding to the desirability of ionic media.

Figures 4 and 5 illustrate the effect of NaCl and KCl concentrations on the rate of spontaneous and peroxidation initiated swelling. Potassium ion appears to stabilize the lysosome more than sodium ion, at 0.3 and 0.6M solution.

Spontaneous swelling in 0.3M KCl was 95.8% of the maximum absorbance while in 0.3M NaCl it was 92% after 9 min. at room temperature. Increasing either the NaCl or KCl concentration increased the extent of swelling but not the rate. The induction period depended upon the medium and was shortened by higher salt concentrations. Figure 6 compares the swelling of the same preparation of lysosomes in 0.175M solutions of KCl and NaCl. Greater swelling occurred in the KCl solutions than in NaCl.

From these data it is possible [illegible]e a medium for study of peroxidation effects of lysosomes usng light scatter[illegible]g rather than enzyme assay as the index of lability. Kidney lysosomes are suspended in 0.175M KCl, 0.02M Tris-HCl, pH 7.0 for study. Work is now in progress to determine the effects of various [illegible]roxidants and antioxidants and membrane stabilizing agents on the lysosomal membrane. A micro oxygen electrode has been supplied to us through the courtesy of Professor Irving Fatt, of the Department of Mineral Technology, so that it is now also possible to determine the oxidation of the membrane simultaneously with changes in light scattering. Oxidative and osmotic effects can thus be separated for the first time. The effect of gas composition on the rate of oxidation of the lysosomal and mitochondrial membrane is likewise under study. Morphological changes in the membrane and general structure of the particle are being determined simultaneously on aliquots preserved for electron microscopy. In addition, the electron micrographs also serve as internal controls of mitochondrial contamination. The latter is currently also determnned by succinoxidase assay.

As a part of the experiment outlined on the effects of vitamin A and tocopherol on the stability of lysosomes from animals exposed *in vivo* to 100% oxygen, we plan to utilize the present swelling assay as a direct test for latent membrane damage. Further, the amount of antioxidants present in the membrane will be

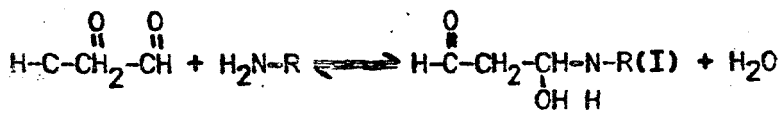
indicated by the induction period before oxygen uptake begins after initiation of oxidation by ferrous ion. The extent of oxygen uptake will be indicative of the unsaturated fatty acids remaining unoxidized in the membranes. TBA measurements on tissues will also serve to indicate if peroxidation has occurred in vivo.

III. Studies of the Reactivity of the Products of Lipid Oxidation. While the previous sections have dealt with the initial effects of lipid oxidation, we have also discovered some interesting reactions of the products of lipid oxidation. As mentioned above, oxidation of polyunsaturated fatty acids such as the essential fatty acids produces a number of substances which react with 2-thiobarbituric acid (TBA) to form colored products. The reaction is an extremely sensitive test of lipid oxidation, and has led to the discovery of the reactive properties of some of the end products of lipid oxidation. These end products react with TBA to form the colored compounds used quantitatively in measuring lipid oxidation and are known as TBRS (2-thiobarbituric acid reacting substances).

Roubal and Tappel (13) have shown that oxidation of polyunsaturated fatty acids in the presence of proteins results in the polymerization of the proteins. They have ascribed the polymerization to the reaction of the protein with the free radical intermediaries occurring in lipid oxidation (fatty acid hydroperoxide free radicals). Appendix III (manuscript submitted to Lipids) presents data illustrating that the polymerization of proteins may be mediated by the end products of lipid oxidation, TBRS, as well as free radical mediated reactions. Polymerization of ribonuclease was observed with pure malonaldehyde, which is the principal TBRS or oxidation end product (14). Additionally, the polymers of RNase produced by lipid oxidation also contained reacted with it the TBRS (probably malonaldehyde).

Appendix IV(manuscript submitted to Arch. Biochem. Biophys.) presents a detailed study of the reaction of malonaldehyde with bovine serum albumin. The reaction between the protein and malonaldehyde appeared to be specific for certain binding sites and to occur at very low concentrations of the aldehyde. The reaction is essentially irreversible at physiological pH ranges and results in an unique spectral absorbance at 282 mμ. The free amino groups of the protein appeared to react with the aldehyde carbonyl group.

Malonaldehyde has been demonstrated to react with imidazol, α and γ-amino, and SH groups but not aromatic groups of amino acids. The reaction was followed spectrophotometrically at 350 mμ, corresponding the malonaldehyde carbonyl absorbance. The reaction was confirmed by gel filtration chromatography using 2-^{14}C-glycine as is illustrated in Figure 7. The molar ratio of malonaldehyde to glycine in the major component was 1.04. A methyol derivative (I) such as below is hypothesized as the reaction product structure:



The spectrophotometrically determined reaction rates with essential amino acids are being confirmed by gel filtration and will be reported in a subsequent publication. In general, SH and ε-amino groups of lysine appeared to react faster than any of the other reactive side groups of amino acids. Malonaldehyde derivatives of proteins probably involve only the amino and thiol gouups. Thiol ester cleavage has not been observed.

Since protein polymerization is a consequence of reaction with both oxidizing lipids and malonaldehyde, we have investigated the general reaction of proteins with cross-linking aldehydes. Bjorsten (14) has discussed the role of cross-linking aldehydes in biological processes such as aging. If extensive lipid oxidation is

occurring during oxygen exposure, then protein polymerization is likely to occur. The homologous series of cross-linking aldehydes, formaldehyde, glyoxal and malonaldehyde, were chosen for study. In summary, the molecular weight distribution of the polymers of RNase depended upon the pH and the aldehyde. RNase was chosen since it is a lysosomal enzyme, of known amino acid sequence and of low molecular weight so that higher polymers might be soluble. Malonaldehyde appeared to react faster than either formaldehyde or glyoxal at either the acid pH (4.0) or the neutral pH (7.6). Formaldehyde produced no polymers in acid solution, but did so in neutral solution. The molecular weight distribution of formaldehyde - RNase polymers tended toward the accumulation of very high molecular weight materials (Figure 7). In acid solution malonaldehyde-RNase polymers were not soluble above 42,000 MW (RNase-trimer) (Figure 8). In neutral solution malonaldehyde-RNase polymers ranged from 150,000 to 28,000 (dimer). Monomeric, dimeric, and trimeric products were clearly visible (Figure 9). Malonaldehyde produced a dimer after only 20 min. reaction. Glyoxal reacted in both acid and neutral solution to produce polymers. Only a slight precipitate formed and soluble higher polymer were found (Figure 10). In neutral solution glyoxal produced polymers of similar molecular weight range as in acid solution. As the reaction proceeded there was a tendency toward the accumulation of higher molecular weight products.

Specific molecular weight ranges of the polymers were collected and assayed for enzymatic activity. Table I presents the activities found. Under acid reaction conditions, formaldehyde-reacted monomer was the most active. Glyoxal monomer and higher polymers contained some activity while the malonaldehyde reacted products were essentially inactive. Under more physiological conditions at pH 7.6 formaldehyde completely inactivated the isolated RNase. Glyoxal-reacted products contained little or no activity. Malonaldehyde-reacted RNase was clearly

separated into two fractions, the active monomer and the inactive dimer and higher molecular weight polymers.

Lysine forms a part of active center of RNase (15). Reactions masking the ε-amino group of lysine-4i eliminate enzymatic activity. The reduction in enzyme activity probably corresponds to reaction between this lysine and the reacting aldehyde. The differences in enzyme inactivation may represent differences in reactivity with pH and the reactivity of the aldehyde. T. W. Kwon (unpublished data) of this laboratory has also found that aldolase is inactivated by malonaldehyde. Enzyme inactivation by malonaldehyde or other aldehydes produced during lipid oxidation is likely if a lysine or another of the reactive amino acid side groups occurs in the active center of the enzyme. Many enzymes fall within this category.

Preliminary experiments in this laboratory also show that malonaldehyde or oxidized lipid produced polymers of bovine serum albumin or of RNase are less readily attacked by proteolytic enzymes than are the native or heat denatured proteins. Reaction of proteins with reactive aldehydes produces three effects: 1) inactivation of enzymes having reactive groups in their active centers; 2) polymerization at high local concentrations of the aldehyde and hence precipitation; and 3) prevention of catabolism by proteases. Thus, the oxidation of lipids in the presence of proteins is undesirable from three standpoints. The stability of the product to the proteolytic degradation involved in the normal catabolism of the cell may be important since inert material might accumulate through this reaction, eventually blocking the functioning of the cell.

The inactivation of RNase by aldehydes and the decrease in brain lysosomal enzyme activity following exposure to 600 and 760 mm Hg. 100% oxygen may be related. O'Malley *et al.* (16) have reported similarly that erythrocyte acetylcholinesterase is inhibited by peroxides and hyperbaric oxygen exposure. The experimental

conditions of O'Malley et al. are certain to produce malonaldehyde as well as hydroperoxides. Malonaldehyde inactivation of brain lysosomal enzymes and erythrocyte acetylcholinesterase underscore the potential importance of this reaction in oxygen toxicity.

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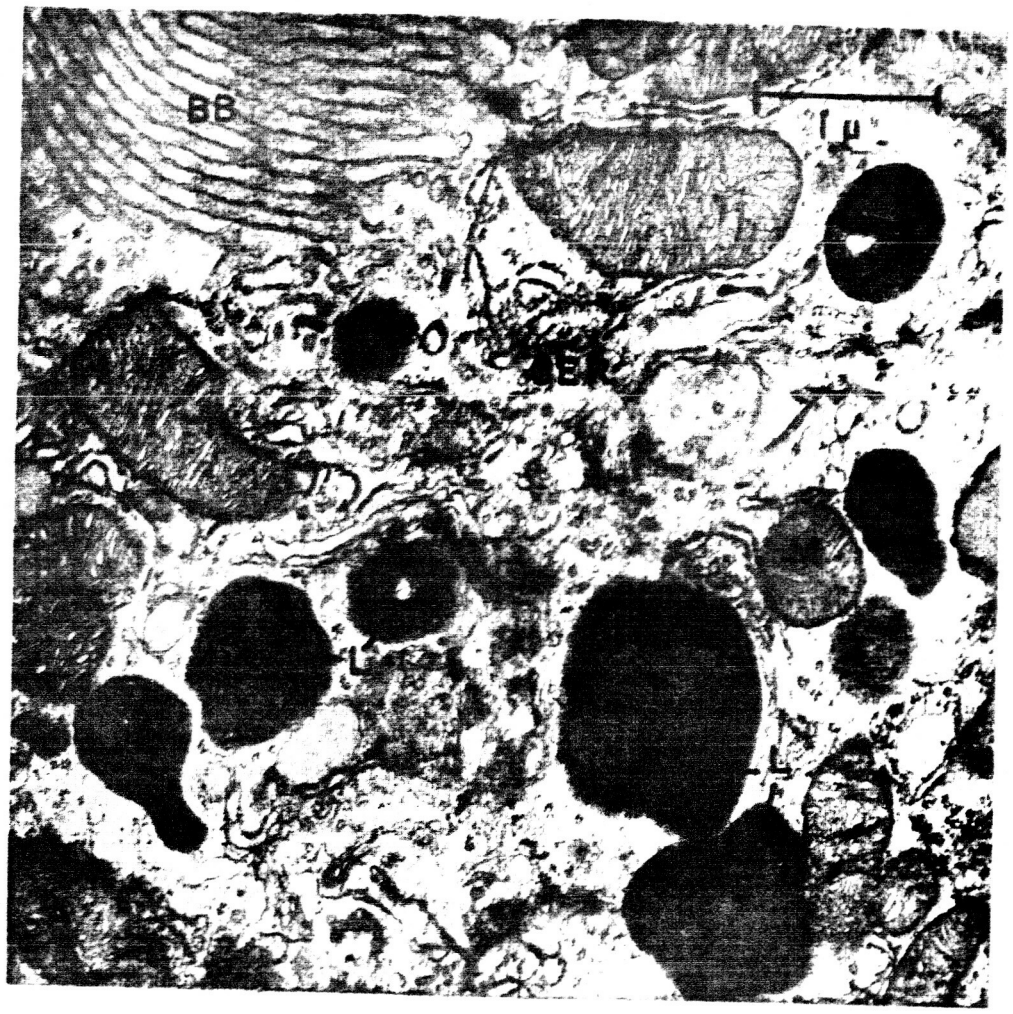
Table I. Inactivation of RNase Enzymic Activity by Cross-linking Agents.

Cross-Linking Conditions	Cross-Linking Agent	Molecular Weight Range	% Activity Compared to Chromatographed RNase Monomer
12 hrs. pH 4.0	FA	14,000	32.5
	GXL	14,000	17.2
		28,000-60,000	5.6
	MA	14,000	0.3
		28,000	0.2
		>60,000	0.1
12 hrs. pH 7.6	FA	14,000-30,000	Inactive
		>60,000	Inactive
	GXL	14,000	0.2
		28,000	0.3
		>60,000	Inactive
	MA	14,000	84.6
		28,000	0.2
		>60,000	0.4

Fig. 1. Rat kidney lysosomes: A. Intact rat kidney, showing large lysosomes (L). Compare with mitochondria (M). Brush-border (BB) and granular endoplasmic reticulum (GER) are shown for orientation. B. Isolated rat kidney lysosomes.


BB
1μ


0.5μ

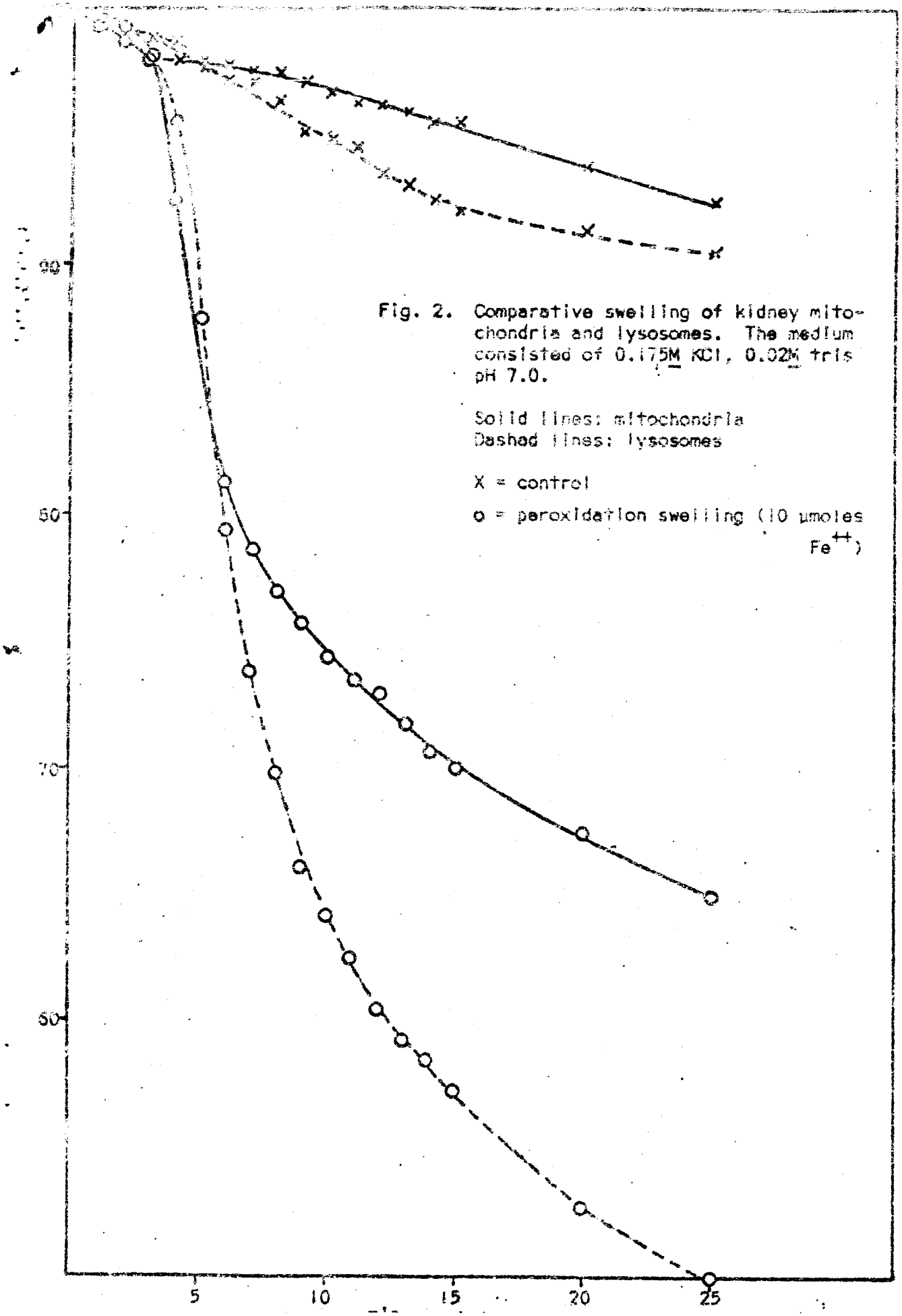


Fig. 2. Comparative swelling of kidney mitochondria and lysosomes. The medium consisted of 0.175M KCl, 0.02M tris pH 7.0.

Solid lines: mitochondria
Dashed lines: lysosomes

X = control

o = peroxidation swelling (10 µmoles Fe^{++})

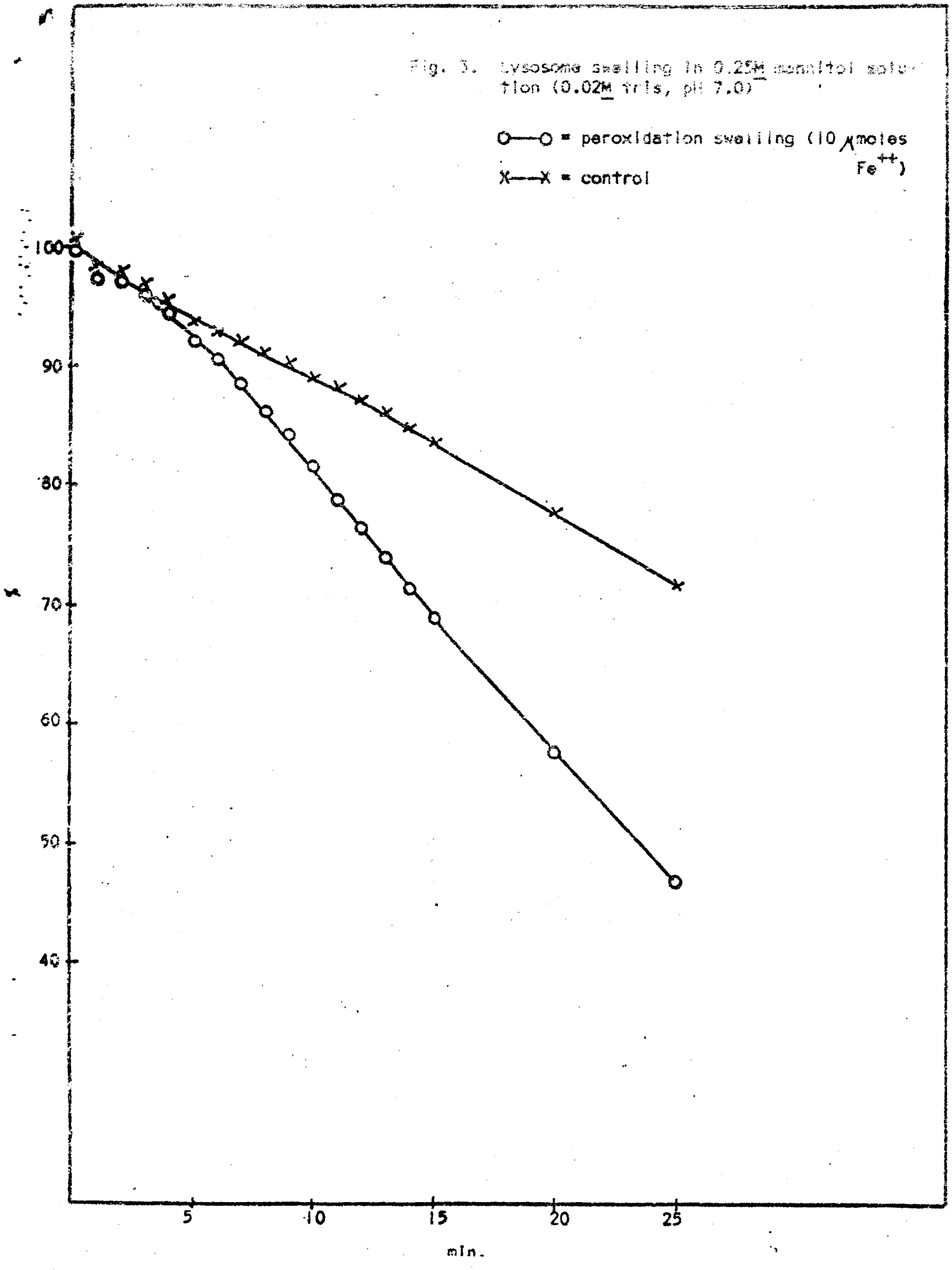


Fig. 3. Lysosome swelling in 0.25M mannitol solution (0.02M tris, pH 7.0)

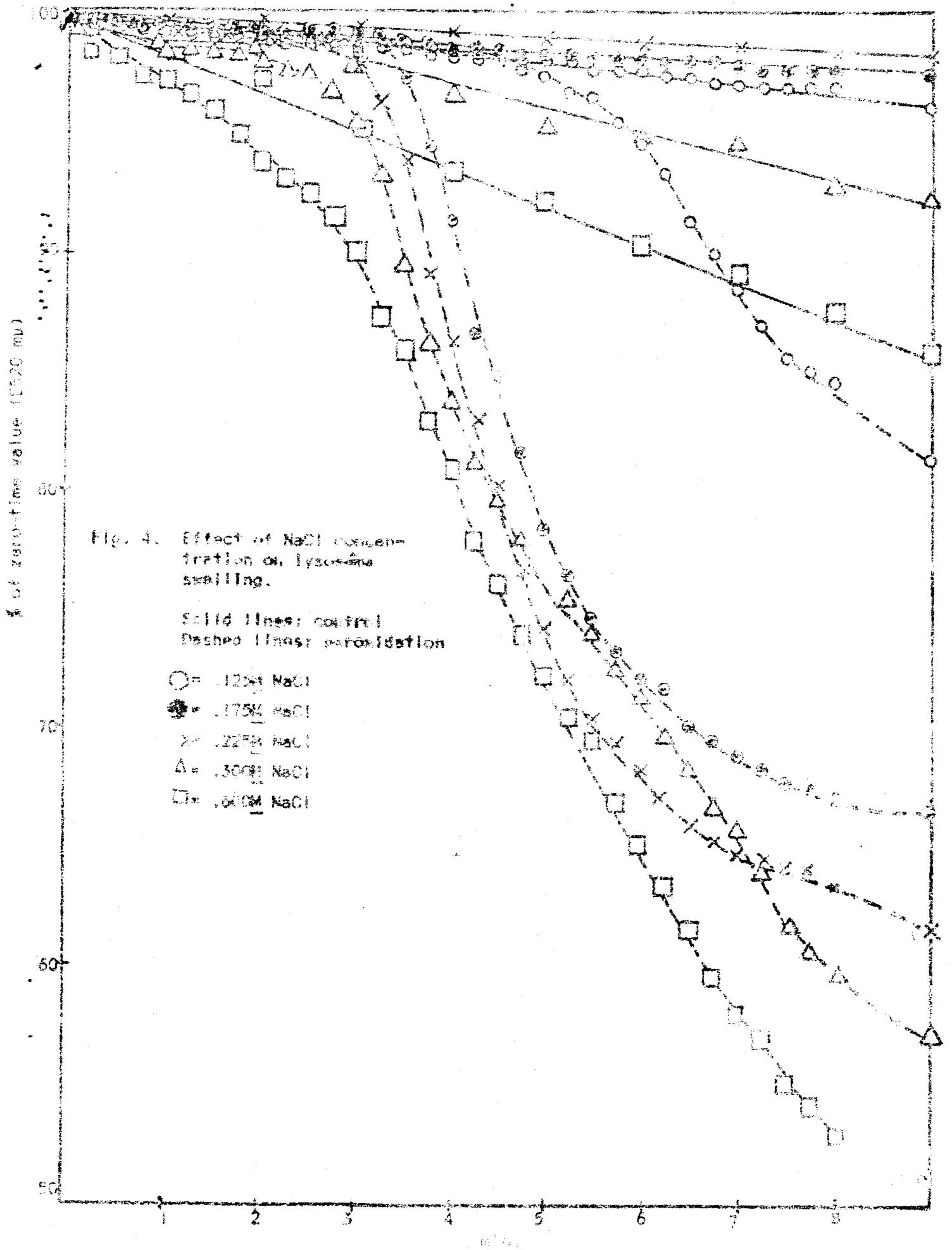


Fig. 4. Effect of NaCl concentration on lysozyme swelling.

Solid lines: control
Dashed lines: peroxidation

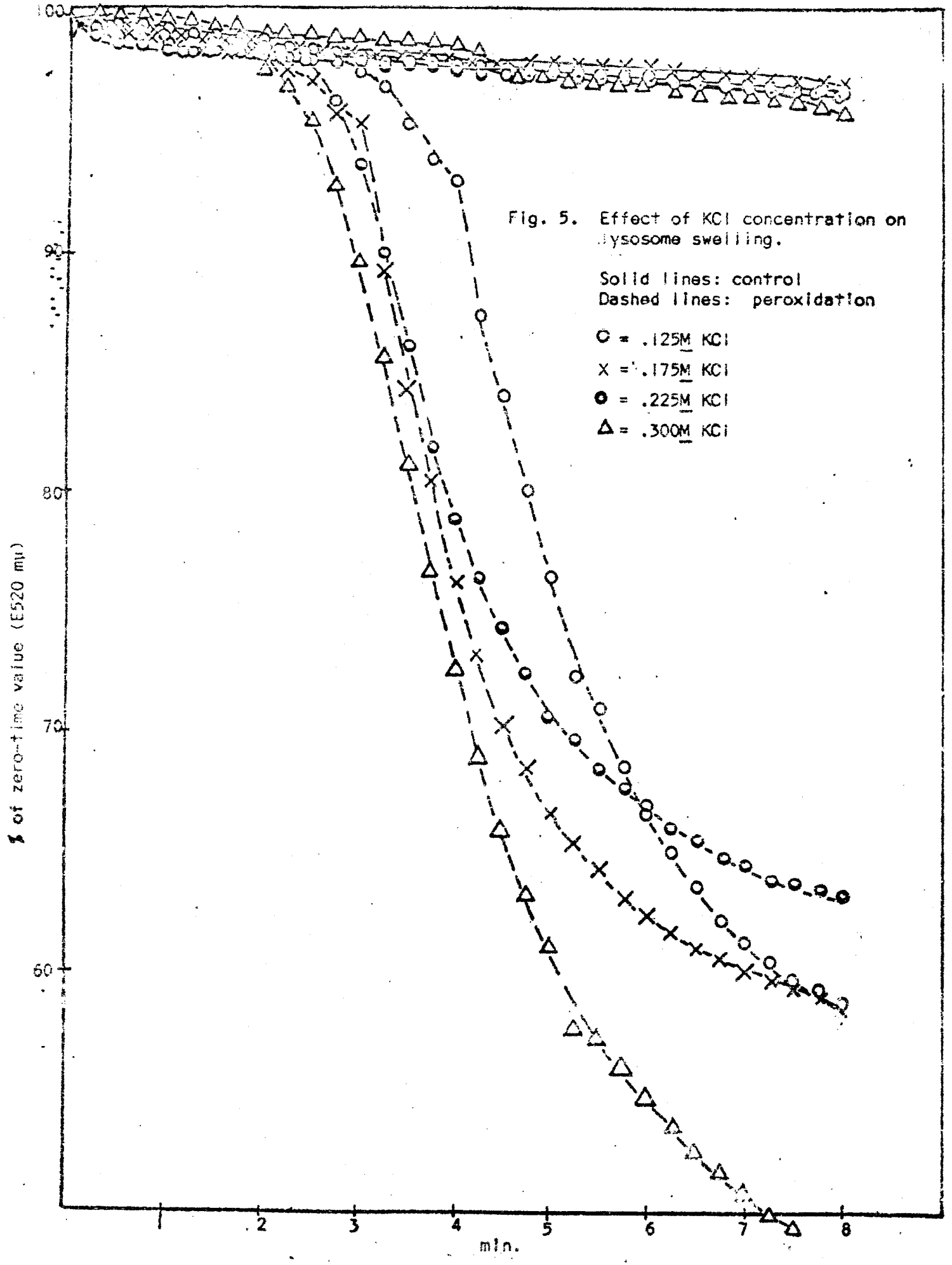


Fig. 5. Effect of KCl concentration on lysosome swelling.

Solid lines: control
Dashed lines: peroxidation

○ = .125M KCl
X = .175M KCl
● = .225M KCl
△ = .300M KCl

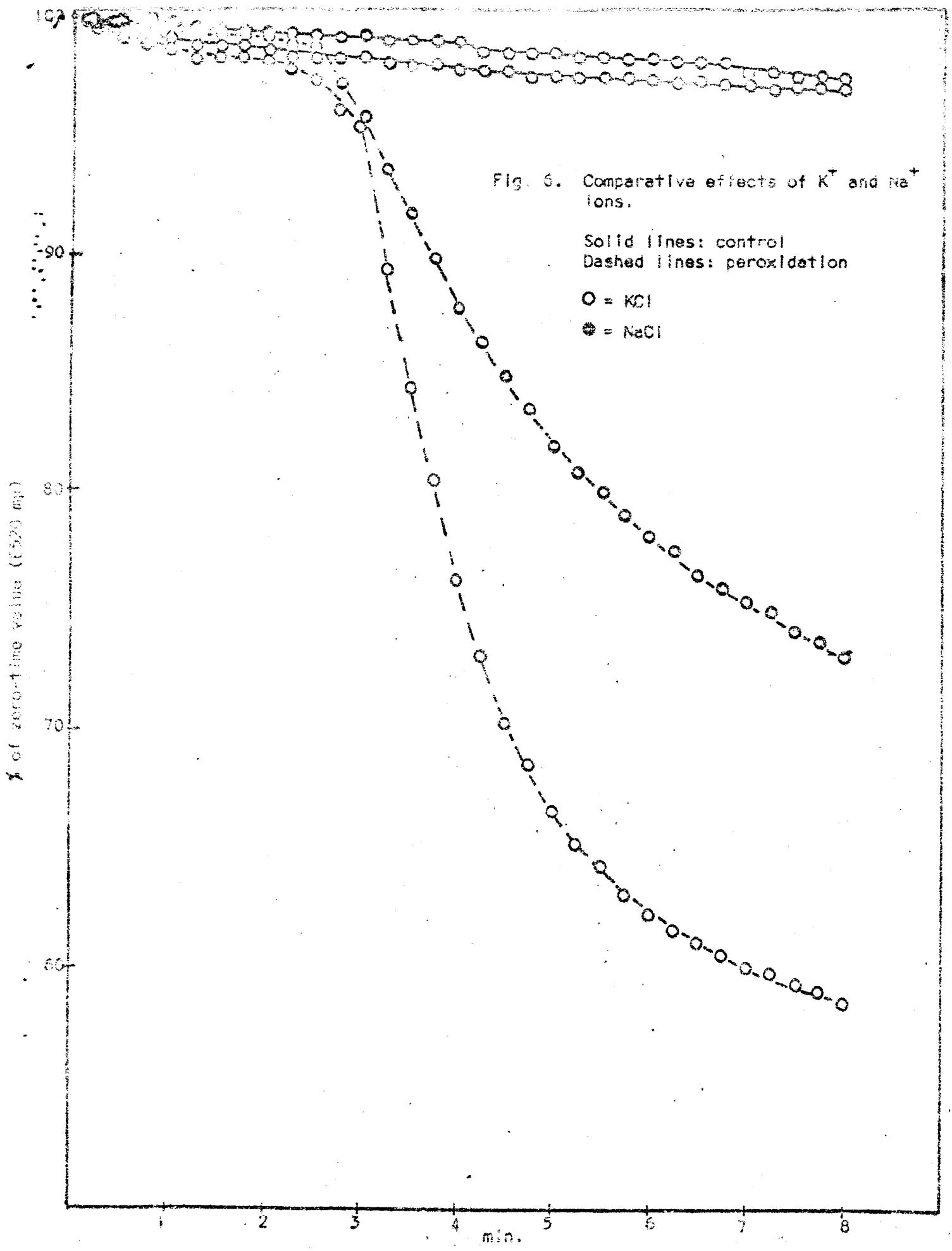


Fig. 6. Comparative effects of K^+ and Na^+ ions.

Solid lines: control
Dashed lines: peroxidation

○ = KCl

● = NaCl

Fig. 7. Gel-filtration separation of glycine-malonaldehyde adduct from glycine and malonaldehyde. 2-^{14}C-glycine was reacted with 10^{-3} M malonaldehyde at pH 7.0.

Circles refer to the absorbance due to the TBA reaction (malonaldehyde content); solid circles to the ^{14}C-content.

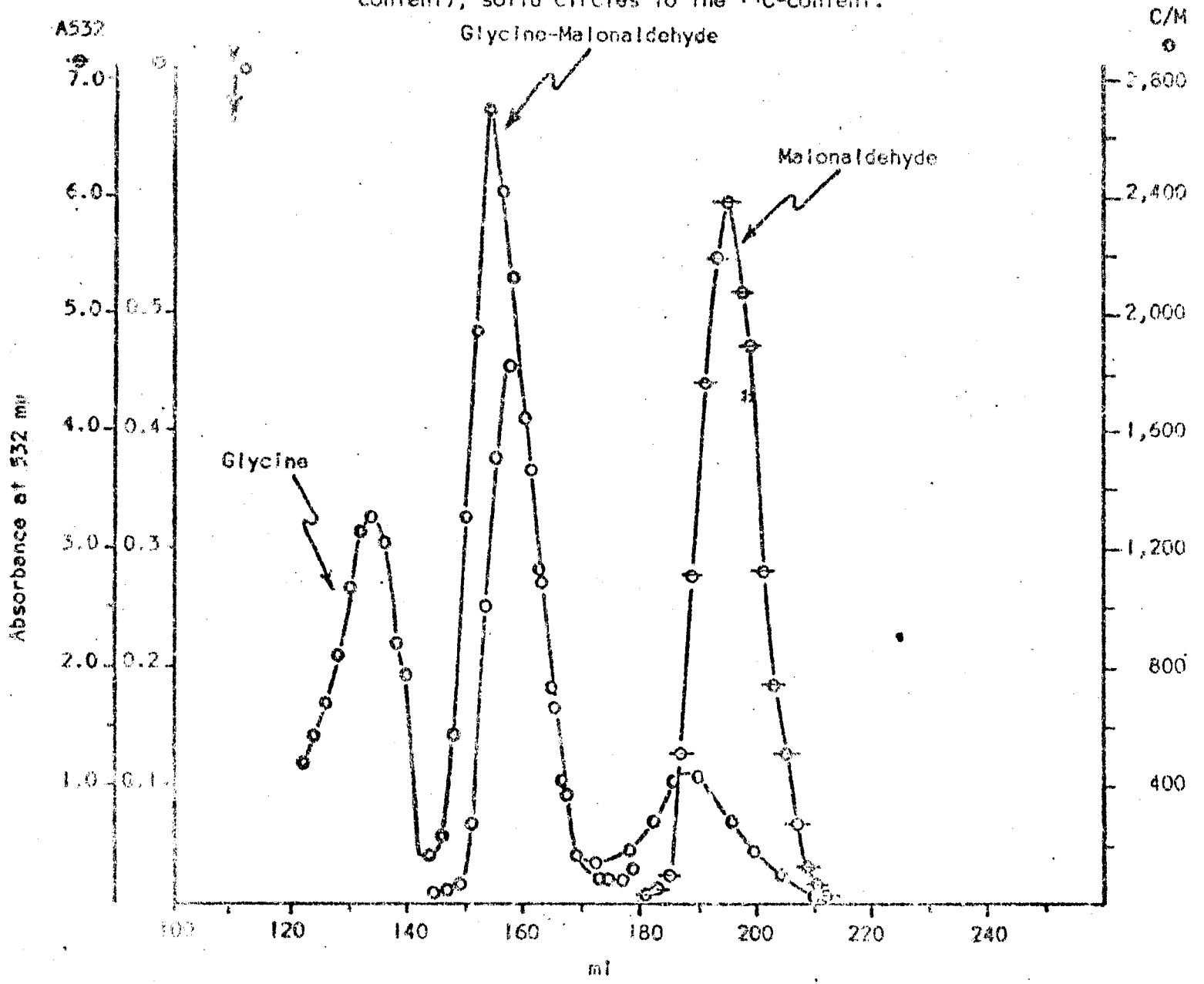


Fig. 8. Polymerization of RNase by formaldehyde at pH 7.6. Gel-filtration separation according to the molecular weight of the polymers.

M corresponds to the monomer; D, dimer; and T, trimer.

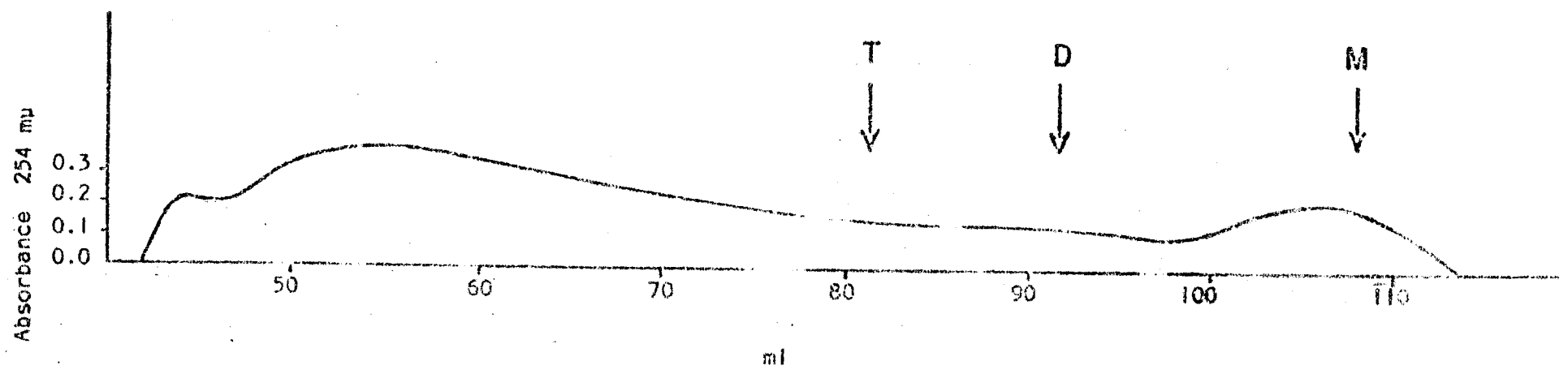


Fig. 9. Polymerization of RNase by malonaldehyde at pH 4.0.
Gel-filtration separation according to molecular weight.

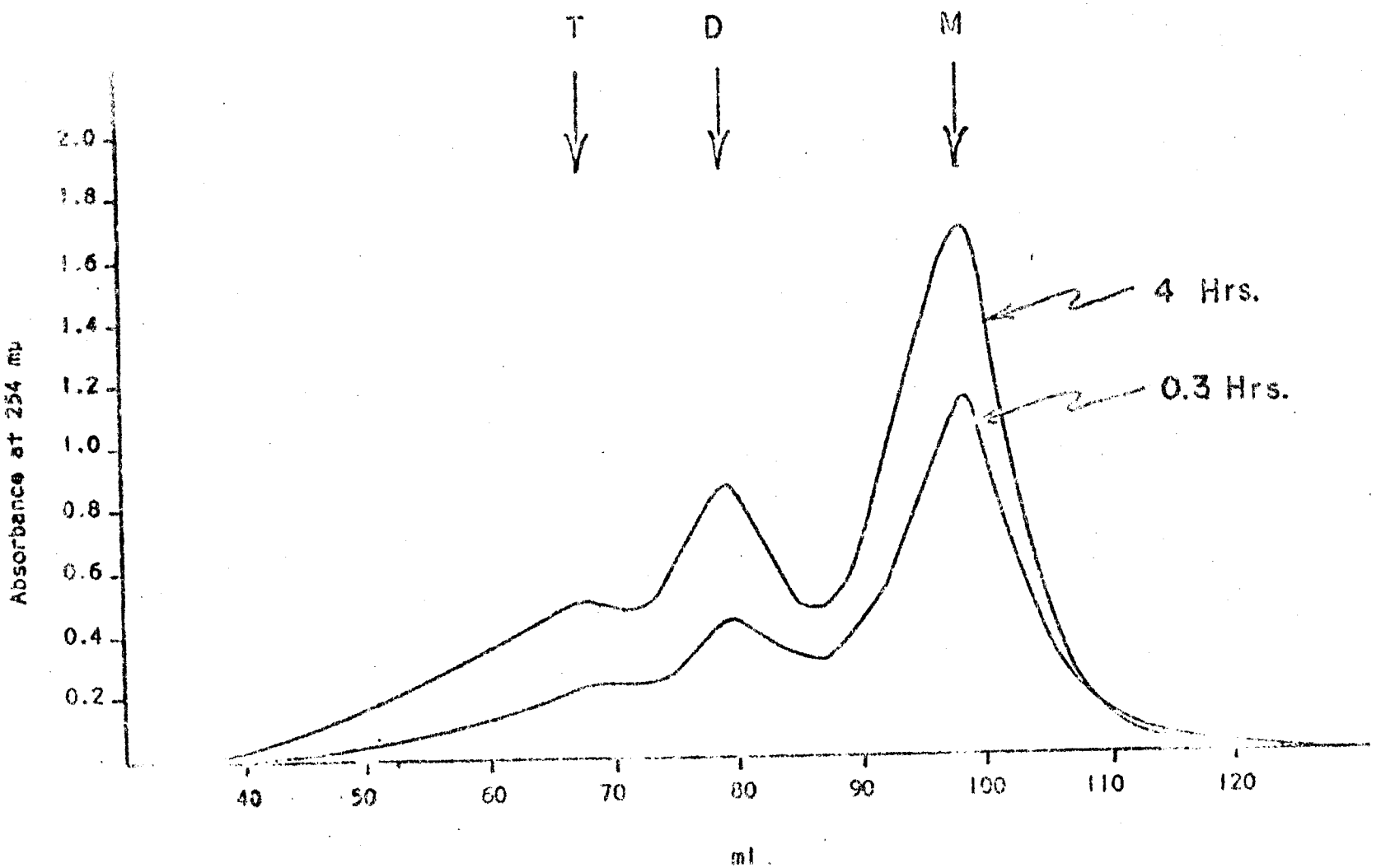


Fig. 10. Polymerization of RNase by malonaldehyde at pH 7.6. Gel-filtration separation according to molecular weight.

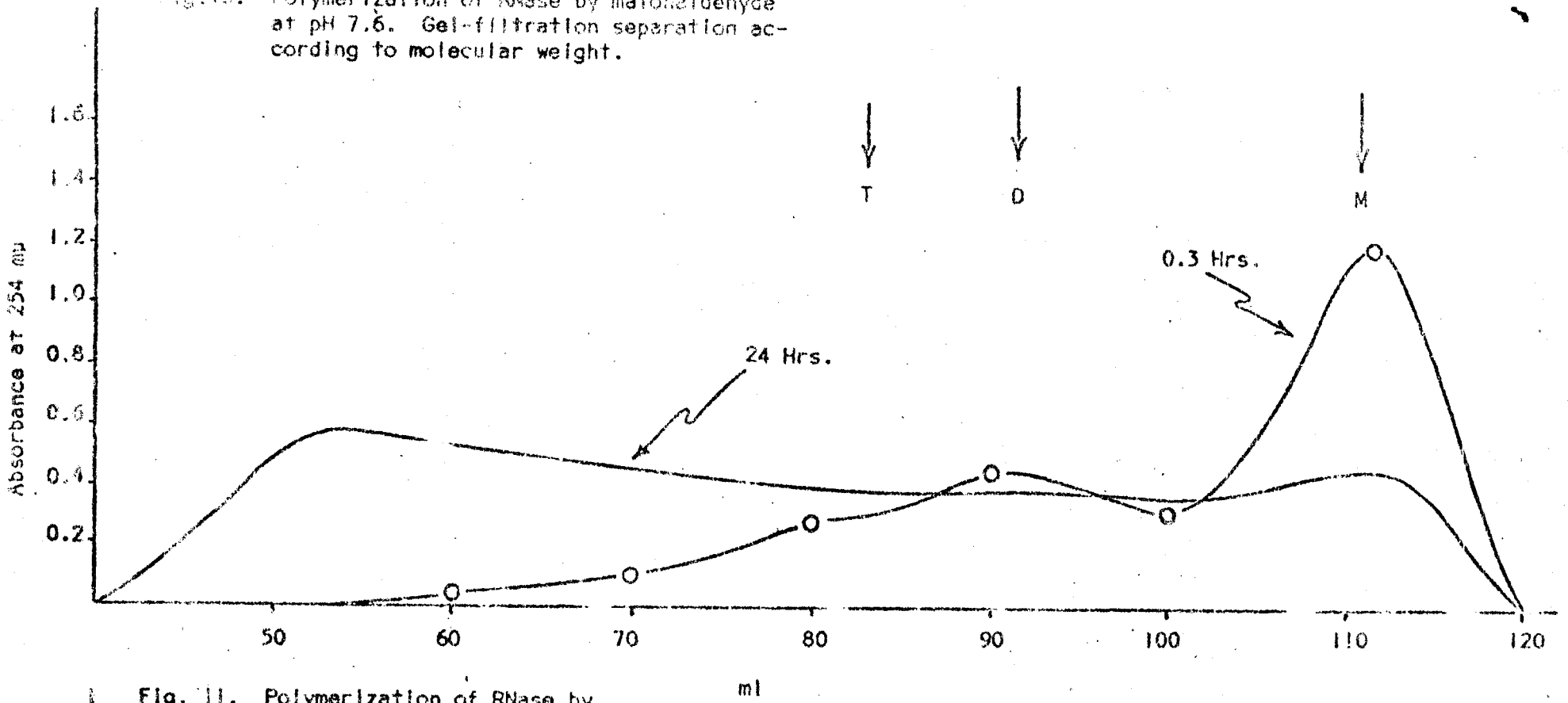
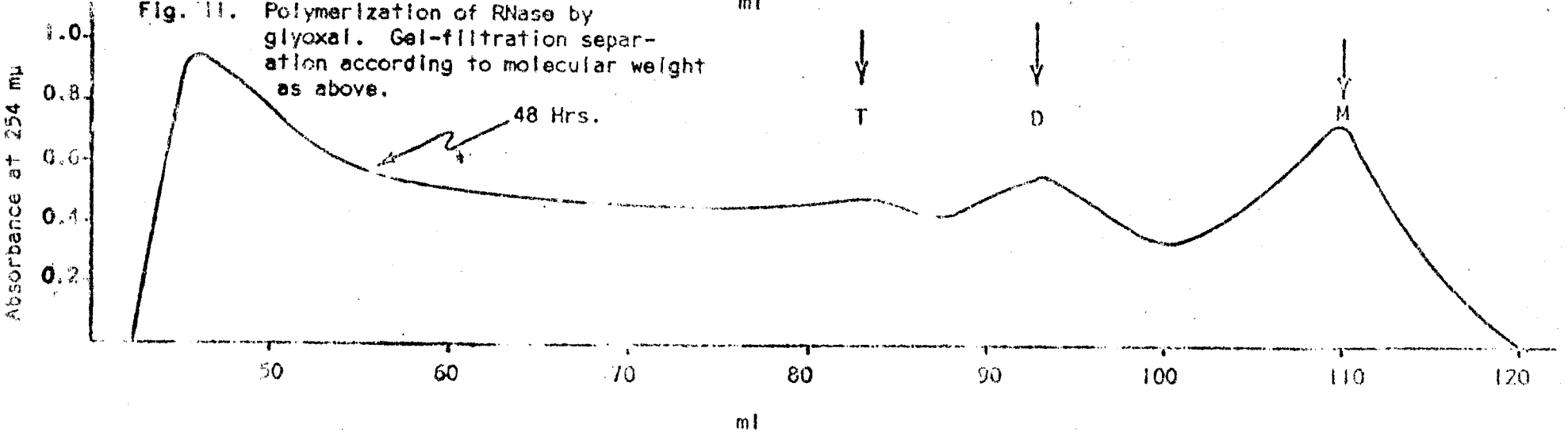


Fig. 11. Polymerization of RNase by glyoxal. Gel-filtration separation according to molecular weight as above.



APPENDIX I

TITLE: Lysosomal Enzymes In Rats Exposed To 100 Per Cent Oxygen

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RUNNING TITLE: Lysosomes and 100% Oxygen

ABSTRACT

Brain, liver and lung tissue homogenates from rats exposed to 600 mm Hg 100 per cent oxygen for 4, 14, and 28 days were assayed for free and total lysosomal aryl sulfatase. Brains of exposed rats showed lower total activity at 4 days, and lower free activity at 28 days than did controls. However, lungs of exposed rats showed significantly higher total activity than controls at 4 and 28 days. Livers of exposed rats showed no significant difference from controls at any time.

Effects of exposure to 760 mm Hg 100 per cent oxygen for 28 days on aryl sulfatase activity did not appear to differ from those of 600 mm Hg. However, studies using a lysosome-enriched pellet from the liver homogenate showed liver lysosomes of rats exposed to 760 mm Hg oxygen to be initially more labile to osmotic shock than those of controls, indicating subtle changes have occured in the lysosomal membrane.

INTRODUCTION

The use of 100 per cent oxygen atmospheres in life support systems has prompted considerable research into its possible undesirable side effects. Considerable speculation exists as to the role of lipid peroxidation in oxygen toxicity (7, 17). Peroxidation is known to cause cleavage of polyunsaturated fatty acids *in vitro* (2). This lipid comprises a major portion of the membranes vital to the normal functioning of the cell.

In 1955, DeDuve postulated the existence of the lysosome, a subcellular organelle containing the acid hydrolases of the cell within a single membrane (1). These enzymes are released by agents which disrupt the membrane: detergents, sonication, lipases, and proteases. While the normal function of lysosomes appears to be the distruction of dead or injured cells (1) and the resorption of useless appendages (15), they have also been strongly implicated in the pathology of muscular dystrophy due to dietary antioxidant deficiency (16).

In view of the possibility of accelerated *in vivo* lipid peroxidation in animals exposed to 100 per cent oxygen, it was of interest to investigate the effect of 100 per cent oxygen on the lysosome. Because of its single membrane the lysosome should be particularly susceptible to disruption by lipid peroxidation, an event which would have disastrous consequences for the cell.

EXPERIMENTAL

Male albino rats, weighing 100-200 grams, were housed in cages specially adapted for regulation of atmospheric pressure and gas composition (9). Control rats were exposed to 760 mm air in all experiments. All animals received Purina stock diet and water *ad libitum*. Weight gain and food intake were measured.

The experiment at 600 mm Hg 100 per cent oxygen was designed to investigate the effects of length of exposure to pure oxygen on the lability of the lysosome. The experiment at 760 mm Hg 100 per cent oxygen was carried out to determine whether effects were more severe at a full atmosphere oxygen pressure and to study lysosome-enriched fractions for possible subtle changes.

After the designated length of exposure the rats were returned to ambient pressure and sacrificed. The brain, liver, and lung were excised and homogenized immediately in ice cold 0.25M sucrose - 1 mM EDTA, pH 7.0. The homogenates were assayed for free and total lysosomal aryl sulfatase at pH 5.0 by the method of Roy (11) with p-nitrocatechol sulfate as substrate. Total enzyme was assayed after disruption of the lysosomal membrane with 0.1 per cent Triton X-100. Protein was determined by the Miller method (8) using bovine serum albumin as standard.

The stability of lysosomes to incubation was measured with a lysosome-enriched pellet obtained from tissue homogenates by centrifugation in the Sorvall refrigerated centrifuge. The homogenate was centrifuged at 900 g. for 10 min.; the pellet was discarded. The supernatant was centrifuged at 7,000 g. for 10 min. to yield the lysosome-enriched pellet. Several preliminary experiments were performed using the lysosome-enriched pellets from liver and brain of control rats to determine the concentration of sucrose in the medium and the pH range providing the greatest stability to incubation at 37° or 0-4°C. From the data obtained the conditions were chosen for incubation of the lysosome-enriched pellets from the livers of experimental and control animals. Portions of the pellet were resuspended in 0.3M and 0.7M sucrose for incubation at pH 7.0, 37°C. The aryl sulfatase released was determined at 0, 30, and 60 min. and the results expressed as per cent of total.

Statistical analysis of the enzymatic activities obtained was carried out using the test for significance of the difference between the control and the experimental groups (4).

RESULTS AND DISCUSSION

Figures 1 and 2 show the weight gain and food intake for the oxygen exposed and control animals at 600 and 760 mm Hg. At both pressures of oxygen the exposed rats initially lost weight and decreased their food intake well below that of the control animals.

After 5-7 days food intake increased and by 14 days of exposure gain in weight had resumed. However, neither food intake nor rate of weight gain reached that of control rats by 28 days. The depressed growth occured without apparent external symptoms and yet, indicated that a pathological situation occured on prolonged exposure to elevated oxygen concentrations.

Table I summarizes the data obtained from assay of aryl sulfatase in the tissue homogenates. The percentage of aryl sulfatase free in the homogenate might be considered an indication of the amount free *in vivo* due to disruption of the lysosomal membrane or at least of relative stability of the membrane to homogenization of the tissue. As shown in Table I there is in most cases little difference in the percentage of enzyme free between exposed and control animals for any length of exposure to 600 mm Hg oxygen. The percentage free of all tissue and the total activity of brain and liver did decrease from 4 days to 28 days of exposure, but this decrease was also exhibited by the controls, suggesting it has a cause other than adaptation to breathing 100 per cent oxygen. Franklin (3) and Dingle, *et al.* (5) have observed a decrease in lysosomal proteases with increasing age. The trend toward lower aryl sulfatase activities may be similarly an age-associated decrease in general lysosomal activity.

The percentage of aryl sulfatase free in the brain homogenates of exposed is, however, significantly lower than that of controls at 28 days, although there is no difference in total activity. At 4 days, the total activity of aryl sulfatase per gram brain in the exposed animals is significantly lower than that of control animals. While the exact cause of these results is not clear, it is possible that the lower activity in the exposed rats is due to damage to free enzymes by lipid peroxides. Roubal and Tappel have observed such damage in vitro (10). These differences in activity were not obtained at 14 days, nor in rats exposed 28 days to 760 mm Hg oxygen.

In contrast, the homogenates of lungs from exposed rats show significantly higher aryl sulfatase activity than controls at 4 days and 28 days. This increased activity is also found in rats exposed to 760 mm Hg oxygen for 28 days, and parallels that seen in muscle in nutritional muscular dystrophy (16). The very high percentage free found for the lungs of control rats at 4 days is not understood. The control value at 760 mm Hg oxygen is also significantly higher than that of exposed rats. These high values are possibly caused by the relatively vigorous homogenization required by healthy lung tissue. Similarly, Dingle, et al. (5) reported that the wide variations in free proteolytic enzyme activities as percentages of total activity obtained in their rat liver and kidney lysosome studies were due to low efficiency in homogenization of these tissues.

Liver differed from brain and lung in that it failed to show significant change in free or total activity of lysosomal aryl sulfatase in the homogenate when rats were exposed to 100 per cent oxygen at either pressure.

The divergence in the response of the brain and lung to oxygen treatment may reflect the ratio of unsaturated lipids to antioxidant in the tissue, as Horwitt (6) has suggested for the relative susceptibility of tissues to lipid peroxidation. It may also reflect the different degree of exposure to oxygen. At these pressures the lung is pathologically the most severely affected tissue.

The increase in oxygen pressure to 760 mm Hg seemed to cause no increase in percentage of aryl sulfatase free in the homogenates of tissues of exposed rats over that found at 600 mm Hg after 28 days exposure. Thus, it appears that the lower pressure of oxygen is sufficient to produce any damage to lysosomal membrane, subject to measurement by this technique, which occurs at pressures up to one atmosphere.

Since the livers of exposed rats appear to be resistant to changes in total activity or percentage of lysosomal enzyme free in the homogenate, it was of interest to determine if more subtle changes in lysosomal membrane stability had occured. For this purpose a lysosome-enriched pellet, free of unknown interfering substances present in the homogenate, was obtained and several preliminary studies on control rats were carried out to determine the best conditions for the investigations of the lability of lysosomes to osmotic shock. The results of these experiments are shown in Figures 3-4. Liver lysosomes were more stable to incubation at 37°C, pH 7.0, in 0.7M sucrose, and least stable in 0.3M sucrose, as measured by release of aryl sulfatase with time (Fig. 3). Liver lysosomes were more stable in the pH range 6.0-7.0 than above or below this range (Fig. 4), and were appreciably more stable at 0-4°C than at 37°C (Table II). Similar results have also been observed by Savant et al. for liver lysosomes (12).

In order to investigate the osmotic lability of lysosomes from livers of oxygen exposed rats conditions were chosen which would give a measureable release of aryl sulfatase in an hour, but under which control lysosomes would be stable enough to allow measurement of subtle changes in lability of the lysosomes. Thus the lysosome-enriched pellets from liver homogenates were incubated at pH 7.0, 37°C, in 0.3M and 0.7M sucrose, but in both media the lysosomes from oxygen exposed rats appeared initially weaker, as shown by a higher inital percentage free. Subsequent increase in availability of lysosomal aryl sulfatase occurs at the same rate as in lysosomes from control animals, perhaps since all incubations

were carried out in air. If the release of lysosomal enzyme is complete on rupture of the membrane, the same rate of release would be expected for the remaining "healthy" lysosomes in the preparation from exposed rats as for the lysosomes from control rats. This observation is in line with that of Shibko et al. (14), that solubilization of enzymes from kidney lysosomes is all-or-none, since the electron dense matrix of the lysosomes is lost all at once, not in graded amounts. However, in the present experiment the suspended lysosome-enriched pellets were not centrifuged before measuring the aryl sulfatase activity; therefore, both increased availability of substrate to the enzyme perhaps still in an intact particle, as well as completely solubilized enzyme were measured.

These studies indicate that there is really no consistent, gross change in the percentage of a lysosomal enzyme free in the tissue homogenate caused by exposure to 100 per cent oxygen under the conditions of these experiments. Similarly, there were no gross pathological changes in the brain and liver of the animals exposed to these pressures. The lung, which showed the greatest increases in aryl sulfatase activity in these experiments, also showed the most severe pathological changes. However, the data do indicate that subtle changes have taken place in the lysosomal structure, making the organelle more susceptible to stressing factors such as osmotic shock. These changes persist after apparent adaption to oxygen exposure, as measured by resumption of growth and food intake, and may cause increased sensitivity to stressing conditions usually well tolerated by normal animals. In view of this possibility further study of lysosomal lability and possible *in vitro* and *in vivo* protective agents is under way in this laboratory.

Acknowledgements

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TABLE I. THE EFFECT OF 100 PER CENT OXYGEN (600 and 700 mm Hg) ON THE ACTIVITY OF LYSOSOMAL ARYLSULFATASE

		BRAIN					LUNG					LIVER				
			S.A.		T.A.			S.A.		T.A.			S.A.		T.A.	
Treatment	Days	%F	F	T	F	T	%F	F	T	F	T	%F	F	T	F	T
600 mm Hg Exposed	4	43.4	6.0	14.0	312.3	708.6	47.7	8.1	17.3**	493.8	891.2**	22.1	3.7	16.6	306.4	1351.5
Control		38.7	6.5	16.8*	343.3	846.6*	75.4*	5.97	88.06	482.9	635.9	32.0	4.0	12.8	344.0	1105.7
Exposed	14	41.9	6.0	15.2	278.8	693.5	39.6	8.3	22.3	438.7	1167.5	17.1	2.0	11.8	176.9	1063.1
Control		43.9	6.9	16.0	295.5	699.5	41.0	8.2	20.7	488.2	1183.7	15.8	2.0	13.5	150.2	979.8
Exposed	28	19.7	2.9	14.0	125.0	607.5	29.2	5.9*	16.6	342.3*	990.1*	11.8	0.9	7.3	79.0	690.1
Control		29.7	3.7	11.7	188.5	616.7	33.3	4.3	12.4	216.6	702.2	12.2	1.1	9.0	101.4	873.3
760 mm Hg Exposed	28	31.0	2.83	8.25	160.76	516.47	25.84	4.56**	17.78**	231.15**	920.14**	15.38	1.15	7.51	130.79	845.39
Control		32.16	3.16	9.90	161.09	506.15	31.55**	2.61	8.36	140.18	451.72	14.74	1.11	7.60	117.61	798.28

All values are means of five or six animals. Specific activity (S.A.): mu moles p-nitrocatechol released/min/mg protein. Total activity (T.A.): mu moles p-nitrocatechol released/min/gram tissue.

* = $p < .05$; ** = $p < .01$

TABLE II. EFFECT OF TEMPERATURE ON THE AVAILABILITY OF LYSOSOMAL ARYSULFATASE

	0-4°C				37°C			
Sucrose conc.	0	8 hr	24 hr	48 hr	0	0.5 hr	1 hr	2 hr
0.3 M	36*	49	54	100	36	37	42	65
0.45 M	24	38	34	78	24	32	36	56
0.7 M	21	35	25	51	21	23	25	34

* Values are expressed as per cent availability of arysulfatase. Lysosomes were suspended in 0.7 M sucrose and were incubated at 0-4°C or 37°C.

Figure 1. Weight gain and food intake of rats exposed to 600 mm. Hg 100 per cent oxygen and to air. Circles represent the oxygen-exposed animals; crosses, the air-exposed. Solid lines represent food intake and broken lines, the body weight.

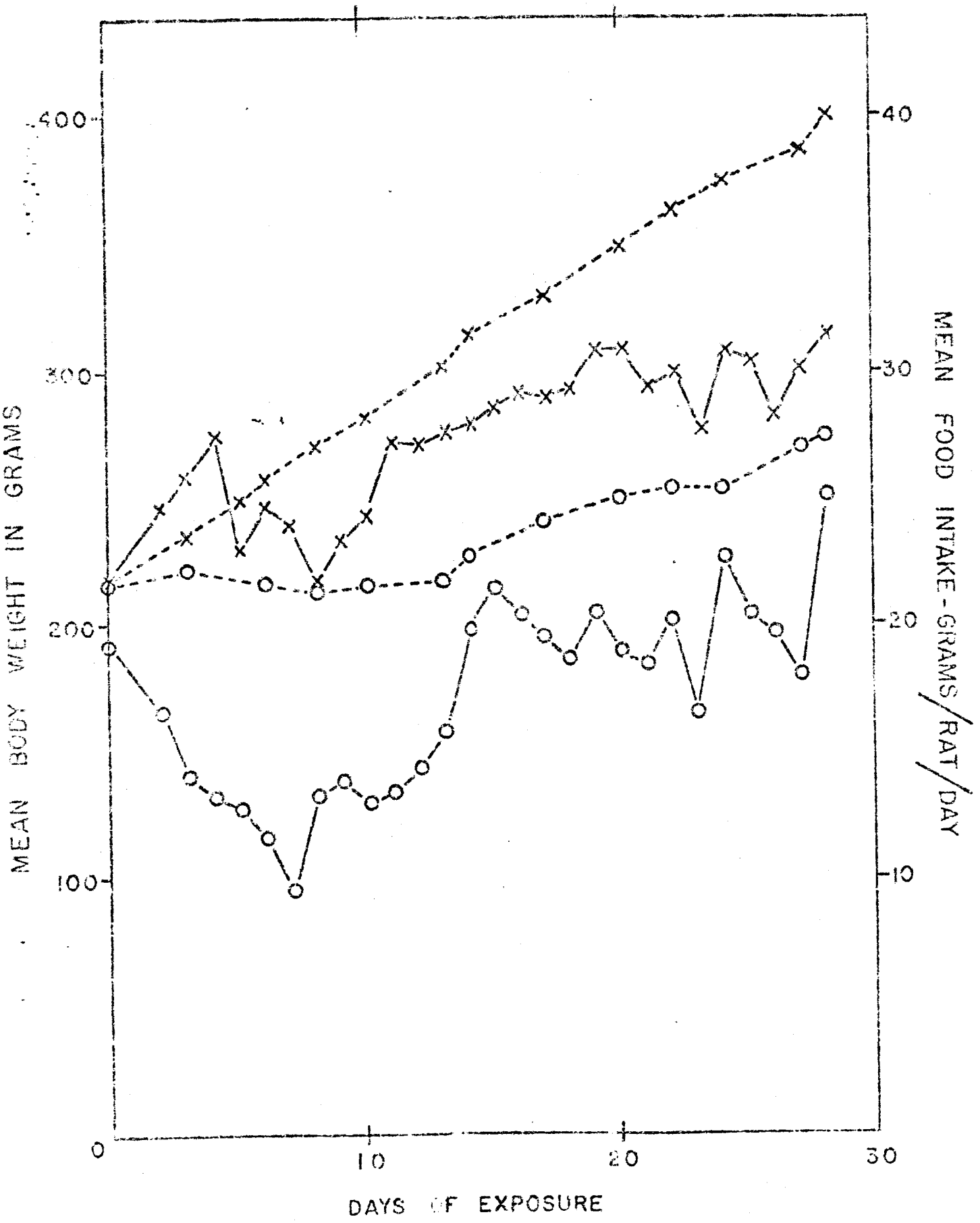

400
300
200
100
0
10
20
30
40
30
20
10
MEAN BODY WEIGHT IN GRAMS
MEAN FOOD INTAKE - GRAMS/RAT/DAY
DAYS OF EXPOSURE

Figure 2. Weight gain and food intake of rats exposed to 760 mm. Hg 100 per cent oxygen and to air. Circles represent the oxygen-exposed animals; crosses, the air-exposed. Solid lines represent food intake; broken lines, the body weight.

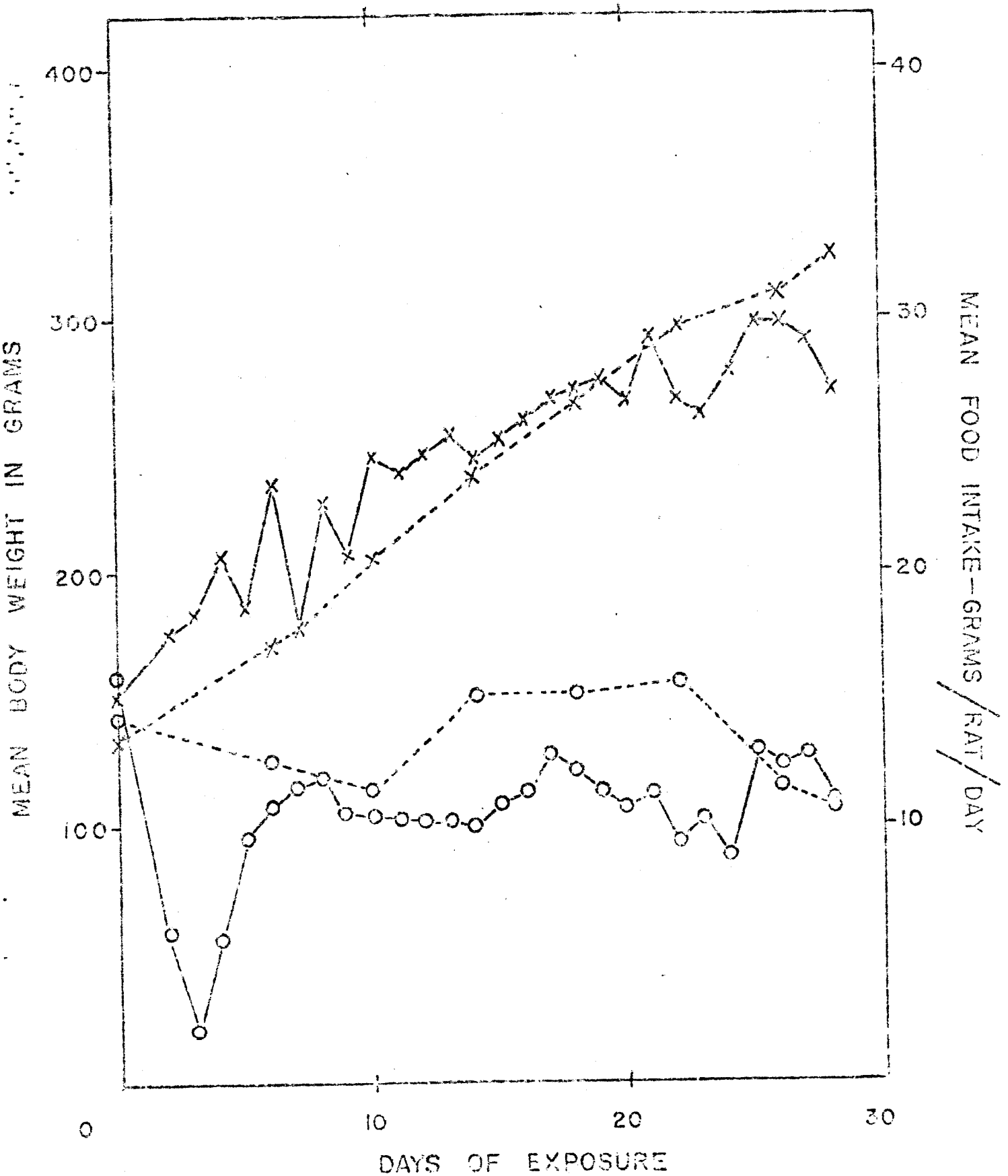

MEAN BODY WEIGHT IN GRAMS
400
300
200
100
0
10
20
30
DAYS OF EXPOSURE
40
30
20
10
MEAN FOOD INTAKE—GRAMS / RAT / DAY

Figure 3. The effect of sucrose concentrations on the stability of rat liver lysosomes. Lysosomes were suspended in 0.3M (Δ); 0.45M (X) and 0.7M (●) sucrose. See text for details.

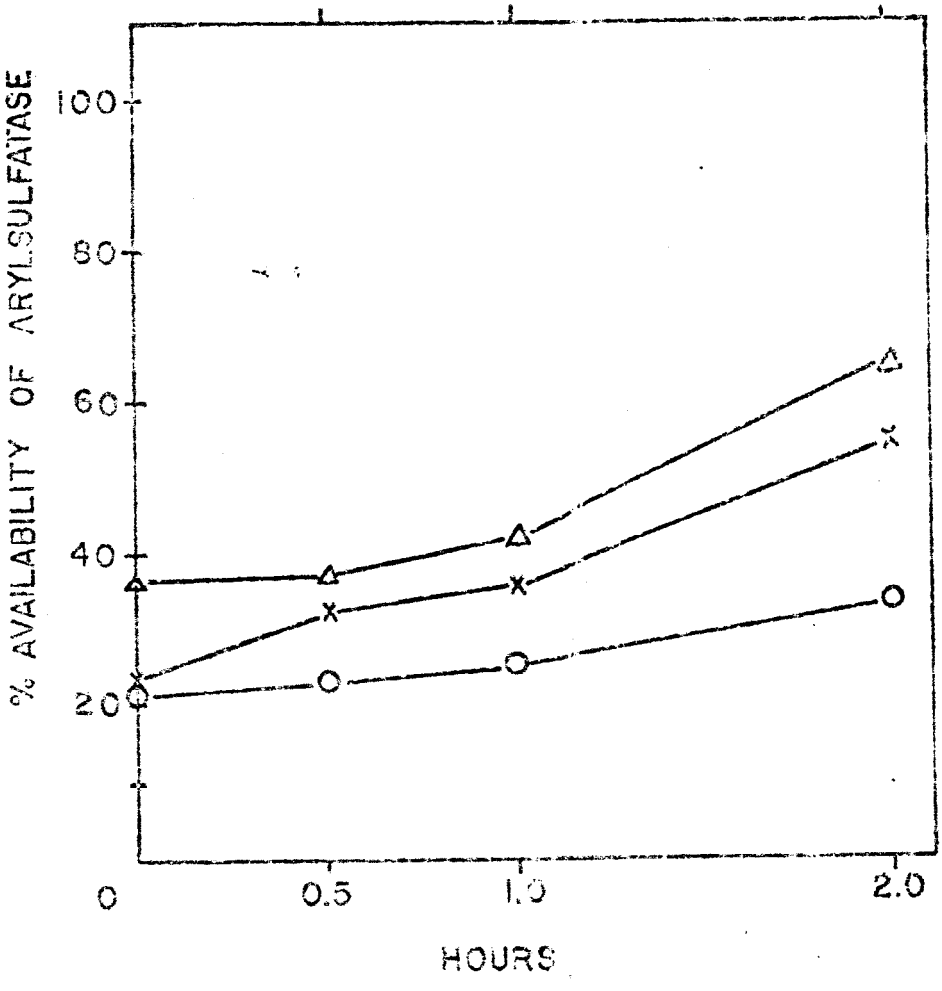

% AVAILABILITY OF ARYLSULFATASE
100
80
60
40
20
0
0.5
1.0
2.0
HOURS

Figure 4. Liability of rat liver lysosomes as a function of the pH. Lysosomes were suspended in 0.7M sucrose containing buffer of the indicated pH and incubated at 37°. From pH 4.4 - 5.2, 0.02M acetate buffer was used; from pH 5.6 - 8.4 , 0.02M Tris-maleate buffer was used. Solid circles represent the "zero" time values; crosses, 0.5 hr. and triangles, 2 hr.

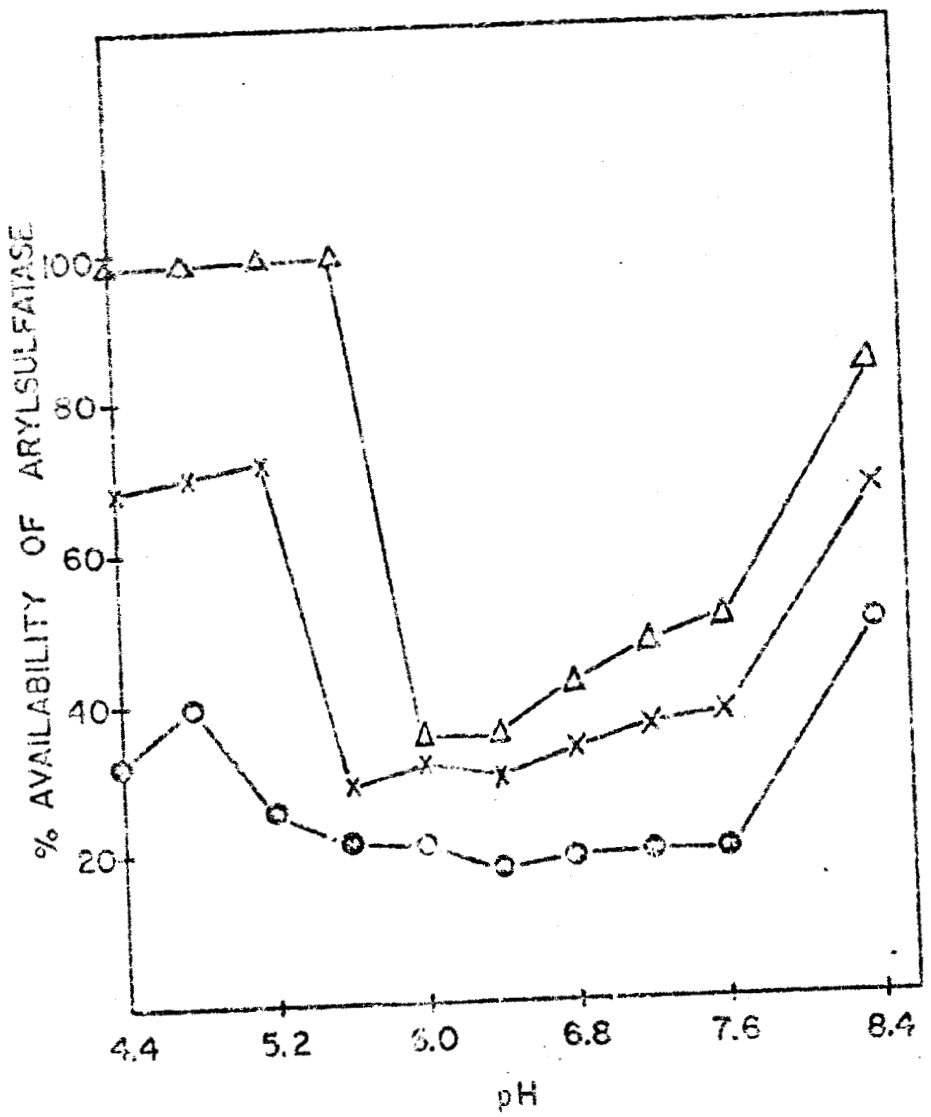
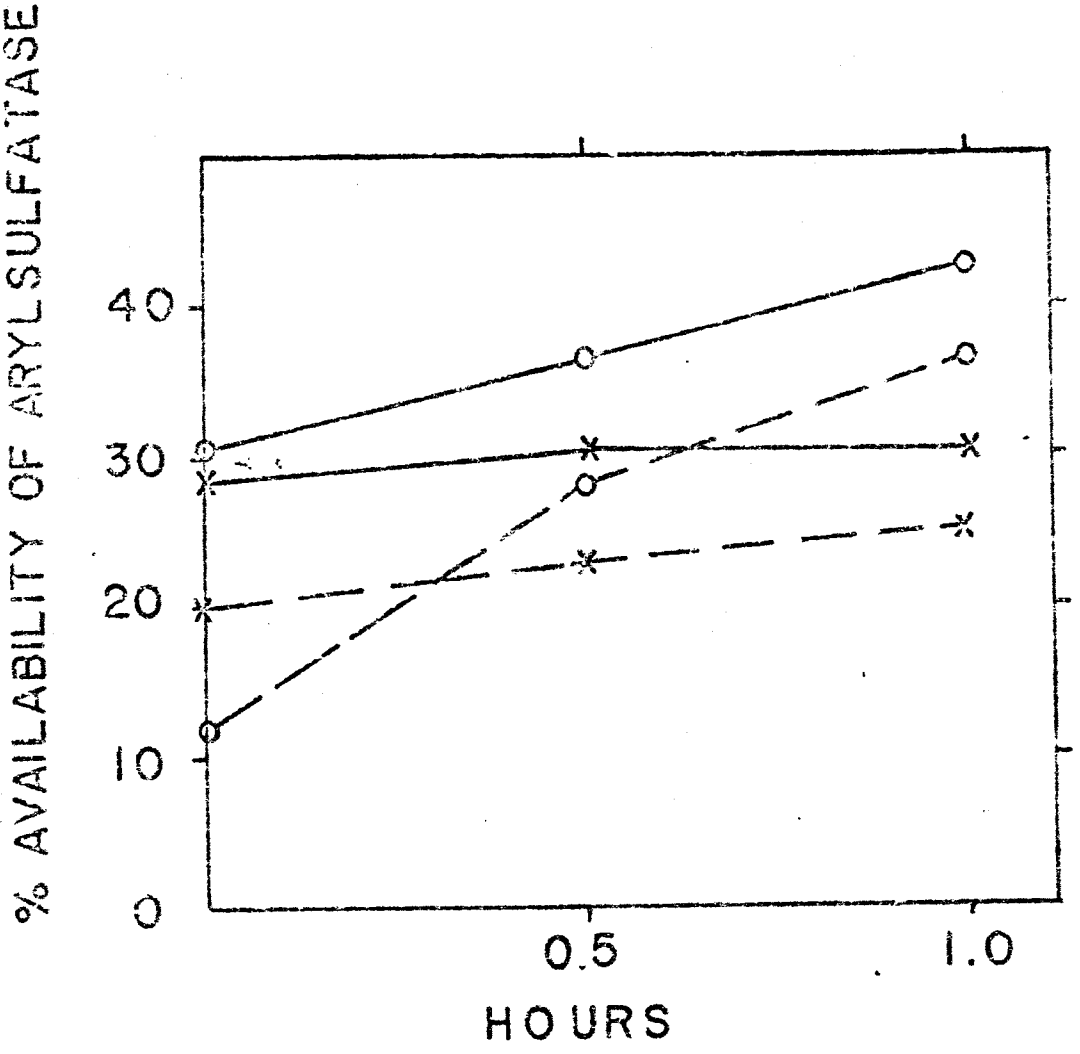

% AVAILABILITY OF ARYLSULFATASE
100
80
60
40
20
4.4
5.2
6.0
6.8
7.6
8.4
pH

Figure 5. Release of arysulfatase from lysosomes of oxygen and air- exposed rats. Lysosomes were suspended in either 0.3M (closed circles) or 0.7M (crosses) sucrose. Oxygen-exposed values are connected by solid lines; air-exposed, by broken lines. Incubation was carried out in air at 37°.


% AVAILABILITY OF ARYLSULFATASE
0
10
20
30
40
0.5
1.0
HOURS

APPENDIX II

COMPARATIVE STABILITY OF RAT LIVER AND BRAIN LYSOSOMES

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The lysosome, a subcellular organelle containing acid hydrolases of the cell within a single membrane, was first described by DeDuve in 1955 (1). Since then, the existance of the lysosome in many animal tissues has been well established (2-9).

It was suggested that the function of the lysosomal membrane is mainly to act as a barrier between the lysosomal acid hydrolases and their substrates (1). Hence the intact lysosomal particles are inactive enzymatically on external substrates unless the membrane is disrupted. Therefore, it was of interest to study the stability of these subcellular particles.

The present communication describes comparative studies of the effects of osmotic pressure, temperature, and hydrogen ion concentration of rat liver and brain lysosomal membranes.

Lysosome-enriched pellets were obtained from rat liver and brain as previously described (11). Arylsulfatase was chosen as an indicator of lysosomal hydrolytic activities. The total and free lysosomal arylsulfatase were assayed according to the method of Roy (12) with p-nitrocatechol sulfate as substrate. Total activity was measured after disruption of the lysosomal membrane with .1% Triton X-100. Measurement of free enzyme activity was used as an index of the lability of lysosomes. The free enzyme activity over total activity was designated as the availability of the lysosomal enzyme arylsulfatase.

As shown in Figures Ia and Ib, decrease in the concentration of the sucrose concentration of the suspending medium for lysosomes from 0.7 to 0.3M resulted in increased availability of both brain and liver lysosomal enzyme arylsulfatase. The results indicated that brain lysosomes were somewhat more susceptible to osmotic pressure changes. Thus, only about 34 per cent of arylsulfatase was released from liver lysosomes while about 58 per cent was released from brain lysosomes after the incubation of lysosomes in 0.7M sucrose at pH 7.0 and 37°C. Increasing the sucrose concentration to 1.2M did not reduce the lability observed at 0.7M. Similar results for the liver lysosomes were also reported by Swant et al. (13). These authors suggested that the instability of lysosomes at reduced osmotic pressure might be due to decreased membrane packing.

Results of the studies on the effect of temperature on lysosomal lability are presented in Table I. Availability of both liver and brain lysosomal enzyme was increased with increased temperature of the incubation medium. The data indicated that lability of lysosome was dependent upon osmotic pressure as well as upon incubation temperature. Here again brain lysosomes showed higher per cent of aryl sulfatase availability under the same conditions. Similar effects of temperature on lysosomes were found by Berthet, et al. (14) using acid phosphatase as an indicator enzyme, and by Swant et al. (13) using arylsulfatase, acid phosphatase, and ribonuclease as indicator enzymes.

Lability of brain and liver lysosomes over a pH range of 4.4-8.4 was studied by measuring the availability of arylsulfatase. The results are summarized in Figures 2a and 2b. Both brain and liver lysosomes showed instability below pH 6.0 and above pH 6.8 (for brain lysosomes) or 7.6 (for liver lysosomes). Instability of lysosomes under these conditions was also dependent upon the length of incubation. Liver lysosomes appeared to be most stable at the range of pH 6.0-

6.8. The most stable pH range for brain lysosomes was the same as that of liver. However, in the case of brain, the pH at which lysosomes were most stable was 6.0 while that of liver lysosome was 6.4.

The lability of lysosomes was dependent upon osmotic pressure as well as upon incubation time. Both liver and brain lysosomes were found to be most stable at a pH range of 6.0-6.8. Brain lysosomes seemed to be more labile than that of liver under the same experimental conditions. The pH stability range of brain lysosomes was narrower on prolonged incubation. Brain lysosomes were also less stable at 1.2M than at 0.7M sucrose. These results, indicating the fragility of brain lysosomes, may explain in part the low yield reported by others attempting purification by sucrose density gradient centrifugation (9).

This work was supported in part by Grant No. NGR-05-003-090 from the National Aeronautics and Space Administration.

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TABLE I

Effect of Temperature on the Availability of Lysosomal Arylsulfatase

Temp.	Sucrose Conc.	Liver (hrs) 0	.5	1	2	8	24	48	Brain (hrs) 0	.5	1	2	8	24	48
0-4°C	0.3M	36*				49	54	100	36				62	76	97
	0.45M	24				38	34	78	38				75	86	86
	0.7M	21				35	25	51	34				67	71	74
37°C	0.3M	36	37	42	65				36	62	84	99			
	0.45M	24	32	36	56				38	45	84	98			
	0.7M	21	23	25	34				34	47	64	58			

* Values were expressed as per cent availability of arylsulfatase. Lysosomes were suspended in 0.3, 0.45, and 0.6M sucrose and were incubated at 0-4°C or 37°C.

Fig. 1a. Effect of sucrose on the stability of liver lysosomes. Lysosomes were suspended in 0.3, 0.45, or 0.7M sucrose and incubated at 37°C. For enzyme assays the substrates were suspended in the above three media.

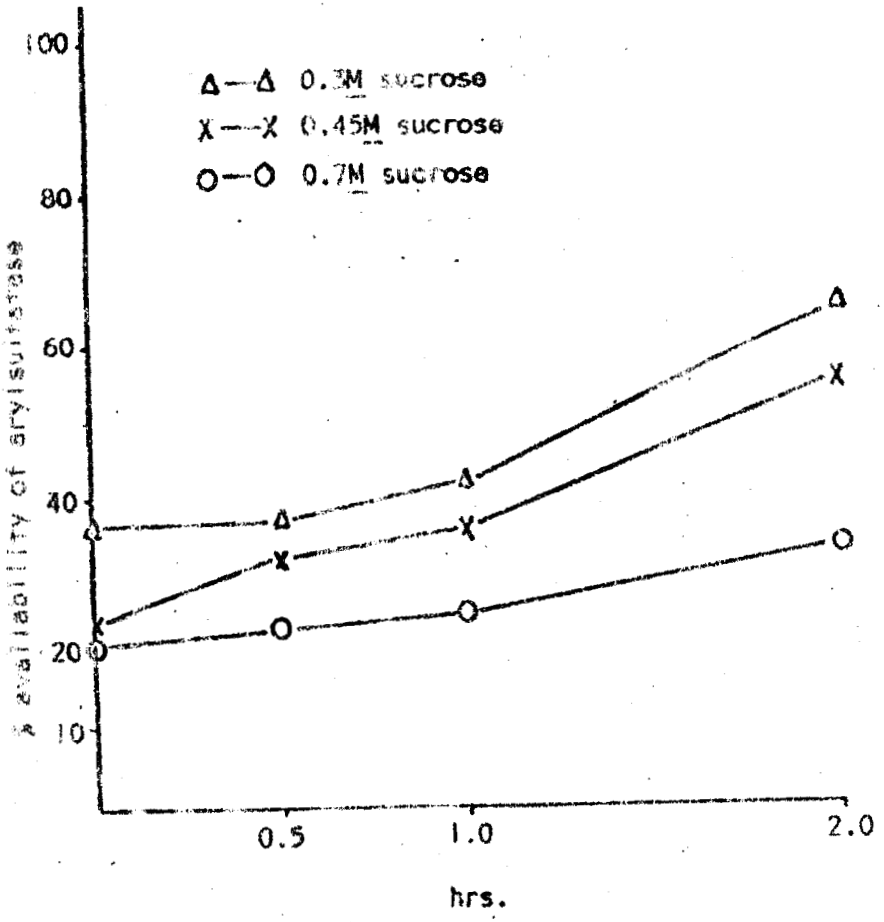


Fig. 1b. Release of arylsulfatase from brain lysosomal fraction at 37°C.

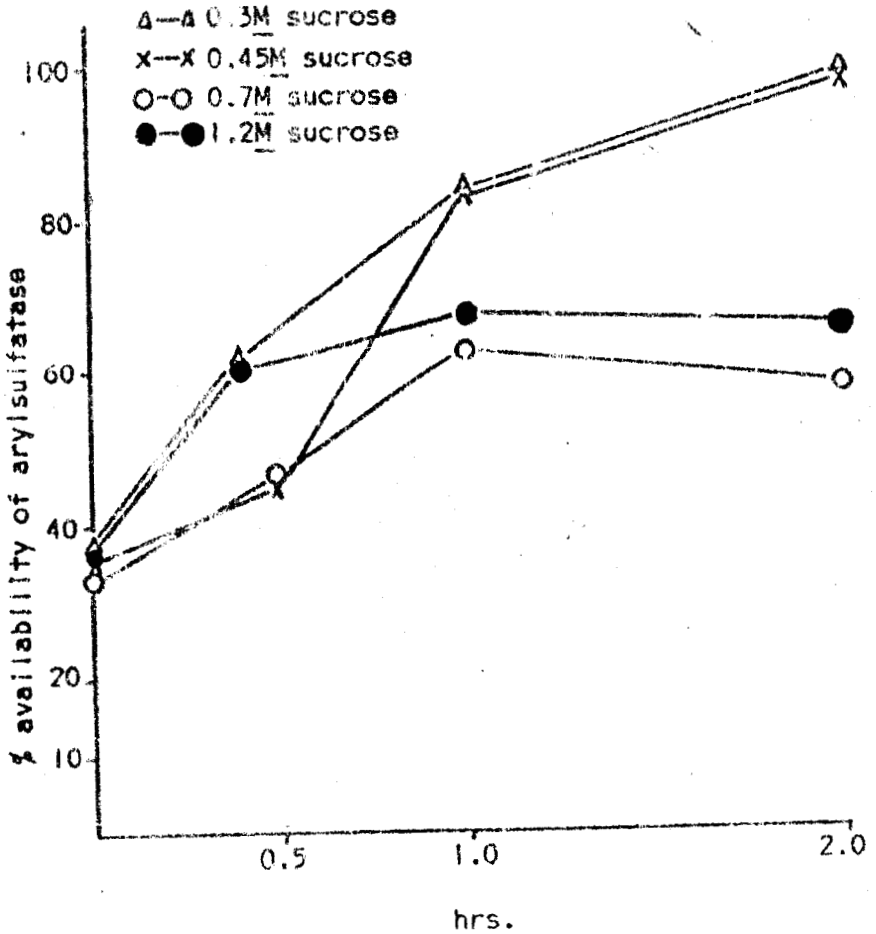
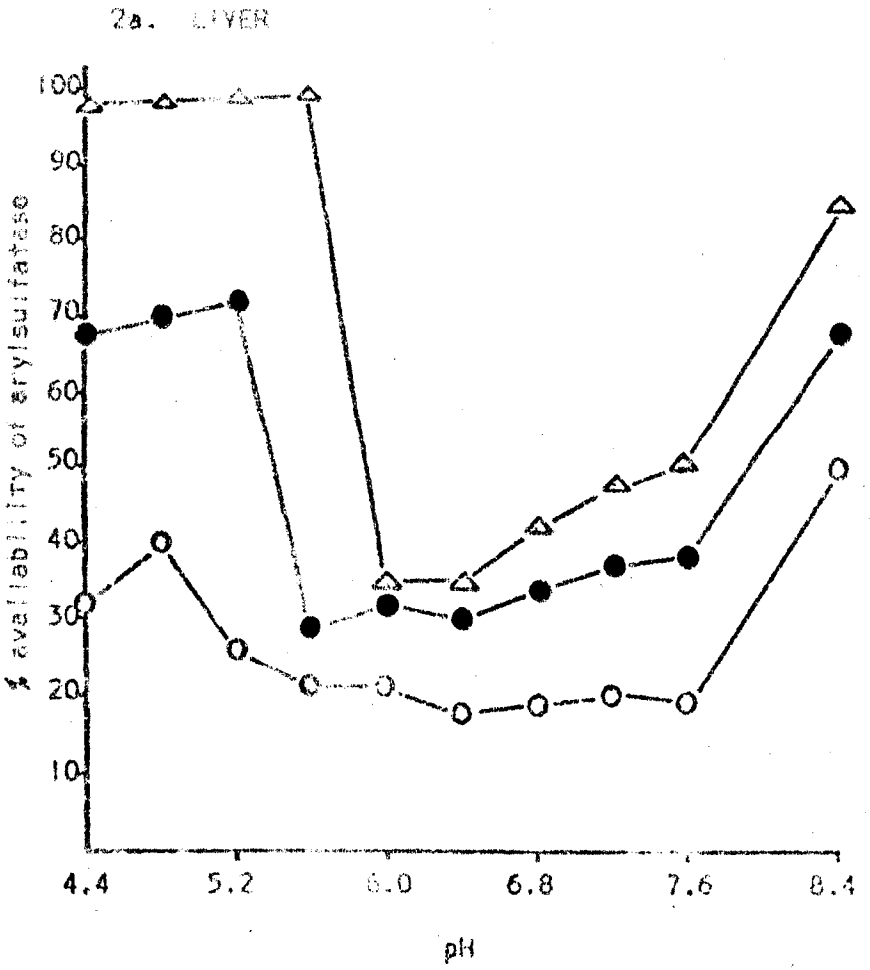
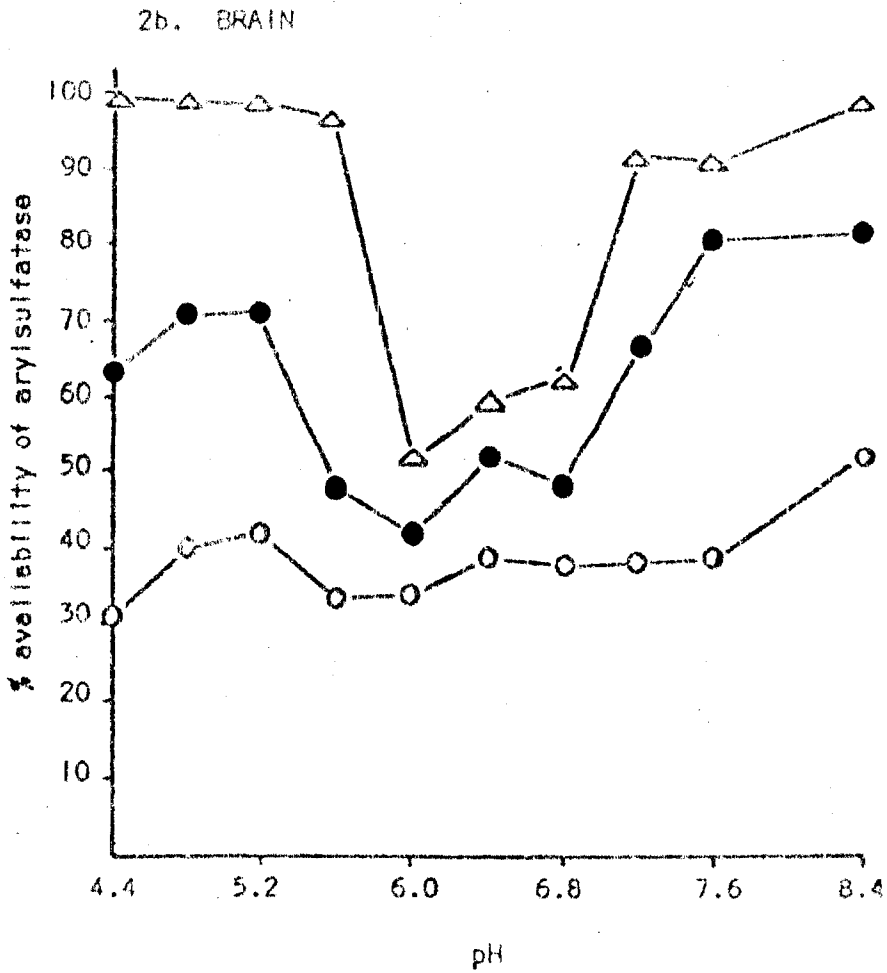


Fig. 2a-2b. Liability of lysosomes as a function of pH. Lysosomal suspension in .7M sucrose containing buffer of respective pH (for pH 4.4-5.0, .02M acetate buffer and for pH 5.6-8.4, .02M tris-maleate buffer) were incubated at 37°C for:

○—○, 0 hr.; ●—●, .5 hr.; Δ—Δ, 2 hr.





APPENDIX III

REACTION OF OXIDIZING LIPIDS WITH RIBONUCLEASE

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The oxidation of polyunsaturated fatty acids produces several 2-thiobarbituric acid-reactive substances (TBRS) of which malonaldehyde appears to be the principal one (1). Kwon et al. (2) have described the reactivity of malonaldehyde (MA) with food constituents and the isolation of a myoglobin-TBRS reaction product from frozen tuna red muscle. Packer et al. (3) have reviewed the subject of oxidation of polyunsaturated fatty acids as a mechanism of biological membrane disruption. They have placed considerable importance on the free radical mechanism of protein damage resulting from the formation of lipid hydroperoxide and other free radicals during oxidation (see also 4,5). While free radicals arising from lipid oxidation may react with membrane proteins, we wish to point out that the TBRS, also products of the oxidation, are in themselves reactive and that TBRS react with proteins and cause their polymerization.

The oxidation of linolenic acid (0.46 g) (Hormel Foundation) was allowed to proceed spontaneously at 30° in the presence of 0.22 g of bovine pancreatic ribonuclease (RNase) (Sigma Chemical Co.) in 2.5 ml of 0.1M phosphate buffer, pH 7.6 in a standard Warburg apparatus. The reaction was stopped when the oxygen uptake as measured manometrically reached an oxygen-to-linolenic acid molar ratio of 0.2. The reaction mixture was extracted exhaustively with cold chloroform and petroleum ether to remove free lipid components. A small amount of insoluble protein was removed by centrifugation. The resulting solution was dialyzed against glass

distilled water for 72 hours at 4° to remove free TBRS. The TBRS reacted with the RNase could only be released by hydrolysis in hot 1N HCl. The protein concentration was determined by the biuret reaction (6) and the TBRS by the 2-thiobarbituric acid reaction (7). Two ml of the yellowish dialyzate containing 43 mg of RNase was analyzed by gel-filtration on a 2 x 98 column of Sephadex G-100 as previously described (2).

RNase (0.340 g) was also reacted with 20mM MA in 5 ml of 0.1M phosphate buffer, pH 7.6, at 30° for 4 hrs. Two ml of the reaction mixture were chromatographed as above.

The relationship between the elution volume and the molecular weight of the polymers was estimated from a plot of the log of the molecular weight of known proteins vs elution volume as described by Whittaker (8). Blue dextran 2000 (Pharmacia Co.) was used to determine the void volume; RNase, trypsin, pepsin, ovalbumin (Sigma), and aldolase were used to calibrate the column.

Figure 1A illustrates the gel-filtration of unreacted RNase, while Figure 1B illustrates that of the RNase reaction product isolated from the oxidized lipid mixture. The elution volumes of the three components correspond to molecular weights of 14,000, 28,000, and 42,000, as would be expected for the monomer, dimer, and trimer of RNase.

While the unreacted RNase containing no aggregates that reacted with TBRS was mostly dimer; some polymers of molecular weight greater than 42,000 were also present. The main component (28,000 MW) of the TBRS reacted RNase contained 0.05 moles of TBRS per mole of RNase, calculated as MA equivalents. No free TBRS were observed. Figure 1C illustrates the gel-filtration of RNase reacted with MA alone. Components corresponding to molecular weights of the monomeric, dimeric and trimeric RNase were found. A small amount of protein of molecular weight greater than 42,000 was also present.

Since the principal TBRS is malonaldehyde (1), it is probably that malonaldehyde accounts for most of the TBRS reacted with RNase. Malonaldehyde alone at 20M concentration produced polymeric forms of RNase similar to those observed on lipid oxidation. The reaction of proteins with TBRS, and particularly malonaldehyde, may account for some of the biological consequences currently ascribed to the free radicals produced by oxidation of polyunsaturated fatty acids (3). TBRS reacted proteins may be of biological importance as a possible source of the "age pigment" as discussed by Bjorksten (4).

This work was supported in part by grant no. NGR-05-003-090 from the National Aeronautics and Space Administration.

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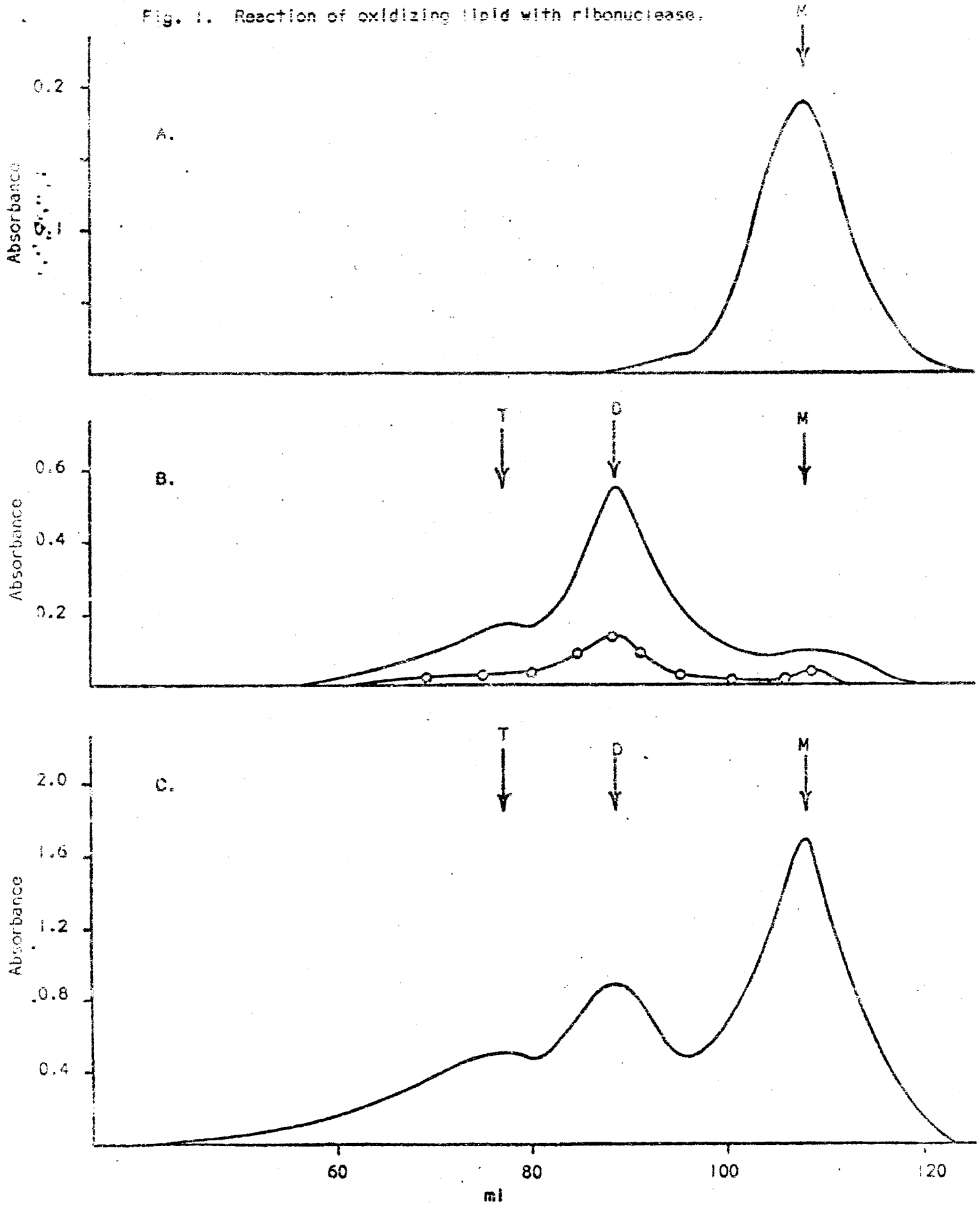
Fig. 1. Reaction of oxidizing lipid with ribonuclease.



APPENDIX IV

TITLE: Reaction of Malonaldehyde with Proteins and Amino Acids. I. Bovine Serum Albumin[1]

RUNNING TITLE: Malonaldehyde and Serum Albumin

AUTHORS: T. W. Kwon, S. C. Fung[2], and D. B. Menzel

FROM: Institute of Marine Resources, Department of Nutritional Sciences, University of California, Berkeley, California

1. This research was supported in part by Grant GM-09899 from the National Institutes of Health, by Grant SC NGR-05-003-090 from the National Aeronautics and Space Administration and from funds administered jointly by the University of California and the Bureau of Commercial Fisheries, U. S. Department of the Interior.

2. California Heart Association Summer Fellow, 1964.

SUMMARY

Malonaldehyde combines with bovine serum albumin to form a stable product. Evidence of product formation was found from dialysis and starch-gel electrophoresis of the reaction product. The reaction product was further characterized by a specific absorption at 282 mμ, and by the disappearance of another absorption band of the aldehyde at 350 mμ. There appear to be 16-17 specific reaction sites in each molecule of the albumin. The reaction was pH, time, and concentration dependent: optimum pH range was between 3 and 5, and the reaction reached apparent equilibrium after 24 hrs at 30°. The reaction was first order with a rate constant of $3.6 \times 10^{-4} min^{-1}$ and the apparent association constant was determined to be $3 \times 10^{3} \underline{M}^{-1}$. Acetylation of the albumin reduced the aldehyde reaction to a large extent. The reaction is believed to involve mainly free amino groups of the protein and the carbonyl group of the aldehyde. Urea (8$\underline{M}$) and Cl^{-}(0.1-0.5$\underline{M}$) reduced the extent of reaction with malonaldehyde by about 40% and 15-30%, respectively. Simultaneous acid (0.1$\underline{N}$ HCl) and heat (100°) treatment was necessary to release quantitatively the aldehyde from the reaction product. The reaction may be of interest as a new means of masking the free amino groups of proteins since it takes place at low concentrations of aldehyde and under relatively mild conditions.

INTRODUCTION

The reaction of aldehydes with proteins has been most extensively studied with formaldehyde. Frankel-Conrat and Olcott (1-4) have shown that the free amino groups of proteins react most rapidly with formaldehyde, followed by the guanidyl, hydroxyphenyl and imidazolyl groups. Darlington and Keay (5), studying the reaction of thioformaldehyde and formaldehyde with mercuripapain, found that reaction with free amino groups was the dominant reaction with both compounds. A similar reaction was found between malonaldehyde (MA) and bovine serum albumin (BSA) in the present study. An unusual pH dependence and a higher reactivity were, however, noted with MA than has been reported for formaldehyde. Like formaldehyde, MA reacts primarily with the free amino groups of the BSA, but a certain degree of conformational specificity appears to be required which is not apparent with formaldehyde. The higher reactivity of MA is reflected in the low concentrations of MA required for reaction and in the rapidity of the reaction (10^{-5} M compared to 10^{-3} M, and 24 hrs compared to 5-7 days).

Our original interest in MA stems from recent investigations in this laboratory into 2-thiobarbituric acid-reactive substances (TBRS)(6), the oxidation products of polyunsaturated fatty acids. Kwon and Olcott (7) have shown that MA is the principal TBRS produced on the oxidation of methyl linolenate. Kwon, et al. (8) have reported the reaction of MA with food constituents, and have isolated a TBRS-myoglobin reaction product from frozen tuna red muscle. Hunter et al. (9) have utilized the production of TBRS as a measure of the extent of lipid oxidation during the irreversible swelling of mitochondria. Packer et al. (10) have demonstrated a light

dependent TBRS producing reaction in chloroplasts and have suggested that this reaction, associated with the irreversible swelling of the chloroplast, is analogous to the above observation (9). The oxidation of the unsaturated lipids of the organelles appear to be associated with the disruption of the integrity of the particles. While the reactions producing MA and other TBRS were not carried out under physiological conditions, they do serve as model systems suggesting the importance of lipid oxidation in the disruption of sub-cellular membranes. We will report elsewhere (11) that a MA-protein reaction product can be isolated from a mixture of oxidizing polyunsaturated lipids and ribonuclease. The toxicity of MA has been reported by Crawford, et al. (12).

While this report describes in detail the conditions of reaction of MA with BSA, it has become apparent to us from studies with individual amino acids that the detailed nature of the reaction is rather complex (13), since MA reacts with other side chains in proteins other than amino groups when they are available. The possible combinations for reaction are further increased by the difunctional nature of MA.

EXPERIMENTAL

BSA and 2-thiobarbituric acid (TBA) were purchased from the Sigma Chemical Co. MA solutions were prepared from acid hydrolysis of its bis-(diethyl acetal)(Kay-Fries Chemicals). All other chemicals were reagent grade and only glass distilled water was used throughout.

Salt-free mixtures of Ma and BSA solutions were adjusted to the desired pH with 1N NaOH or HCl. Final concentrations of BSA ranged from 5×10^{-5}

6A

to 1×10^{-4}M (assuming a molecular weight of 67,000 [20]); that of MA from 2.5×10^{-5} to 2×10^{-3}M. Following incubation at 30° portions were removed and dialyzed for at least 72 hrs against glass distilled water at 4°. The water was replaced at 24-hr intervals. Acetylated BSA was also reacted with MA under these conditions. Acetylation of the BSA and determination of the amino groups before and after acetylation was conducted according to the procedures of Fraenkel-Conrat (14). Protein concentrations were determined by the biuret reaction (15) and the MA concentration by the TBA reaction (5). Spectroscopic investigations were conducted with a Cary Model 15 recording spectrophotometer. Horizontal starch-gel electrophoresis of the dialyzed reaction product was conducted in 10mM succinate buffer, pH 5.4 at 5 V/cm and at 4°. Sedimentation velocity experiments were performed in a Spinco Model E ultracentrifuge equipped with a phaseplate as a schlieren analyzer. All of the sedimentation velocity experiments were conducted at a constant temperature (22-24°) with rotor speeds of 59,780 rpm. Solutions of both unreacted BSA and reaction product (6:1 MA/BSA molar ratio) were adjusted to initial protein concentrations of 7 mg/m. of 0.2M NaCl and 0.01M Tris-HCl buffer, pH 7.0. Serial weight dilutions were made from the initial solutions and the protein concentrations calculated. The observed sedimentation coefficients were corrected to values corresponding to a solvent with the viscosity and density of water at 20° ($s_{20,w}$).

RESULTS

Unreacted MA was separated easily from BSA by dialysis. Up to 14 moles of MA per mole of BSA were retained on dialysis, while control dialysis experiments showed the complete removal of the MA. Incubation for up to 80 hrs failed to increase the amount of MA reacted with BSA above that found at 24 hrs. The reaction thus appears to reach equilibrium within 24 hrs and to be essentially irreversible, between pH 3 and 11.

The absorption spectrum of the reaction product isolated in this manner showed a characteristic shift from 278 mμ, the maximum of the native albumin, to 282 mμ. The absorption spectra of BSA and the reaction product are shown in Figure 1. The increased absorbance of the reaction product at 282 mμ was proportional to the molar ratio of MA combined with the BSA (Figure 2), and may be ascribed to the formation of the reaction product. Difference spectra of native and reacted albumin were likewise altered. The base-neutral difference spectrum of the reaction product showed two maxima at 238 and 290 mμ with a ratio of 7.0, while the native albumin showed maxima at 246 and 297 mμ with a ratio of 4.9.

Starch-gel electrophoresis separated the reacted from the native BSA. Table I illustrates the relative rates of migration of the native and a series of reacted BSA preparations. Greater reaction with MA resulted in higher anionic migration.

The sedimentation properties of the reacted BSA were measured by velocity experiments with a reaction product having a molar ratio of MA to BSA of 6:1. Plots of the sedimentation coefficients ($s_{20,w}$) of the

reaction product and of the unreacted BSA against the protein concentration fell on the same line. The sedimentation coefficient extrapolated to infinite dilution ($s^0_{20,w}$) was 4.4 S for the combined data. About 5-10% higher aggregates were detected in both cases and were neglected in the calculations. The sedimentation studies indicated no conformational change in the reacted BSA. The molecular weight appears to be essentially unchanged, as there was no significant increase in the higher aggregates at the concentration of MA examined.

The stability of the reaction product was examined under the conditions of Table II. Hydrolysis in hot 90% glacial acetic acid was sufficient to recover quantitatively the MA reacted under these conditions. Such hydrolysis corresponds to the conditions required for the quantitative estimation of free MA by the TBA reaction. Prolonged incubation (10 days) of the reaction mixture reduced the quantitative recovery of MA to 73%, indicating that a secondary reaction may be occuring.

The reaction of MA with BSA was pH dependent. The molar ratio of MA reacted with BSA was highest between pH 3 and 5 and was much lower at other pH ranges (Figure 3). The addition of Cl^- to reaction mixtures also reduced the amount of MA reacted with BSA (Table III), indicating competition for the reactive sites. Acetylation of BSA markedly reduces the reactivity with MA as can be seen in Table III.

An initial mixture of MA and BSA has an absorption band at 350 mμ, characteristic of the carbonyl group of MA (16). The 350 mμ absorption band corresponds to the $n \rightarrow \pi^*$ transition of the unpaired carbonyl electrons. This carbonyl absorption band disappears as the reaction proceeds,

indicating that the carbonyl group is involved in the reaction. The absorption spectrum of the isolated reaction product is also devoid of this absorption band. Together with the stability properties of the reaction product and the reduced reactivity of acetylated BSA, these observations strongly suggest that the reaction involves the free amino groups of BSA and the carbonyl groups of MA.

The rate of reaction was followed at pH 4.0, the pH of maximum interaction, by the dialysis technique described. The reaction was relatively slow with a first order rate constant of 3.6×10^{-4} min^{-1}. The rate constant determined under dynamic conditions by following the disappearance of the MA carbonyl absorption at 350 mμ was 4.3×10^{-4} min^{-1}.

The extent of reaction was concentration dependent. Above final concentrations of 1.5mM MA a yellowish precipitate appeared, indicating either a decreased water solubility or formation of a polymeric product. The extent of reaction above this concentration of aldehyde is thus uncertain. We have shown previously that the reaction is essentially at equilibrium after 24 hrs and irreversible. Keeping these restrictions in mind and neglecting corrections for electrostatic factors, it is possible to apply the relationship derived by Klotz (17) to this reaction. This relationship is given by:

$$1/r = \frac{1}{nk(A)} + \frac{1}{n}$$

where r is the number of combined MA molecules per molecule of BSA; (A), the molecular concentration of the free MA at equilibrium; n, the number of binding or reactive sites in the BSA molecule; and k, the apparent association constant. The association constant is taken in this case to represent

not the binding of free MA to BSA but the subsequent covalent reactions resulting in the irreversible combination. A plot of 1/r vs 1/(A) gave a straight line (Figure 4). From this plot and the alternative plot of r/(A) vs r, the association constant was calculated to be $3 \times 10^3 M^{-1}$. The number of reaction sites was calculated to be 16.6 sites per BSA molecule.

DISCUSSION

Formaldehyde reacts with the free amino groups of proteins and amino acids to form methylol derivatives (1). Reactions of formaldehyde with side-chain groups of proteins are not, however, restricted to the amino groups (1-4). Yet, the free amino groups of proteins appear to react more readily with formaldehyde than other side chains do. Acetylation thus decreases the reactivity with formaldehyde and thioformaldehyde as it decreases the reactivity with MA.

The formation of methylol derivatives can be reversed by acid, liberating the free aldehyde. The carbonyl absorption band of MA at 350 mμ could not be detected in the spectrum of the reaction product. Indeed, the rate of disappearance of this carbonyl absorption band corresponded to the rate of appearance of the reaction product. It is thus probably that the reaction involves mainly the covalent reaction of the carbonyl groups of MA with the free amino groups of BSA. Anderson and Jencks have shown that the rate-limiting step in semicarbazone formation is the acid catalyzed dehydration step (21). By analogy, the pH-dependence of the reaction between BSA and MA may involve an acid catalyzed dehydration. The general acid-catalysis is seen in the region from pH 3-7. The lower reaction

below pH 3 may represent a reduction in the available carbonyl groups of the aldehyde by the formation of an intramolecular hydrogen-bonded MA species. Above pH 3, the enolic MA (β- hydroxyacrolein) and its enolate anion exist in equilibrium with each other (16); therefore, the relative concentration of the anion is practically constant above ph 3. The observed pH dependence of the reaction is thus the combination of the available carbonyl groups of Ma and the acid-catalysis of the reaction.

Chloride ion is known to be a weak competitor for BSA binding sites (17). In our experiments, the addition of KCl reduced the MA reaction. The Cl^- inhibition of the MA reaction may be due to both a true competitive association and a non-specific electrolyte effect. The reduced reaction in the presence of urea suggests that a certain conformational requirement amy be present in the reaction. BSA contains 60 lysine and 23 arginine residues per molecule (18). While Tanford, et al. (22), have shown that all of the amino groups of BSA titrate normally, we have determined that between 16 and 17 sites are involved in the MA reaction, indicating that all of the free amino groups of BSA are not available for this reaction. The possibility that other side chain groups may also be involved in the reaction sites remains. Studies are now in progress investigating the relative reactivities of MA with amino acids and the nature of the reaction product formed.

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Table I. Electrophoretic Migration of Unreacted and Mereacted BSA.

Molar Ratio of MA to BSA	Time of Reaction (hrs)	Migration (cm)
None	—	3.00
2.5	2	3.75
4.0	7	4.00
6.0	24	4.50

Reaction with MA was carried out at pH 4.9 with 1mM MA at 30°. The reaction product was dialyzed against the electrophoresis buffer for 24 hrs. Electrophoresis was carried out in 10mM succinate buffer, pH 5.4, at 5.0 V/cm 24 hrs at 4°. Anionic migration of the center of the band was measured directly on amidoschwartz-stained gels.

Table II. Stability of the MA-BSA Reaction Product (6:1 MA/BSA molar ratio).

Solutions of the reaction product were adjusted to 1 mM in protein concentration and the conditions described below.

Treatment	%MA release
45% Glacial Acetic Acid, 35 min at 100° [a.]	100
0.1 N HCl, 0 min at 25°	9
50 min at 25°	13
5 min at 100°	100
5% TCA, Ice cold	17
5% TCA and 1 N HCl, Ice cold	17

a. Conditions identical to the TBA reaction used to determine MA quantitatively.

Table III. Effect of Acetylation, KCl and Urea on the MA-BSA Reaction.

The reaction mixture consisted of 1mM MA and 0.1mM BSA, pH 5.0 and was incubated at 30° for 24 hrs. The products were assayed for MA after dialysis against water for 72 hrs.

Additions (Final Concentrations)	% MA Found Reacted
None	100
Acetylated BSA[a]	18
0.1M KCl	85
0.5M KCl	73

[a] 13% of the original free amino groups remained unacetylated.

Figure 1. Ultraviolet Absorption Spectra of BSA and MA-BSA Reaction Product.

A. Spectrum of the reaction product (6:1 MA/BSA molar ratio)

B. Spectrum of unreacted BSA. Protein concentrations were 5×10^{-6} M in both cases.

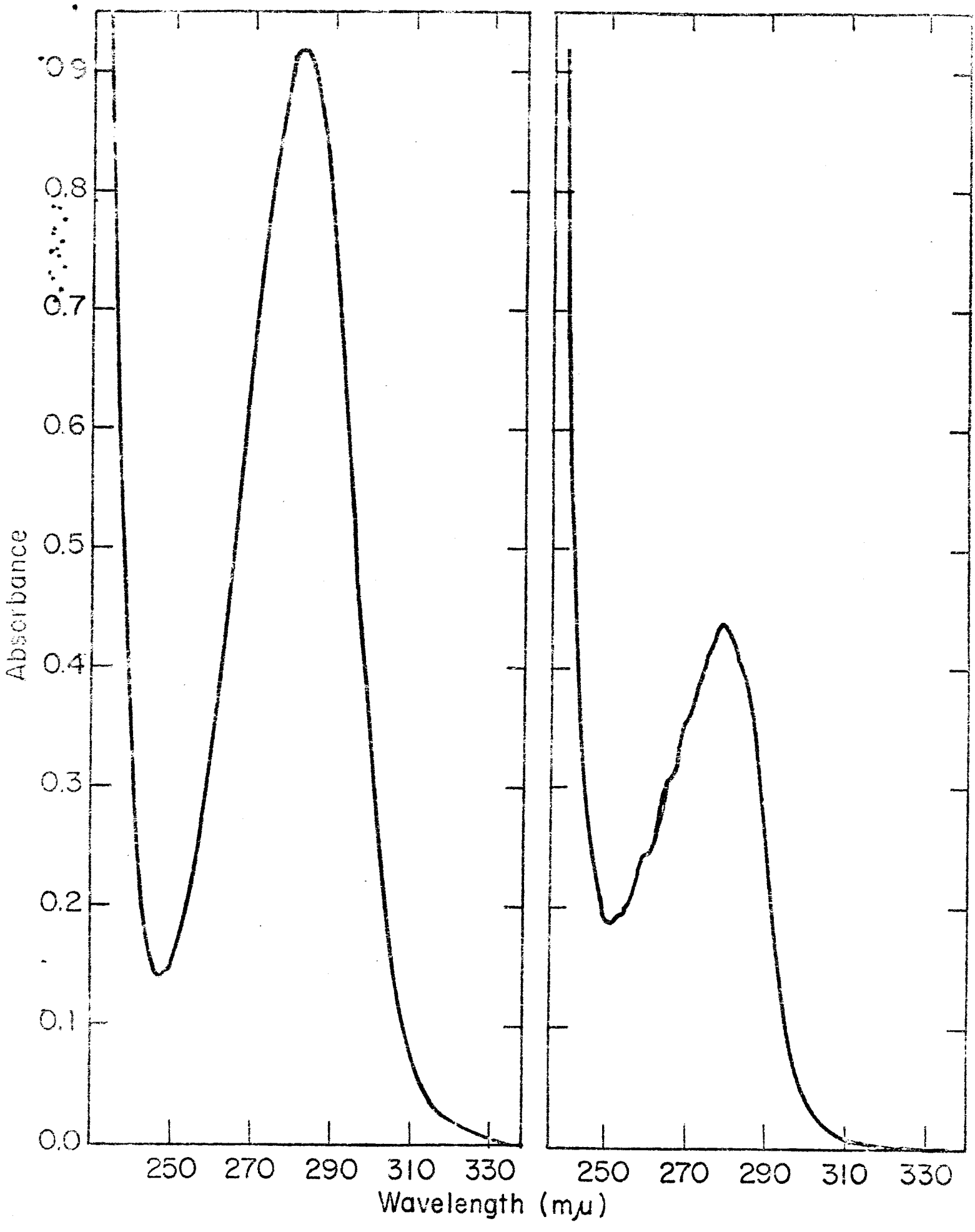

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance
250
270
290
310
330
250
270
290
310
330
Wavelength (mμ)

Figure 2. Relationship between the increased absorbance of the reaction product at 282 mμ and the molar ratio of combined MA to BSA in the product. The absorbance was measured against unreacted BSA at the same concentration (5×10^{-6} M).

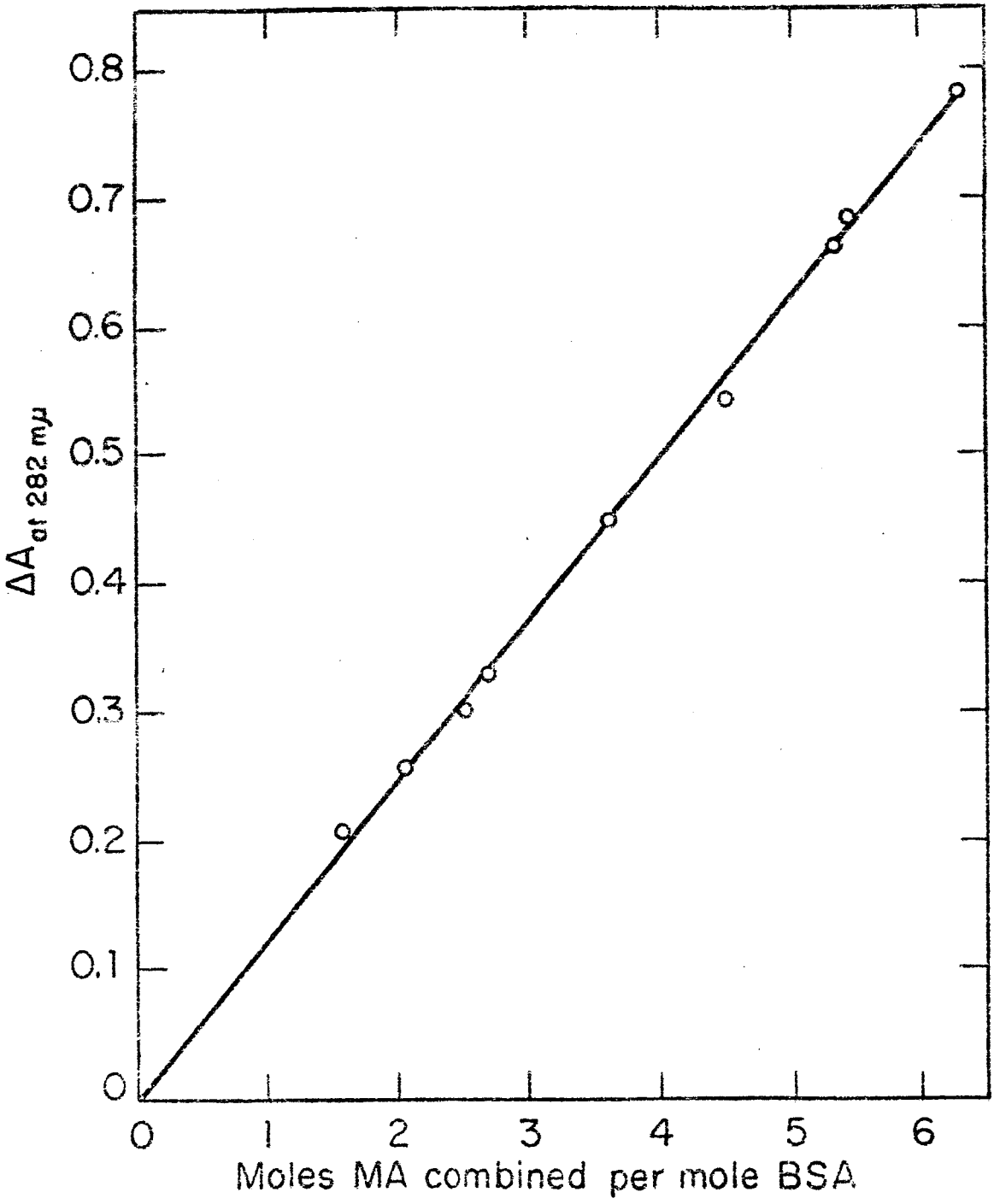

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
$\Delta A_{at\ 282\ m\mu}$
0
1
2
3
4
5
6
Moles MA combined per mole BSA

Figure 3. The pH dependence of MA-BSA reaction product formation.

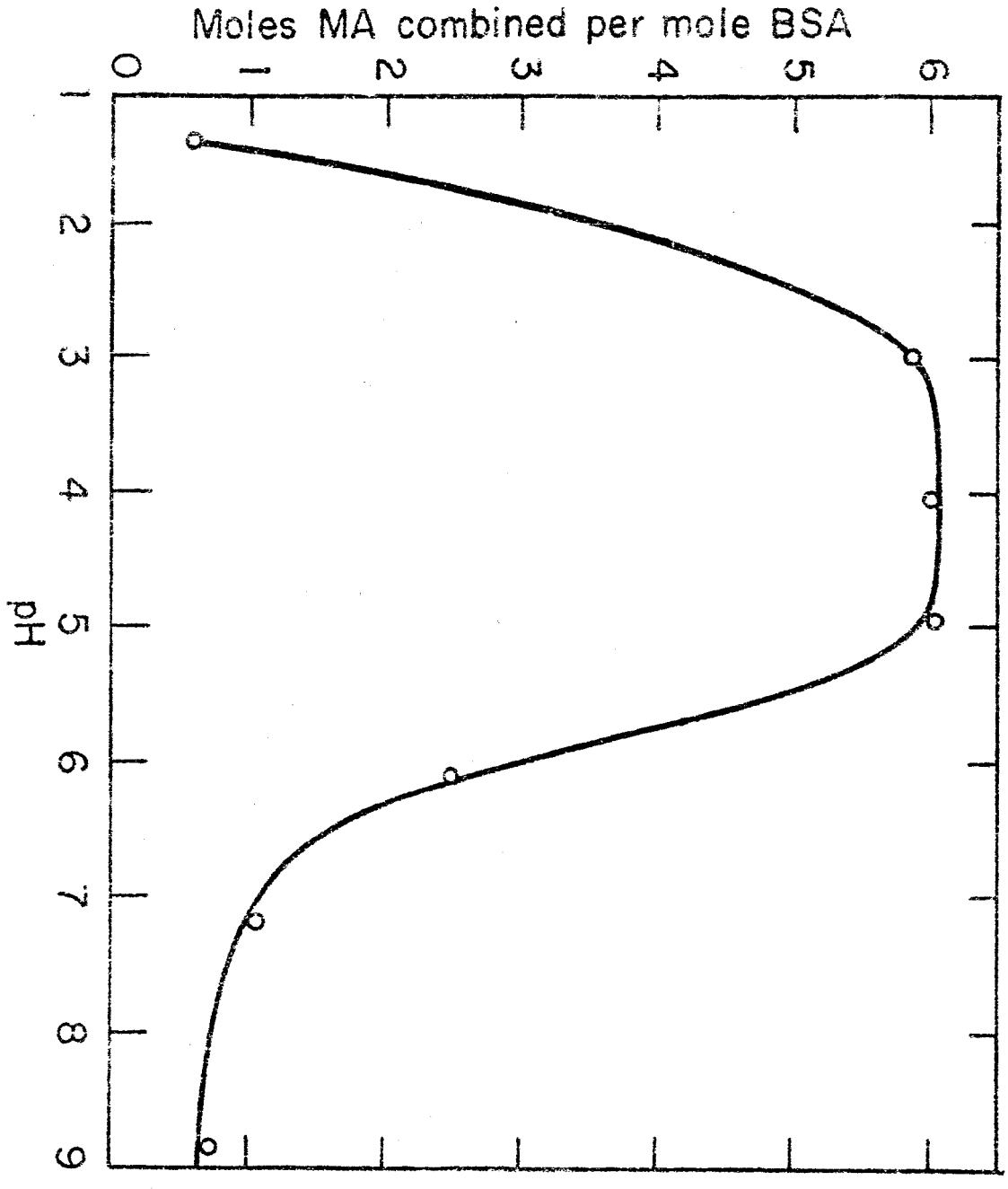
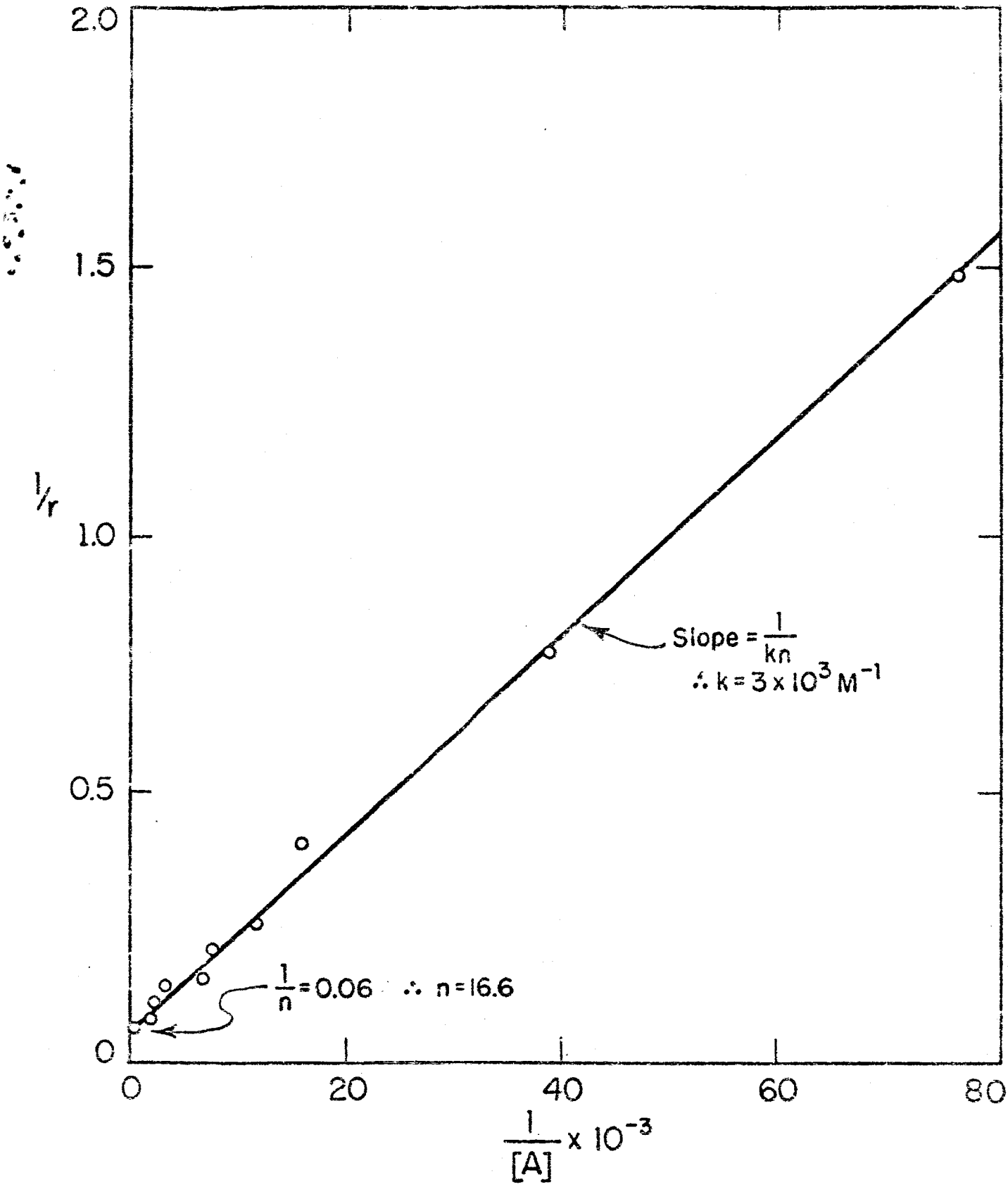

Moles MA combined per mole BSA
0
1
2
3
4
5
6
pH
1
2
3
4
5
6
7
8
9

Figure 4. Graphic solution of the number of sites reacting with MA in BSA. The molar ratio of MA to BSA of the reaction product is $\underline{r}$ and the abscissa is the reciprocal of the free MA concentration. The intercept corresponds to 16.6 reactive sites per mole, while the slope of the line corresponds to an apparent association constant of 3×10^3 $\underline{M}^{-1}$. See text for details.


2.0
1.5
1.0
0.5
0
1/r
0
20
40
60
80
1/[A] x 10^-3
Slope = 1/kn
∴ k = 3 x 10^3 M^-1
1/n = 0.06
∴ n = 16.6