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Title IN VITRO INHIBITION OF LIPASE ACTIVITY BY MALONALDEHYDE, FORMALDEHYDE AND PROPIONALDEHYDE

Abstract approved _____
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The reactions of aldehydes with proteins are of nutritional and physiological significance. The nutritive value of food materials can be impaired by a reaction necessitating additional dietary protein, and enzymes have been shown in vitro to be inhibited by reactions with aldehydes.

Aldehydes present may arise from the autoxidation of lipid materials. A diversity of carbonyl products can occur. Several autoxidizing food lipid systems have been analyzed by other investigators, and the carbonyls present isolated and identified. In most cases the short chain aldehydes predominated.

Reactions between aldehydes and proteins have been studied mainly with formaldehyde. Reactions between the aldehyde and many protein functional groups have been suggested including amino,

amide, guanidyl, sulfhydryl and imidazole. The amino groups of the protein have received the most attention. The reactions proposed have included: a. methylolamine formation followed by condensation forming a cross link, or b. Schiff's base formation. The reactions have been found to be pH specific with the pH of maximum reaction depending upon the particular protein used.

Possible reactions of dialdehydes with proteins have been little explored. Of interest are the possible reactions of malonaldehyde, one of the dialdehydes occurring in autoxidizing lipid systems. Herein, the action of malonaldehyde has been compared to that of formaldehyde, considered a reactive aldehyde, and to propionaldehyde, its monoaldehyde counterpart. The enzyme lipase was used as both the protein and the system of analysis for following the course of the reaction.

Lipase activity was determined by potentiometrically titrating the fatty acids liberated from an olive oil emulsion. The commercial bovine pancreatic lipase preparation used was found to have an activity optimum near pH 9.0. The relationship of the amount of enzyme present to the amount of fatty acids liberated was determined and found to be linear.

Maximum stability of the control enzyme appeared between pH 6.0 and 6.5, while maximum lability in the presence of MA-Na (the

sodium salt of the enolic form of malonaldehyde) was also near pH 6.0. Formaldehyde and propionaldehyde, however, both exhibited a non-pH-specific inhibition of lipase.

Of the three aldehydes reacted with lipase at pH 6.0 and analyzed at pH 9.0, MA-Na was the most inhibitory. The reaction appeared to be two stage, the first being rapid and a function of the MA-Na concentration. The second stage was slower, and the rate was related linearly to the MA-Na concentration.

Methanol-free formaldehyde did not inhibit lipase as effectively as MA-Na. Low concentrations (0.01 M) of formaldehyde had no apparent effect on the enzyme while concentrations above 0.05 M produced only slight changes in the degree of inhibition.

Propionaldehyde showed only slight inhibition of lipase activity. Its reaction was two stage, with the second stage paralleling the inhibition seen in the control due to factors other than the aldehyde present.

Reactions such as these, especially for the more reactive aldehydes such as malonaldehyde, may occur *in vitro* and *in vivo* with other proteins and could be of important nutritional or physiological significance.

IN VITRO INHIBITION OF LIPASE ACTIVITY BY MALONALDEHYDE, FORMALDEHYDE AND PROPIONALDEHYDE

by

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INTRODUCTION

The reactions of carbonyl compounds produced by autoxidizing lipid materials should be of nutritional and physiological significance. These carbonyls have been shown to affect both nutritive value and biological activity of protein materials.

Attempts to determine the type of reactions occurring between carbonyls and various protein materials have been conducted mainly with formaldehyde, considered a very reactive aldehyde. Only a few other aldehydes have been thus studied. Dialdehydes, a major classification of carbonyls formed from autoxidizing lipid materials, have received little attention.

In this work a dialdehyde, malonaldehyde, which can be produced from the autoxidation of polyene fatty acids, is compared with formaldehyde and propionaldehyde, its monoaldehyde counterpart. The inhibition of the enzyme lipase is used as the basis of comparison. The aldehydes are allowed to react with the enzyme and the degree of inhibition is followed through the course of the reaction.

The experiment was designed to show the effects of pH on the aldehyde-lipase reaction and the relative velocities of the reactions using the three aldehydes.

LITERATURE REVIEW

The reaction of carbonyls with proteins has been of particular significance since carbonyls have been recognized to be a product of autoxidizing unsaturated lipid materials. The possibility of a reaction between carbonyls and the functional groups of their environment, be it food stuffs or *in vivo* compounds, is of nutritional and physiological importance.

Physiological and Nutritional Significance of Aldehyde-Protein Reactions

Certain aldehydes of metabolic origin have been shown by Milch (61) to induce hardening of gelatin sols. Those which produced cross links inducing the hardening were aliphatic, water soluble aldehydes of three or less carbon atoms. The α carbon in each case possessed a reactive electrophilic substituent, viz., a carbonyl, hydroxyl or alkene group, or a reactive hydrogen atom. Other fat, carbohydrate or protein metabolites examined were ineffective with the exception of the C_4 α,β-unsaturated aldehyde, crotonaldehyde. The active aldehydes, acrolein and crotonaldehyde, at 0.15 M were also found to produce lesions in canine aortic wall (62, p. 104-123). Saline, propionaldehyde, methanol and glycerol did not produce such lesions. Harman (36) has hypothesized that these

highly reactive substances from the autoxidation of lipids may initiate aortic lesions in other species through cross linking.

The nutritional value of foods containing aldehydes may be impaired by reactions between functional groups. Considerable work has been done on the available lysine content of foods. It has been hypothesized by Carpenter (11) that once ϵ-amino groups of lysine are tied up by reactions within a product, as with carbonyl groups, the lysine may be nutritionally unavailable. In cereal proteins where low lysine content already limits the protein value, any further reduction due to reaction is of importance. Prior to the development of Carpenter's method (11) of lysine analysis using 2,4-dinitrofluorobenzene, the available lysine, that not reacted, was often overestimated by determinations involving acid hydrolysis.

A high correlation between the free ϵ-amino content of lysine and protein quality was found in rat repletion tests using cottonseed meal by Baliga et al. (3), and using fish meal in chick growth studies by Kellenbarger (45). Martinez et al. (53), in studying the effects of gossypol, a polyphenolic dialdehyde, on available lysine in cottonseed meal also found a high correlation between rat repletion data and free ϵ-amino groups of lysine and a poor correlation with total lysine. Formaldehyde, when used as a preservative of fish meal, depressed its available lysine content as determined both chemically

and biologically by Olley and Watson (66). It was hypothesized that formaldehyde reacted with the α- and ε-amino groups of the protein forming methylolamines which condensed with other groups forming methylene bridges.

Autoxidized lipids and their concomitant carbonyls have been shown to have adverse effects in animal diets. Rats when fed rancid fat had an increased protein requirement judged to be due to direct damage to the nutritional value of the protein (34, p. 421-440). In feeding autoxidized menhaden oil triglycerides to rats as ten percent of their diet, Rasheed et al. (69) found steatitis, enlarged livers and high levels of malonaldehyde in blood and excreta. In using autoxidized clay-bleached menhaden oil at 15 percent of the rat diet, anorexia, steatitis, lowered hemoglobin levels and death occurred. As the level of oxidation increased the symptoms became more extensive or severe.

It is this type of evidence that is indicative of the significance of the reactions of autoxidized lipids and their carbonyls with protein both in vivo and in vitro.

Lipid Autoxidation Theory

The carbonyls which may be present in a food product due to autoxidation of lipid material could originate either from primary

oxidation or from secondary reactions of primary reaction products as reported by Lillard (49, p. 1-10). According to the generally accepted free radical mechanism of Farmer et al. (25), lipid autoxidation progresses through hydroperoxide formation to hydroperoxide decomposition, and subsequent polymerization and secondary reactions (44, p. 79-89). The free radical mechanism is based on hydrogen lability of the unsaturated system. The unsaturated systems are divided into four groups of decreasing hydrogen lability. These are, according to Farmer et al. (25):

I. $-CH=CH-CH_2-CH=CH-$ III. $-CH_2-CH=CH-CH_2$

II. $-CH=CH-CH_2-CH_2-CH=CH-$ IV. $-CH=CH-CH=CH-$

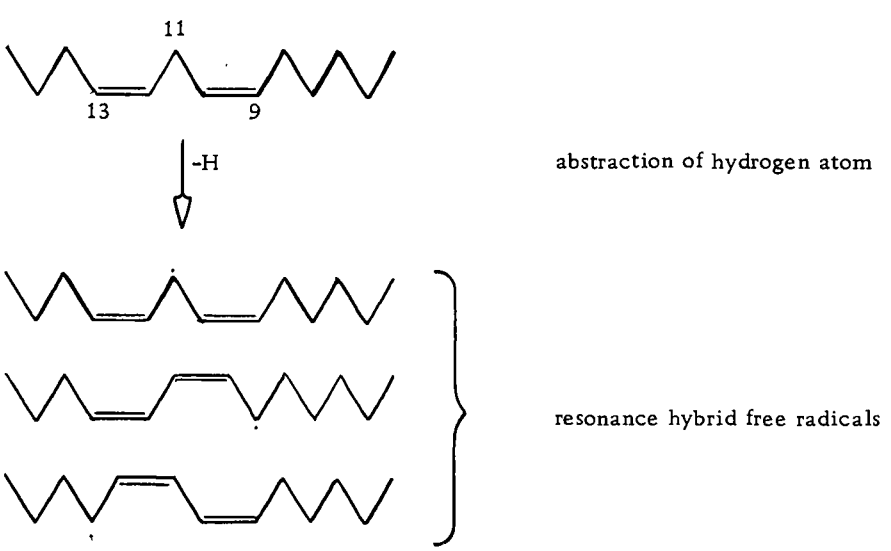
The pentadiene system, Group I, with the α-methylene flanked by two double bonds has the highest hydrogen lability. The methylene group, interrupting the double bonds, hinders dissipation of energy through resonance. Group IV, the conjugated system, has the lowest hydrogen lability due to resonance stabilization; energy is dissipated throughout the entire system.

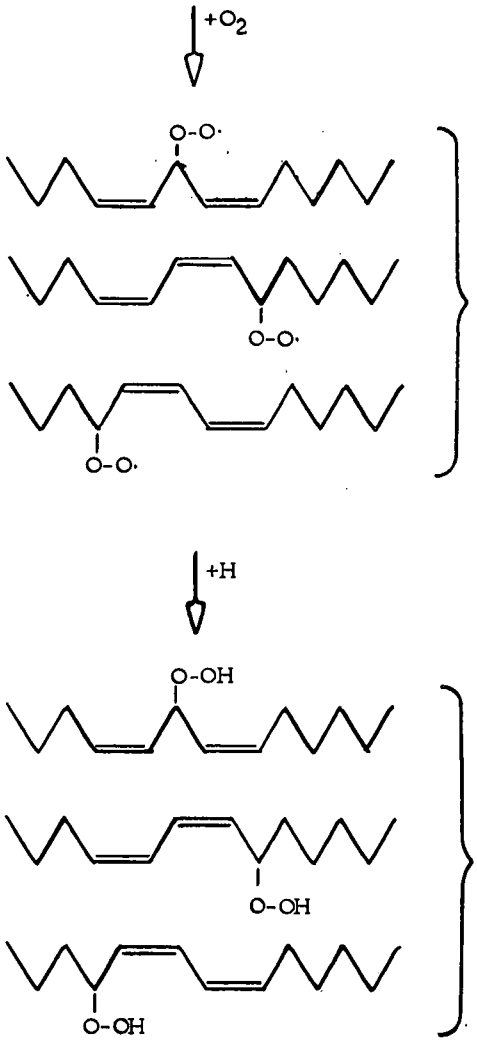
A general statement of autoxidation theory (49, p. 7) based on the pentadiene system, Group I, calls for excitation of the system by an external energy source. The energy is expended through the escape of an electron which takes a proton with it to form a hydrogen atom. The free radical formed on the α-methylene carbon sets up

resonance isomers with the adjacent unsaturated groups. An oxygen molecule can then be absorbed by the free radical to form a peroxide free radical which then accepts a hydrogen atom to form a hydroperoxide. The hydrogen atom usually is abstracted from another fatty acid ester, thus forming a free radical to propagate the chain.

Autoxidation of Linoleic Acid

Linoleic acid, containing Farmer's Group I, has a high rate of autoxidation and is a major source of autoxidation problems (49, p. 11), the polyethenoic acids being the main source of oxidative rancidity in fats (85, p. 14) The oxidation of linoleic acid, a non-conjugated diene, follows the general scheme shown below which was formulated by Bolland and Koch (9) based on Farmer's theory (25).





addition of oxygen

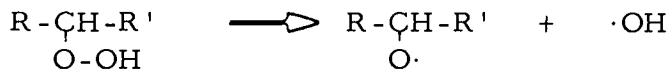
three possible peroxy radicals

addition of hydrogen atom abstracted from another linoleate molecule

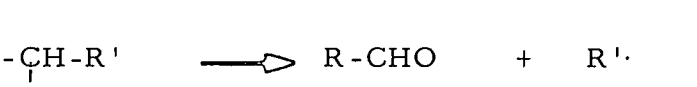
three possible hydroperoxide products, two of which are conjugated

Secondary Reaction Products

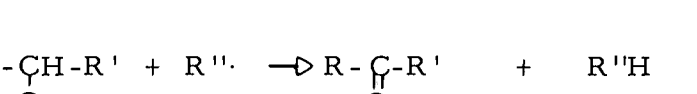
Secondary products of lipid autoxidation believed formed from hydroperoxide decomposition include aldehydes, alcohols and unsaturated compounds highly susceptible to further oxidation. Hydroperoxides decompose according to the scheme of Bell et al. (6), presented by Badings (2), to the alkoxy and hydroxy free radicals.

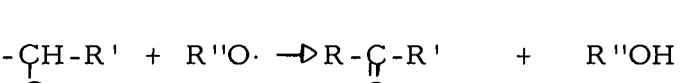


These free radicals then react in one of several ways:

1.  aldehydes and alkyl radicals

2. abstraction of hydrogen from another molecule produces an alcohol and a free radical

3.  chain termination, carbonyl formation

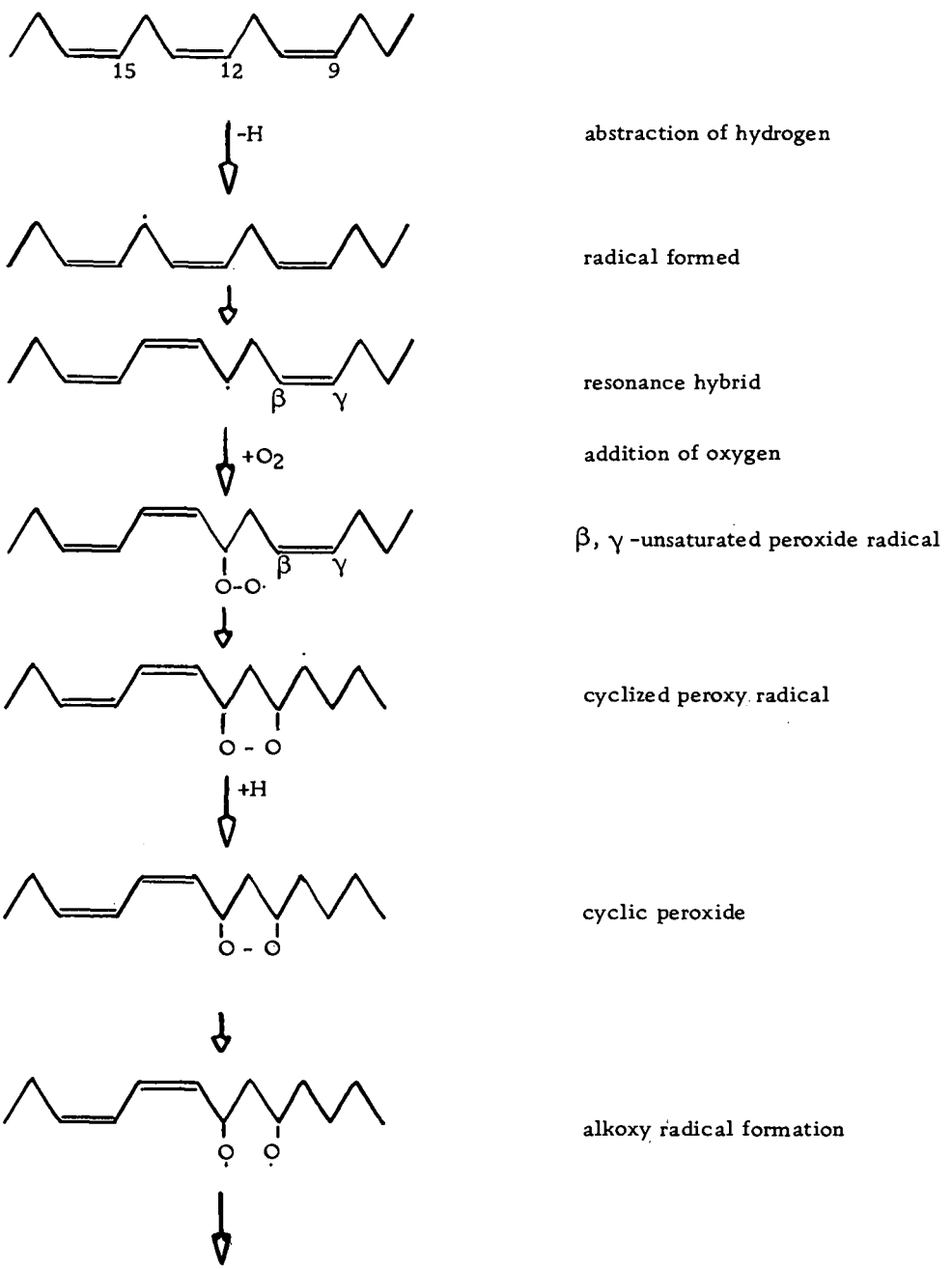
4.  carbonyl and alcohol production with chain termination

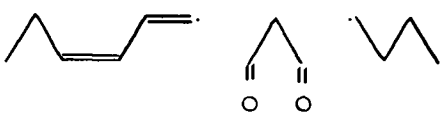
Many carbonyls of concern in food products are formed by these mechanisms. However, adequate answer for the genesis of malonaldehyde, the three carbon dialdehyde, and other dialdehydes is not provided by this mechanism.

Genesis of Malonaldehyde

Another mechanism based on the oxidation of linolenate was proposed by Dahle et al. (16). It is shown on page ten. Linolenate, which oxidizes by a mechanism analagous to that of linoleate, forms four conjugated peroxy radicals of which two are β, γ-unsaturated. It was hypothesized that the β, γ-unsaturated peroxide radicals could cyclize to form a five membered peroxide ring. Cleavage

of this ring would yield malonaldehyde. Linoleate, which does not form β, γ-unsaturated peroxide radicals, does not produce malonaldehyde in the early stages of oxidation thereby supporting this theory (16).





formation of dialdehyde

Carbonyls in Autoxidizing Lipids

From the above mechanisms and their reaction products a multiplicity of reactive compounds is produced. The carbonyls produced have been isolated and determined in several oxidizing food lipid systems. Autoxidized pork fat yielded many carbonyls, most of which were nonvolatile and/or bound according to Gaddis et al. (31, p. 495-506). Fifteen volatile monocarbonyls were identified in autoxidizing salmon oil by Yu et al. (99). Yet, volatile carbonyls usually consist of less than three percent of the total measurable carbonyls, the greater part of the determinable carbonyls being nonvolatile according to Lillard and Day (50). Twenty-six monocarbonyls including alkanals, alk-2-enals and alk-2, 4-dienals, in addition to the dicarbonyl, malonaldehyde, were isolated and identified in autoxidizing salmon oil by Wyatt and Day (98). Short chain carbonyls predominated with methanal and propanal most prevalent. The oil which contained 1102 mg malonaldehyde per kilogram oil (0.0153 M), as determined by the 2-thiobarbituric acid method of Sinnhuber and Yu (81), had a peroxide value of 171. From it 0.032 M methanal, 0.011 M ethanal, 0.030 M propanal, 0.010 M butanal

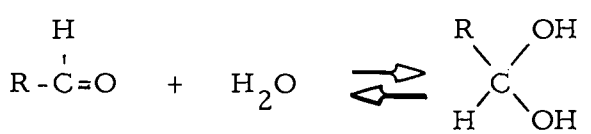
and smaller amounts of other aldehydes were isolated. The amounts varied with the level of the oxidation (98).

An impressive list of reactive entities within a food system could be presented if the carbonyl compounds which have been isolated and identified were added to those which may be present but have not yet been elucidated.

Aldehyde-Protein Reactions

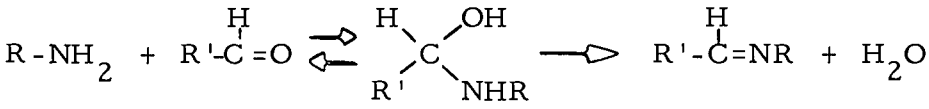
Determinations of the sites and mechanisms of reactions between aldehydes and proteins have been the objective of many studies using varied substances and techniques.

Recently infrared evidence has been obtained by Milch (61) that the aldehydes capable of reacting with proteins exist in aqueous solution in the hydrated form in a manner quite analagous to that of hydrated formaldehyde (methylene glycol). Nonreactive aldehydes (i.e., non-cross linking) appeared to exist almost exclusively in the free aldehyde or carbonyl configuration.

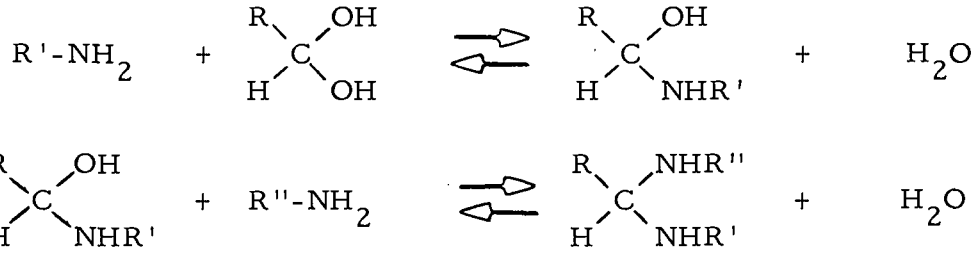


Some of the recent work has been based on the formation of Schiff's bases which are the products of reactions between primary

amines and aldehydes. This reaction is assumed to proceed via an intermediate amino alcohol (32, p. 260-262; 61; 82).



However, much of the contemporary work points to a primary reaction between a hydrated aldehyde and an amino group, as the ϵ-amino group of lysine, forming a methylolamine, which then condenses with methylene bridge (assuming formaldehyde) formation (27, p. 2673-2844; 60; 61).



Formaldehyde Reactions with Protein Materials

In general the reaction of formaldehyde with a protein leads to a decrease in water solubility and an increase in resistance to the action of enzymes and chemical agents according to Walker (90, p. 312-313). Reactions of many functional groups within a protein with formaldehyde appear likely. Pearse (67, p. 53-56), as a histochemist, presents an extensive list of possible reactions of formaldehyde including: active hydrogen, amino, imino, amide,

guanidyl, hydroxyl, carboxyl and sulfhydryl groups, and aromatic hydrogen. Even to his list additions of indole and imidazole groups and phenolic nuclei have been made (90, p. 312-317). These reactions are governed by the relative reactivity of the groups concerned and the conditions of temperature, pH and perhaps ionic strength.

Loeb (51, p. 116-127), in 1913, reported the methylation in neutral media of the amino acid glycine by formaldehyde forming methyleneamino acetic acid, CH_2=N-CH_2-COOH, which was not recrystallized. In acid media, methylenediglycine, CH_2(NH-CH_2-COOH)$_2$, was formed. In alkaline solution no condensation was reported.

The pH of the reaction media has been shown to influence the reacting groups of a protein. Fraenkel-Conrat et al. (26; 28, p. 827-842) ascertained that primary and secondary amino groups form methylol derivatives with formaldehyde over the pH range 3 to 8 even at low temperatures, while amide, guanidyl and indole groups require elevated temperature, or acid or alkali before reacting. Secondary amines require a strong acid catalyst (26). In an extensive study of the reactions of commercial formaldehyde with various proteins, Fraenkel-Conrat and Olcott (27, p. 2673-2684) reported that the primary reaction was the formation of methylolamine

followed by cross linking with primary amide or guanidyl groups. The reaction proceeded between pH 3 and 8, within 24 to 48 hours, to give condensation products with the general structure:

$$R\text{-}NR'\text{-}CH_2\text{-}NH\text{-}\overset{O}{\overset{\|}{C}}\text{-}R''.$$

Representative compounds were isolated in pure form. The reaction, however, was low on the acid side of the isoelectric point. N-alkyl amines including peptides, R-NH-CO-R', did not react in this manner. Also observed was gelation of the acid-formaldehyde reaction mixtures of gliadin, zein and polyglutamine by small amounts of primary amines or ammonia probably by the introduction of cross links of the following structure between the numerous amide groups of these substances.

$$-CH_2\text{-}\underset{R'}{N}\text{-}CH_2-$$

The cross linking of bovine fibrinogen by dilute commercial formaldehyde was studied in 1963 by Mihalyi (60). The reaction appears to be two stage at pH 7.2. The first is an equilibrium reaction in which the hydroxymethyl derivative is formed which then, in the second phase, condenses. The cross links were believed formed between ϵ-amino groups and amide groups of the fibrinogen. At pH 6 the reaction did not occur.

Similar studies using formaldehyde and various proteins show

the dependence of the reaction upon experimental conditions, particularly pH. There is disagreement however as to the groups reacting which may be attributed to the use of different proteins and reaction conditions. Middlebrook (59) found formaldehyde cross linking between amino, and glutamine or guanidyl groups. In another study by Middlebrook and Phillips (58) lysine residues of collagen and keratin were reacted quantitatively with one percent formaldehyde solutions buffered at pH 8.0. The guanidyl residues also reacted, and at pH 5 cysteine reacted rapidly with formaldehyde. Kinnear and Naismith (46) examined with the ultracentrifuge the cross linking of groundnut proteins by formaldehyde in the pH 6 to 11 range. Evidence for the formation of dimers, trimers and tetramers of the parent molecule in the conarachin II fraction was obtained at pH 8.0. Further studies on protein-formaldehyde reactions have included those on groundnut (37; 38; 89), human hemoglobin (35), fibrinogen and thrombin (24, p. 735-765) and collagen (33). It is possible other aldehydes would act similarly.

Reactions between Other Aldehydes and Amines

The reactions of other aldehydes, including α,β-unsaturated aldehydes, with amines have been reviewed by Sprung (83, p. 297-398) and Layer (48, p. 485-510). Milch (61) postulates for dialdehydes

reactions similar to those he presented for monoaldehydes. They are of two types:

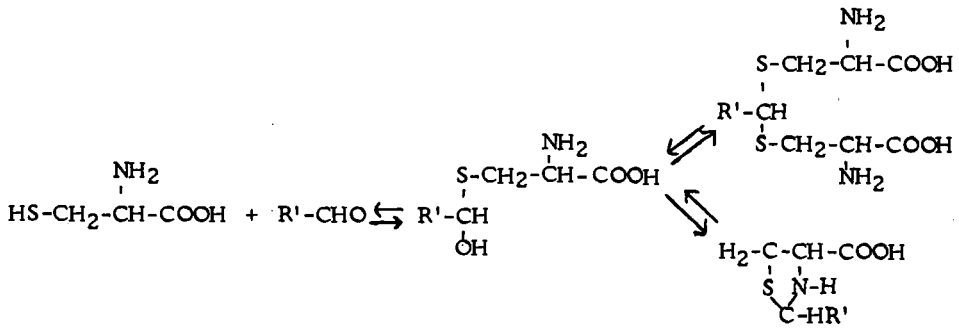
a. Intermolecular cross linking with methylene bridge formation.

b. Schiff's base formation between adjacent chains.

$$R\text{-}NH_2 + OHC\text{-}\ldots\text{-}CHO + H_2NR' \rightleftharpoons R\text{-}N{=}C\text{-}\ldots\text{-}C{=}N\text{-}R' + 2H_2O$$

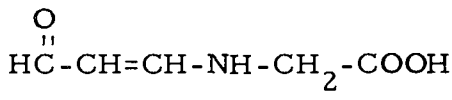
Acrolein and crotonaldehyde were found by Decroix et al. (17) to be more reactive with keratin than the saturated aldehydes of the same chain length. Purified casein, after reaction with acetaldehyde, had reduced formol-titratable groups and was resistant to the action of trypsin and papain.

The reactions between amino acids and various aldehydes have also been studied. The reaction of sulfhydryl compounds with carbonyls has received attention by Bergel and Harrap (7). They proposed a general reaction leading to the formation of a hemimercaptal intermediate followed by dimercaptal formation or ring closure leading to a thiazolidine derivative, depending upon the amount of sulfhydryl compound present.



The system of Bergel and Harrap (7) employed cysteine and pyridoxal phosphate. The reaction proceeded over the pH range 4 to 10 and maximally at about pH 8.0.

The formation in acidic solution of a malonaldehyde, glycine compound of the following structure has been proposed by Crawford et al. (15).



The reaction between malonaldehyde and 2-thiobarbituric acid has been demonstrated to form a compound of the empirical formula $C_{11}H_8N_4O_4S_2$. Sinnhuber et al. (80) propose that the crystalline pigment is a condensation product of one molecule of malonaldehyde with two of 2-thiobarbituric acid with the probable elimination of two molecules of water.

Robert and Penaranda (71; 72) have examined the reactions of various amino acids with acetaldehyde and propionaldehyde. The reactions with acetaldehyde were two stage. The first, a rapid, bimolecular reaction led to a Schiff's base type compound. A slower second reaction followed which led to a mixture of products of increased acidity. The reactions of propionaldehyde with amino acids were more ambiguous. The amino nitrogen level of the tryptophan, propionaldehyde reaction mixture, for example, reached a minimum and then returned to the initial level.

Protein-Oxidized Lipid Reactions

In reactions of oxidized lipids with proteins Tappel (86) noted yellow-brown copolymers formed by the oxidation of unsaturated fats in the presence of proteins. Matsuo (56, p. 330-332; 57) noted formation of orange-brown polymers when autoxidized cuttlefish fatty acid ethyl esters were added to ovalbumin solution. Large amounts of protein were incorporated into the copolymers with subsequent loss of some of the physical and chemical properties of the protein. Tappel (87) found the hematin compounds, hemoglobin, cytochrome c and hemin to be catalysts for the copolymer formation. Further study using a reaction system of linolenic acid, cytochrome c and oxygen by Desai and Tappel (19) showed cytochrome c to decrease in solubility as oxidation products added. Paper chromatography of the amino acids from the protein hydrolysate showed loss of histidine, serine, proline, methionine and cystine. Narayan and Kummerow (64; 65) in complexing oxidized linoleic acid and egg albumen found optimum complex formation to occur with one percent protein, pH 7.0, at 60°C.

Enzyme-Carbonyl Reactions

Enzymes have been used as both the protein and as the analytical

system to follow the progress of a reaction with carbonyls. Lipase has been used by several investigators. Weinstein and Wynne (92, p. 649-660) found formaldehyde, acetaldehyde, butryaldehyde, aldol and benzaldehyde inhibitory to lipase at pH 8.9. Milk lipase, Schwartz et al. (78) reported, was inhibited by formaldehyde, the degree of inhibition being dependent upon the concentration of the reagent and time. Organic peroxides inhibited in a similar manner according to Wills (93). Succinyl peroxide was found to be a powerful inhibitor of -SH enzymes. A reaction with cysteine or methionine was suggested, although reaction with other groups was not excluded. Succinyl peroxide at 1×10^{-3} M caused 58 percent inhibition of pancreatic lipase, one of 18 enzymes used. Urease, a -SH enzyme, was completely inhibited within a few minutes. Reversal of inhibition was possible only if time of contact was very short. The enzymes could usually be protected by reaction with a reversible -SH reagent. Variations in inhibition were found with both pH and the type of buffer used. Singer and Barron (79) concurred that lipase could be partially inactivated by tying up the -SH groups. Monty (63) however, postulates that inhibition of lipase was due to alterations of the surface of the emulsion droplets rather than to alteration of the enzyme. He used a fat soluble aldehyde, decyl aldehyde, adding it to the substrate at pH 8.0, rather than to the enzyme solution. It has

also been found (39) that lipase is much less capable of hydrolyzing emulsions of oxidized oils than of normal fresh oils.

Riordan and Bier (70, p. 327-336), studying the effects of commercial formaldehyde on trypsin, found the native enzyme to be readily inactivated in alkaline media. Acylation of the free amino groups led to an increased stability of the enzyme by the prevention of methylene cross linking with formaldehyde. The reactions appeared to be of two types, one rapid, followed by a slower one. Commercial formaldehyde was used at one, three and six percent concentration.

Emulsions of oxidized linoleic or linolenic acids dissolved in ethanol were found by Wills (94;95) to inhibit several other enzymes. The -SH enzymes were more rapidly inhibited than enzymes without -SH groups, and sulfhydryl compounds protected the enzymes. Oxidized methyl linolenate (8) inhibited succinoxidase and cytochrome oxidase. Incubation of washed tissue suspensions or mitochondria found the above enzymes inactivated parallel with the rise in the amount of 2-thiobarbituric acid reacting material (malonaldehyde) produced according to Bernheim et al (8). Tappel (87) found similar results with succinoxidase and DPNH-cytochrome c reductase.

Jonsson and Lagerstedt (42) found ribonuclease activity inhibited by a 0.4 M commercial formaldehyde solution. The reaction

with the crystalline enzyme was rapid initially, followed by a comparatively slow secondary phase of inactivation. The reaction was very pH dependent with almost 80 percent of the activity inhibited at pH 7.0, but only a few percent at pH 2.4. The reaction was also demonstrated with rat pancreas tissue.

Certain Chemical and Physical Properties of Malonaldehyde

Specific information on malonaldehyde has been appearing since it was recognized as being one of the products of lipid autoxidation and a material producing a chromophore with 2-thiobarbituric acid (80; 81; 100). The instability of free malonaldehyde, reported by Hüttel (41) and later by Arnold and Šorm (1), has led to the use of derivatives. The sodium salt of the enolic form of malonaldehyde, as prepared by Protopopova and Skoldinov (68), has the advantage of being readily water soluble. However, most studies on the properties of malonaldehyde have been done using the hydrolysis products of one of the acetal derivatives. Mashio (54; 55) found the ultraviolet spectra of malonaldehyde to be pH dependent and to be characteristic of an α,β-unsaturated carbonyl compound suggesting that it exists in the enol form. The ultraviolet absorption observed at 245 mμ, below pH 4.5, has been related to intramolecular bonding of the enolic hydrogen by Saunders and May (77). Above pH 4.5

dissociation of the enolic hydrogen results in spectral changes.

Certain Chemical and Physical Properties of Lipase

Pancreatic lipase, a lipolytic enzyme, was used in this study. Lipase has been reported by Sarda et al. (74) to be one protein or a group of very similar proteins which act catalytically on the hydrolysis of emulsions of esters. Its active center is formed only when the protein is absorbed at an interface (20, p. 35; 21; 22, p. 370-372). The enzyme appears more active in the presence of sodium taurocholate, possibly due to altered surface properties of the substrate (14; 18; 20, p. 47-49; 30, p. 719-730) or a direct activating effect on the enzyme (5). It requires no coenzyme according to Marchis-Mouren et al. (52). Sulfhydryl groups have been reported necessary for enzymatic activity by Wills (96;97) and Barron (4).

The isoelectric point at pH 5.0 to 5.5 has been determined by Sarda and collaborators (75; 76). The pH of optimum activity has been placed between 7 and 9 depending upon the substrate, the purity of the enzyme and the buffer system used (5; 10; 91). Baskys et al. (5) reported a pH optimum of 8.0 to 8.1 on a highly purified preparation while the impure enzyme had an optimum at pH 8.9 to 9.1. Pancreatic lipase is heat labile, being completely inactivated after ten minutes at 55°C. Its optimum reaction temperature is about 40°C,

but usually 37 to 38°C is used for activity analysis Kates reports (43, p. 171). The molecular weight has been postulated to be quite low (52), and Chandan et al. (12) have found the molecular weight of milk lipase to be about 7,000; however, comparison of the various lipases may not be valid.

EXPERIMENTAL

Lipase Determinations

General Method

The bovine pancreatic lipase used was a commercial preparation (Nutritional Biochemicals Corporation #448). The enzyme activity analyses were performed using, with slight modification, the method of Marchis-Mouren et al. (52) employing a potentiometric titration of the fatty acids liberated from an olive oil emulsion in the presence of sodium taurocholate. The emulsion was prepared by rapidly blending for ten minutes in a chilled container 165 ml of cold ten percent gum arabic solution, 15 g crushed ice and 20 ml olive oil. Ten ml of the emulsion were placed in a beaker with 0.3 ml 0.1 M sodium taurocholate solution and sufficient water to bring the final volume to 30 ml. The beaker was placed into a constant temperature bath held at 37°C. The electrodes of a Beckman Zeromatic pH meter were inserted and a stream of nitrogen bubbled through the mixture. The pH was brought to 9.1 to 9.2 by the addition of 0.1 N NaOH, and after five minutes temperature equilibration a volume of enzyme solution was added. As soon as the pH fell to 9.0 a stop watch was started. Small amounts of 0.1 N NaOH were

added to keep the pH in the range 8.9 to 9.1, and the amount of base required to maintain the pH was recorded. The titration was carried out over a five minute period for which the rate of acid release has been shown to be constant for a given amount of enzyme and proportional to the amount of enzyme present (52). The titrations were recorded as ml of 0.1 N NaOH used and converted to μ equivalents of acid liberated. The activity of an enzyme solution was first determined immediately after preparation by adding a portion of the enzyme solution to an emulsified substrate sample and titrating over the five minute period. Subsequent analyses of the enzyme solution were made at specific times using the same procedure. Due to difficulty in obtaining reproducible results from day to day, due to emulsion variations, the results of the subsequent analyses were reported as percent of the daily initial analysis.

Determination of the pH Optimum of Lipase

The enzyme was dissolved in cold 0.1 M buffer of the desired pH and brought to volume with cold buffer. Enzyme concentration was 0.08 percent w/v. A one ml portion of the enzyme solution was removed and added to the emulsion which was then titrated at the desired pH. Tris buffer was used at pH 7.5 to 8.0, borate buffer at pH 8.0 to 9.3 and phosphate buffer at pH 8.8.

Effect of Lipase Concentration on Rate of Hydrolysis

The desired amount of lipase was dissolved in cold 0. 1 M phosphate buffer at pH 6. 0. Samples of this were removed and analyzed at pH 9. 0 according to the modified method of Marchis-Mouren et al. (52) described above. The lipase concentration was varied from 0. 02 to 0. 1 percent w/v (five to 25 mg per 25 ml total volume).

Effect of Certain Aldehydes on Lipase Activity

Method of Analysis

Lipase was dissolved in cold 0. 1 M phosphate buffer at pH 6. 0, the aldehyde added and the solution brought to the proper volume with additional cold buffer. In the time studies, a sample of this enzyme solution was first analyzed while still cold. The enzyme solution was then incubated at 37°C with 1 ml portions being removed at one half, one, two, four and six hours for analysis.

Preparation of Aldehydes

The aldehydes used were malonaldehyde, formaldehyde and propionaldehyde. The sodium salt of the enolic form of malonaldehyde (MA-Na) was prepared by the method of Protopopova and Skoldinov (68) from 1, 1, 3, 3-tetraethoxypropane in this laboratory.

Methanol-free formaldehyde solution was prepared from paraformaldehyde by refluxing, and analyzed for formaldehyde content using the sodium sulfite method given by Walker (90, p. 382-384). The solution was stable at room temperature for at least three weeks. For comparison a commercial formaldehyde solution containing ten percent methanol was also used. Propionaldehyde was an Eastman Organic product and was redistilled prior to use. A stock solution of one to 50 dilution was used as the source of propionaldehyde.

The levels of aldehydes used were in the range of those isolated from autoxidized salmon oil by Wyatt and Day (98).

RESULTS AND DISCUSSION

pH Optimum of Lipase

The optimum pH of pancreatic lipase was found to be near pH 9.0 under the conditions used (Figure 1). This is in agreement with Borgström (10) and Weinstein and Wynne (92). All subsequent analyses were then performed with the pH adjusted to 9.0 with 0.1 N NaOH, although the enzyme may have been prepared at a different pH.

Relationship of Lipase Concentration to Rate of Hydrolysis

Increases in lipase concentration from 5 to 25 mg per 25 ml volume produced an equivalent, linear change in the rate of release of fatty acids from the emulsified substrate (Figure 2). This then allowed the assumption that the μ equivalents of fatty acid titrated in any subsequent analysis were a linear measure of the amount of active lipase present. From these data 20 mg lipase per 25 ml solution volume (0.08 percent w/v) was chosen for future analyses.

Relative Inhibition with pH by the Aldehydes

It would be expected that the reaction of a protein with the aldehydes would be dependent upon the particular ionic state of the

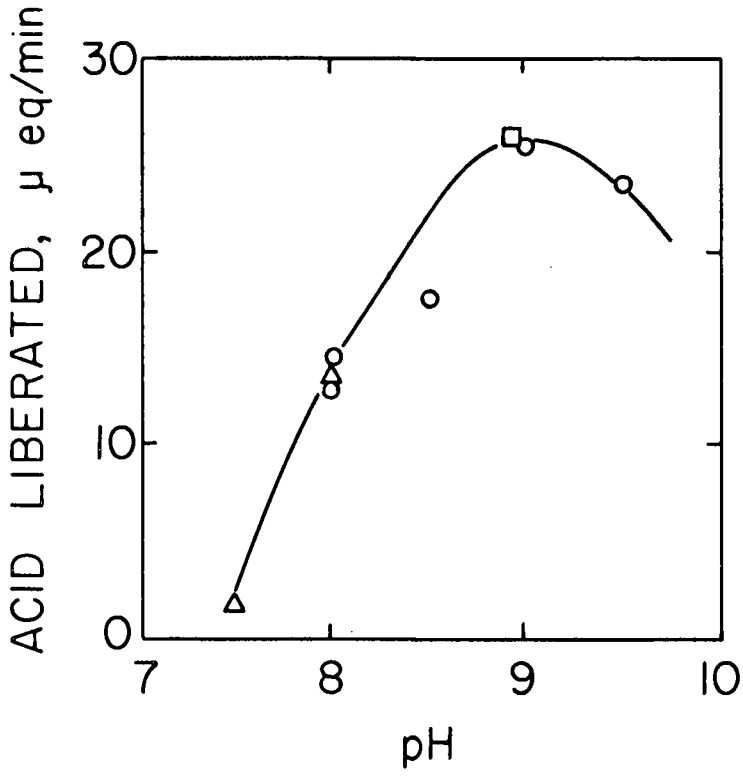


FIGURE I.- pH Optimum of Lipase. 0.1 M Buffers - Tris (△), Borate (○), Phosphate (□).

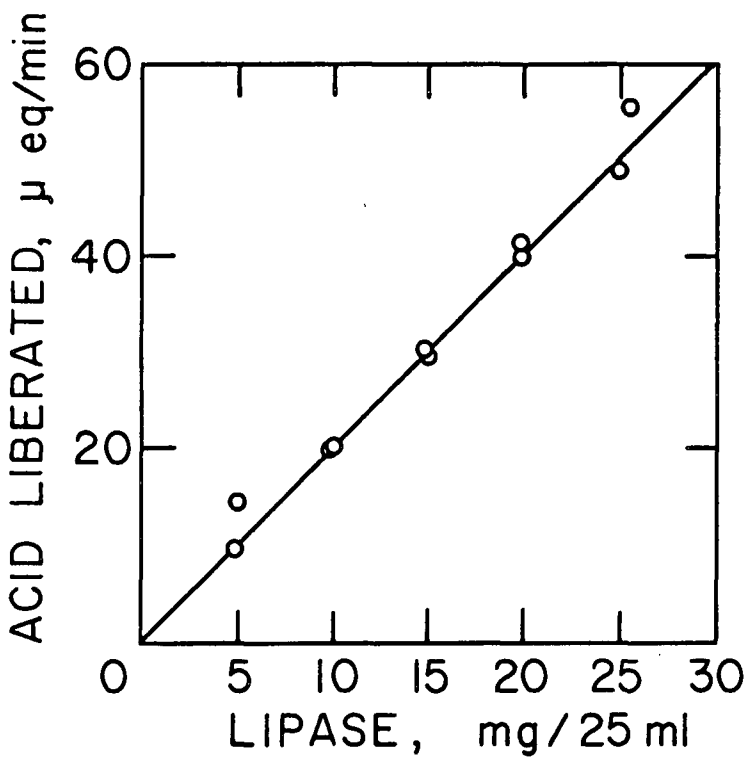


FIGURE 2.- Relationship between Lipase Concentration and Rate of Liberation of Fatty Acids.

protein, for different ionic forms may react at differing rates. Thus, at a given pH the aldehyde reactions would be equally affected if they were reacting at the same site. However, the inhibition of lipase varied with both the aldehyde and the pH (Figure 3) indicating the reaction was not proceeding in the same manner in all cases. Since formaldehyde and propionaldehyde show a different pH inhibitory curve than MA-Na, it is possible that they may be attacking different sites on the enzyme. An ionization of an inhibitor could also have affected the rate.

The control sample, containing no aldehyde, produced with pH a regular, bell-shaped curve. The inactivation in the alkaline region may be due to contaminating proteases in the enzyme preparation as well as to pH. Optimum stability of the control, at 37°C, was found to range from a pH of 6.0 to 6.5.

Of the three aldehydes MA-Na was the most inhibitory at pH values below 7. However, at pH 9 the sample containing MA-Na was more stable than the control possibly due to inhibition of contaminating proteases. MA-Na was even more inhibitory below pH 7 than equal concentrations of formaldehyde which is considered to be highly reactive. Formaldehyde was more inhibitory generally than propionaldehyde, possibly due to the smaller molecular size and the corresponding greater activity due to less steric hindrance of

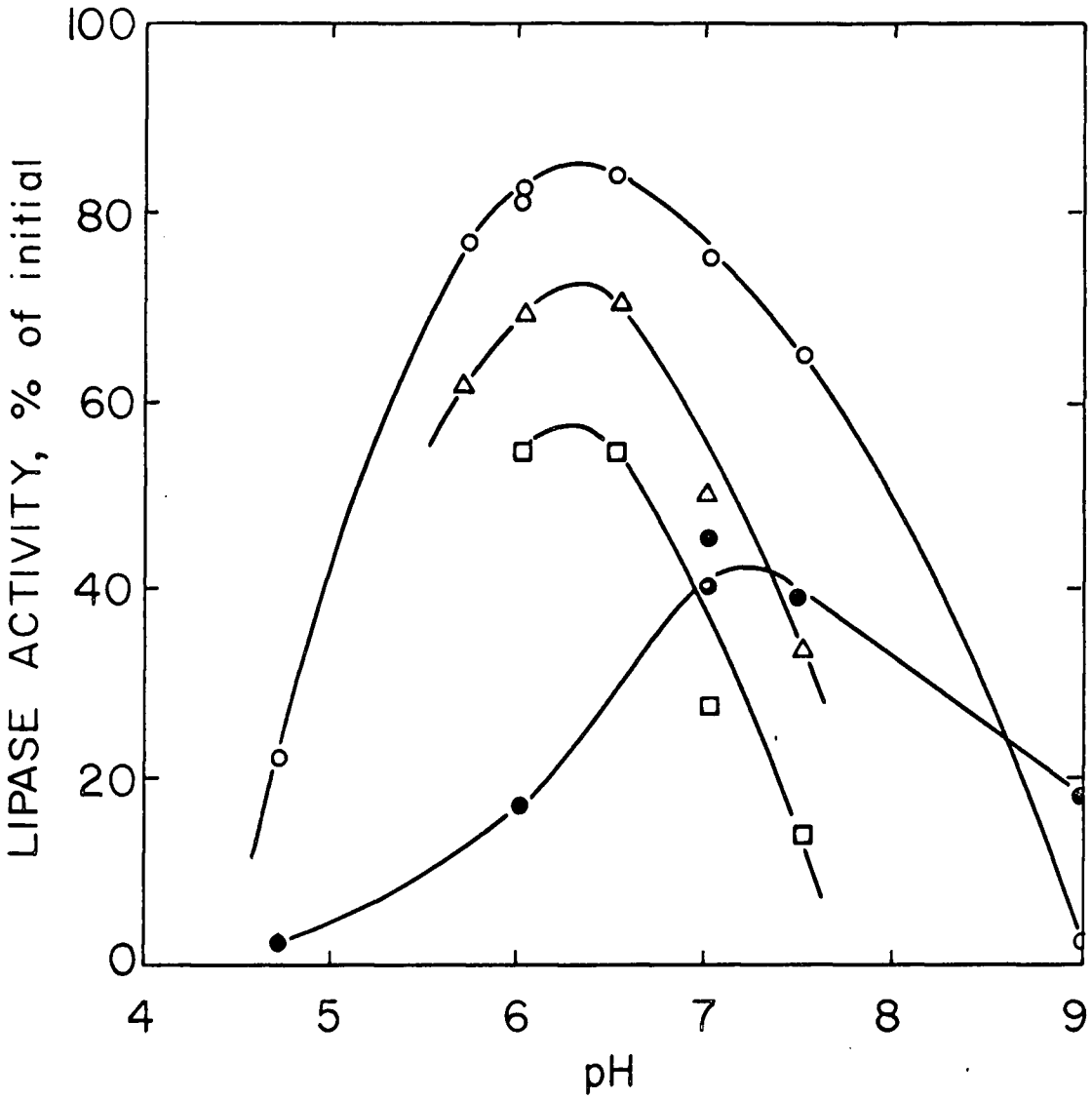


FIGURE 3.- Effect of pH on Lipase Stability after Four Hours Contact with 0.05 M Aldehyde. MA-Na (•), Formaldehyde (□), Propionaldehyde (△). Control (○).

the former. No specific pH gave formaldehyde or propionaldehyde a significantly greater inhibition for the range tested. Their inhibition curves are grossly similar to the control.

That maximum inhibition occurred near pH 6 while optimum activity was found at pH 9 may indicate that the aldehydes were not reacting with the active center of the enzyme in its active form.

With both optimum stability of the enzyme and maximal inhibition by malonaldehyde occurring near pH 6, this area was chosen for the remainder of the work. It is of interest that during active digestion in the human small intestine the pH values range from 4.5 to 5.1 in the duodenal region, to 5.9 to 6.5 in the ileum (40, p. 363; 47, p. 252; 84, p. 899).

Lipase Inhibition by MA-Na

The inhibition of lipase by MA-Na appears to follow first order kinetics (Figure 4). The rate constant, k, of the reaction, when plotted against the concentration, indicates a linear relationship between the rate of inhibition and the concentration of MA-Na used (Figure 5). Extrapolating the data in Figure 4 back to zero time indicates that the data do not pass through the origin. The deviation from the origin appears to be a function of the MA-Na concentration; however, this deviation also appears in the control (Figure 6).

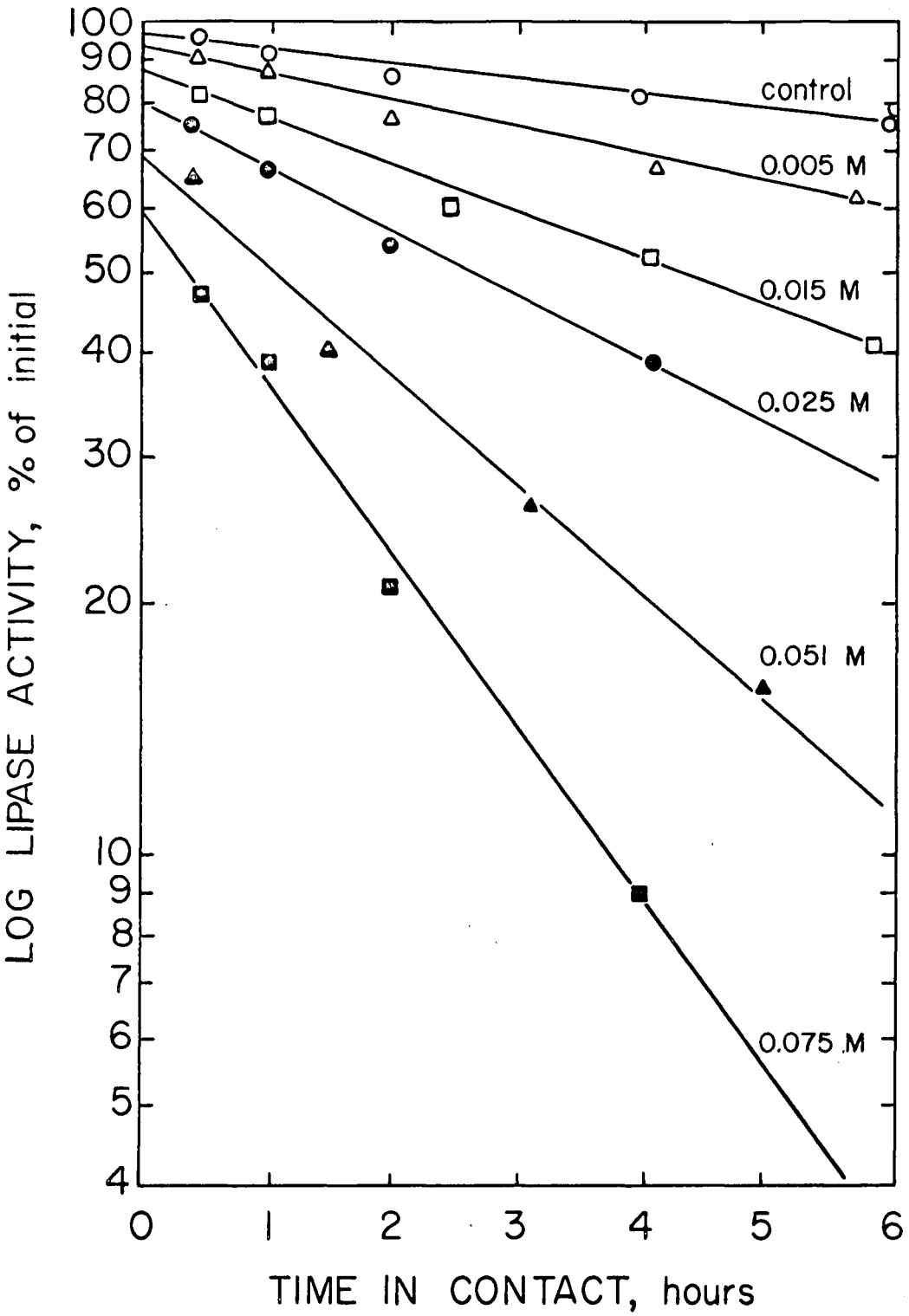


FIGURE 4.- Lipase Inhibition by MA-Na.

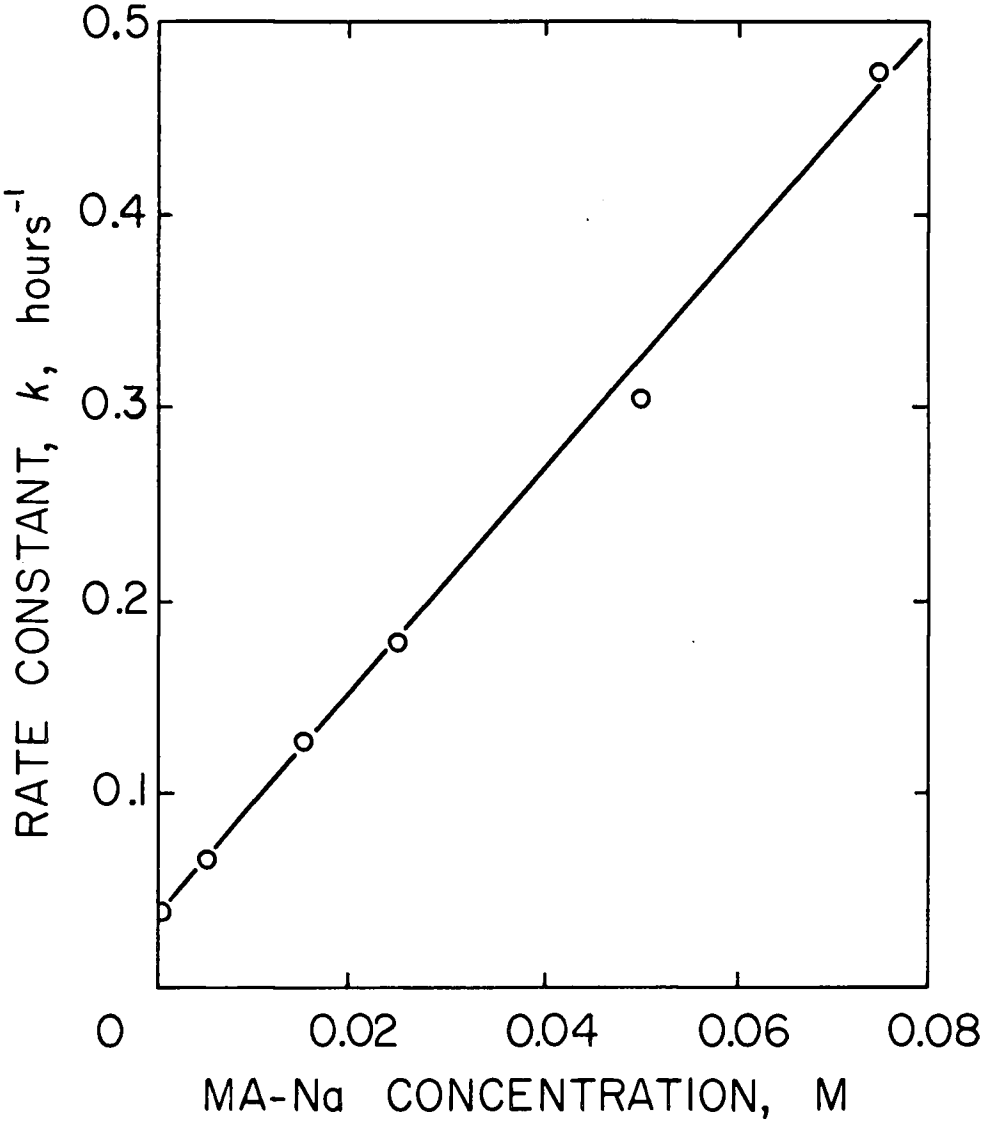


FIGURE 5.- Effect of MA-Na Concentration on Rate Constant of Reaction with Lipase.

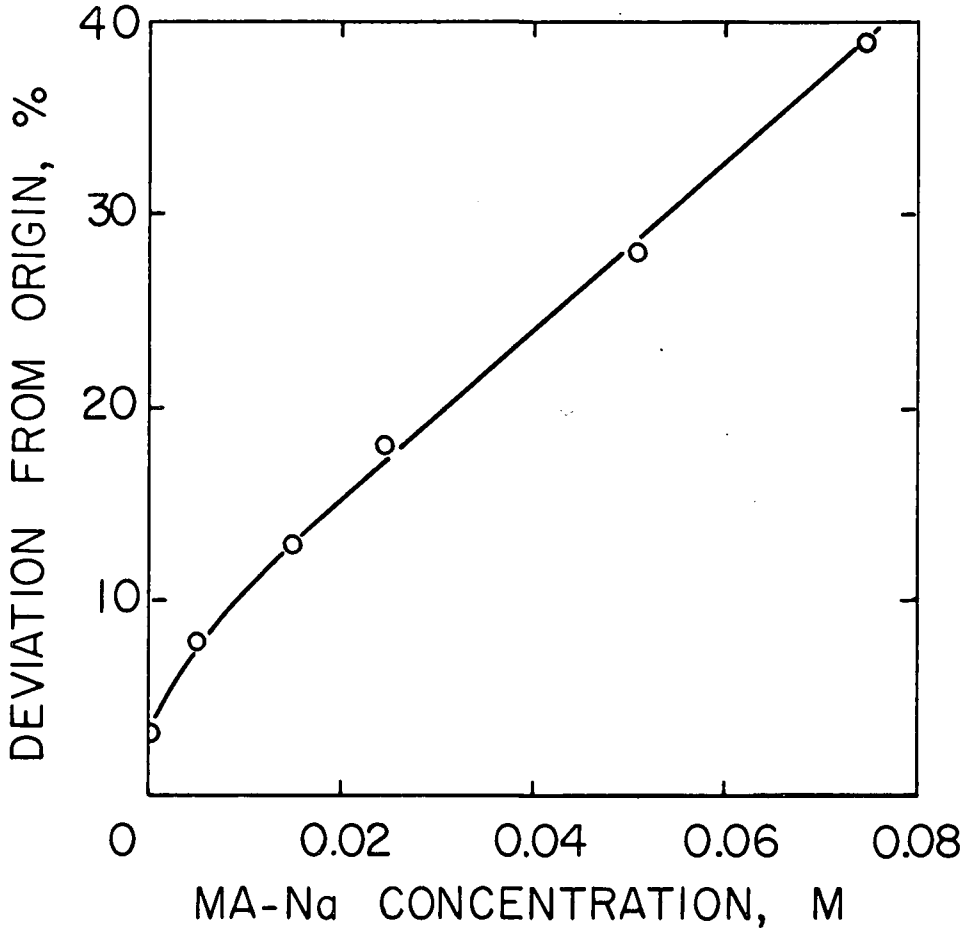


FIGURE 6.- Deviation from Origin with MA-Na Concentration.

This deviation may indicate a very rapid initial reaction which is followed by a slower reaction. This type of two stage reaction has been previously discussed by Mihalyi (60) and Fraenkel-Conrat and Olcott (27, p. 2673-2684). Details on the initial rapid reaction would have to be obtained using other methods of analysis.

A possible explanation for the reaction of MA-Na with lipase would include an initial bonding of the carbonyl, perhaps in the enol form, with an amino group, followed by a reaction with an amide to form either an inter- or intramolecular cross link. Another residue which may be involved in the second reaction would be the guanidyl of arginine. This second reaction of MA-Na would be expected to proceed more slowly due to the lower degree of reactivity of the second carbonyl function due to resonance stabilization and the steric factors involved. A reaction with sulfhydryls would also be possible (7). The non-linearity of the plot in Figure 6 of the deviation from the origin possibly is due to the accentuated effect of thermal inactivation at low MA-Na concentrations.

The action of MA-Na appears to be of an irreversible type which is "characterized by a progressive increase with time, ultimately reaching complete inhibition even with very dilute inhibitor, provided that the inhibitor is in excess of the amount of enzyme present" (23, p. 366-367). This would require an amount of inhibitor

dependent upon the number of sites of inhibition. The effectiveness of the inhibitor is then expressed by a velocity constant which determines the fraction of an enzyme inhibited in a given period of time by a certain concentration of inhibitor (23, p. 366-367).

Lipase Inhibition by Formaldehyde

Commercial formaldehyde containing ten percent methanol was shown to be much more inhibitory at equal concentrations than formaldehyde prepared without methanol from paraformaldehyde (Figure 7). Previous work on formaldehyde reactions should be considered in view of the type of formaldehyde used. When the formaldehyde is specified as meeting National Formulary or American Chemical Society specifications it contains six to 15 percent methanol as preservative (90, p. 66 and 393-394). The remainder of this work was conducted with methanol-free formaldehyde.

At low concentrations methanol-free formaldehyde appeared to be much less inhibitory than equal concentrations of MA-Na (Figure 8). Concentrations of 0.01 M had little effect on lipase activity. At concentrations greater than 0.05 M increases in concentration did not produce commensurate changes in the degree of inhibition, possibly due to the enzyme nearly reaching a saturation of groups reacting with this aldehyde. The reaction could, as with MA-Na, be two

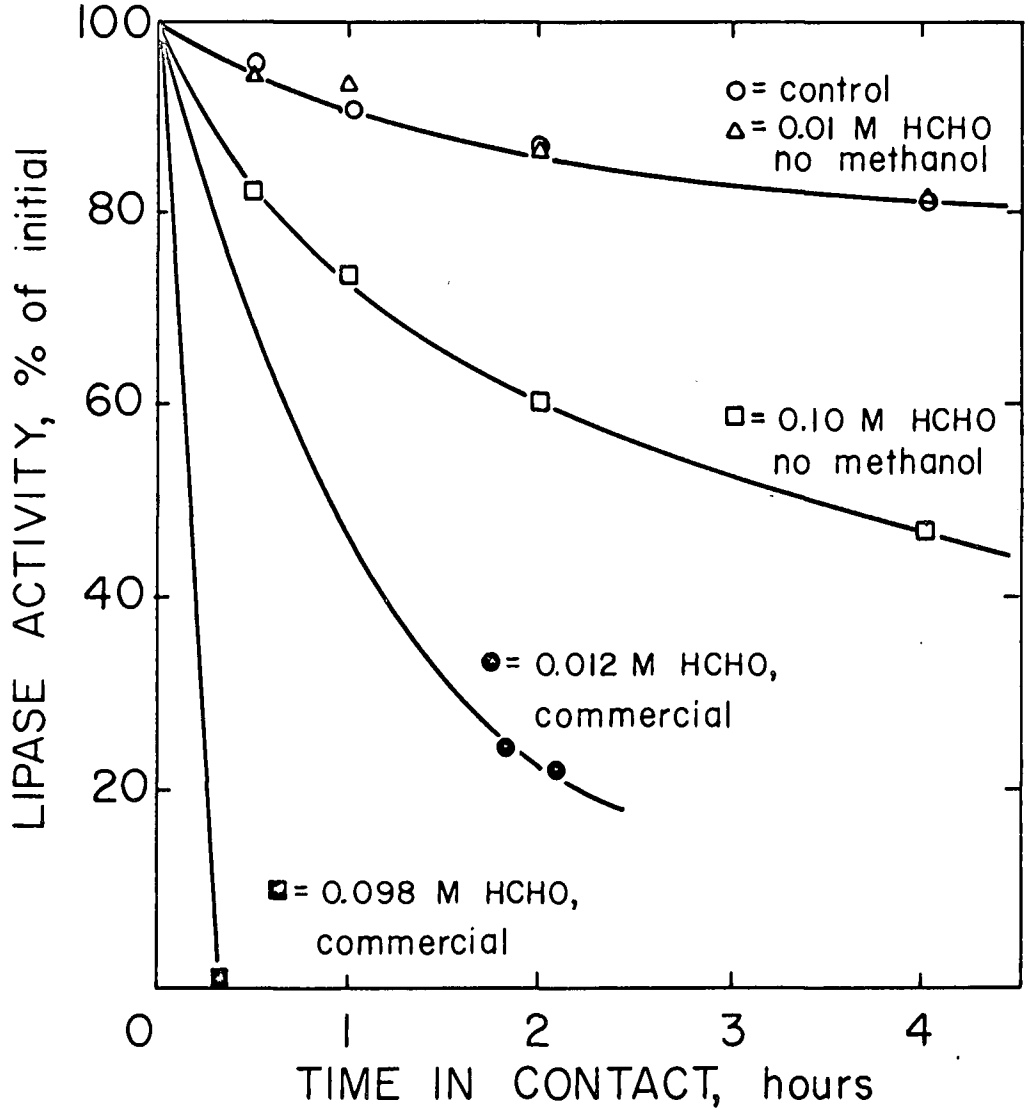


FIGURE 7.- Effect of Formaldehyde with and without Methanol on Lipase Activity.

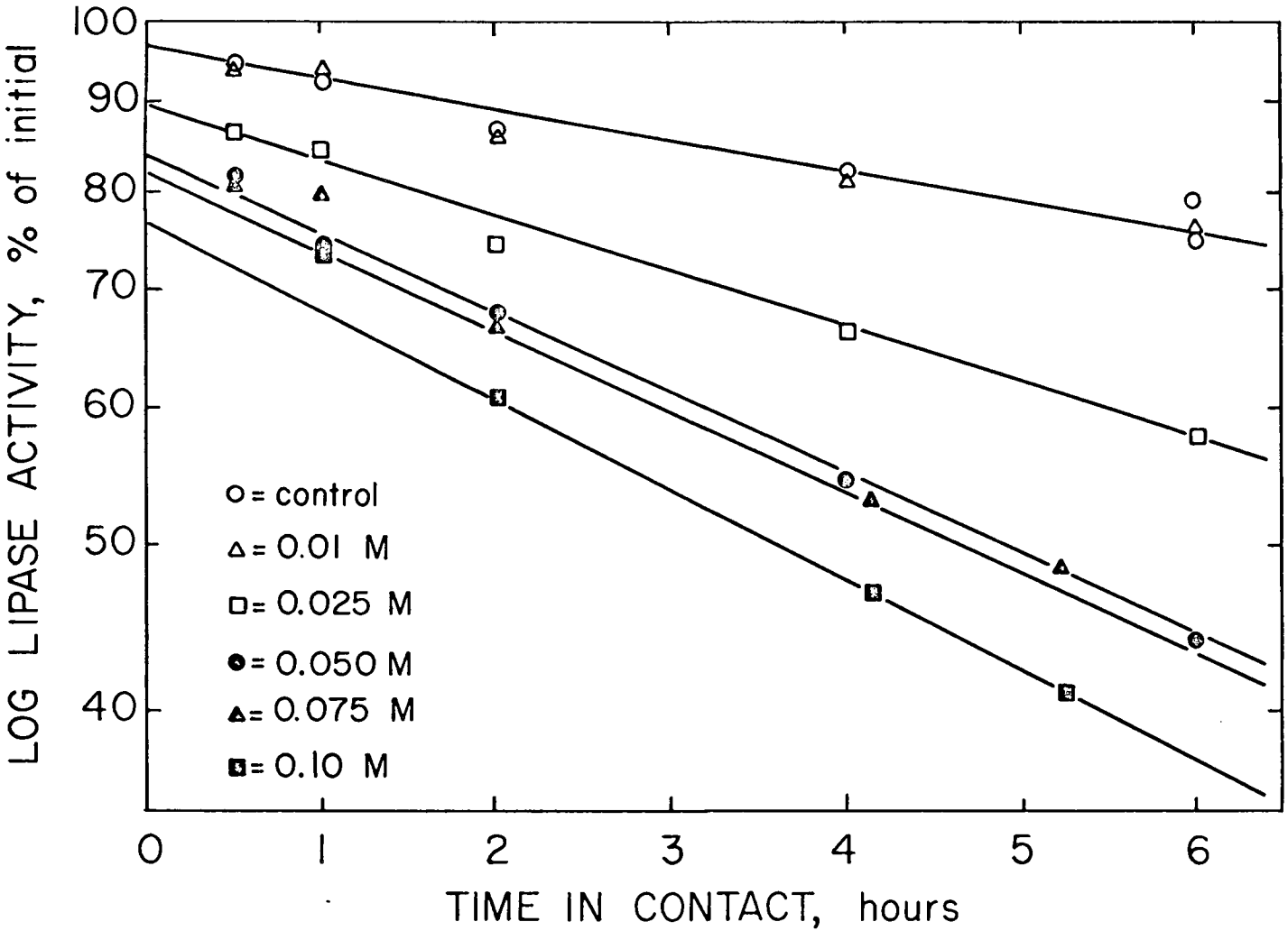


FIGURE 8.- Lipase Inhibition by Formaldehyde.

stage, but the data are not as definitive. The reaction may be more ambiguous due to the lower degree of specificity of formaldehyde.

The primary reactions of formaldehyde with protein have been given (27, p. 2673-2684) as methololamine formation by reaction with primary amines followed by methylolamine reaction with amides to form cross links. It has been further stated that simple methylol derivatives are the primary products of such reactions (90, p. 314).

Lipase Inhibition by Propionaldehyde

The action of propionaldehyde on lipase differs from that of both formaldehyde and MA-Na. From Figure 9 it can be seen that the curves of the inactivation of lipase by propionaldehyde passed through the origin, and that the reactions were proceeding at a much lower rate. The curves also show a break point occurring after about one and a half to two hours of reaction. Variations in the propionaldehyde concentration did not change the time needed to reach the change in velocity at the break point. The amount inhibited at the break point did depend upon the aldehyde concentration, as did the total inhibition at the end of six hours.

The early portion of the reaction may represent a reaction of a Schiff's base type with a particular functional group imparting some inhibition, perhaps of a steric nature. The latter portion of the

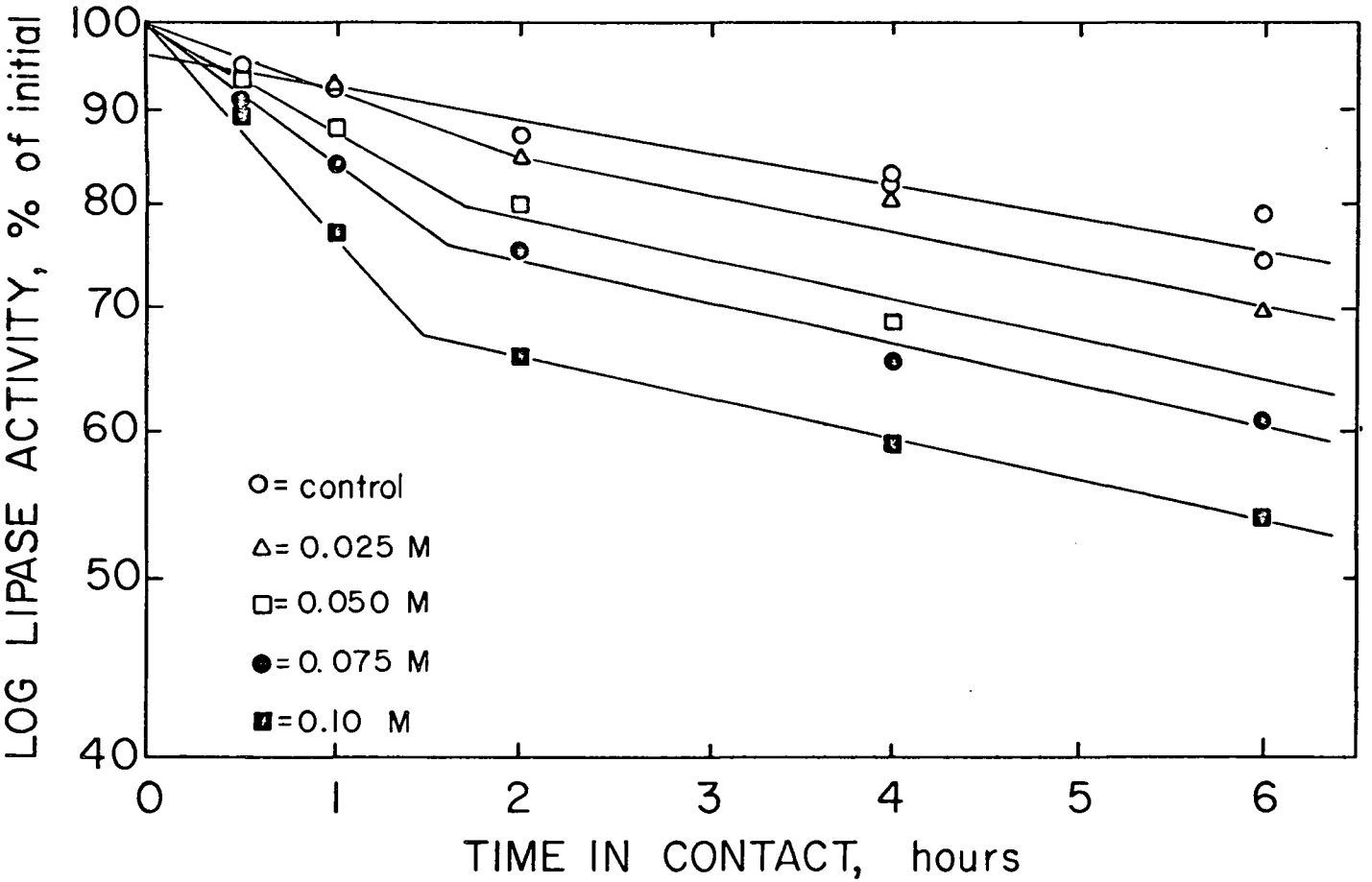


FIGURE 9.- Lipase Inhibition by Propionaldehyde.

curve where the lines are nearly parallel may represent predominantly a continuation of the thermal inhibition seen in the control.

It is evident that the inhibition imparted by propionaldehyde is not as great as that with MA-Na, its dialdehyde counterpart, for any comparable concentration.

Relative Inhibition of Lipase by MA-Na, Formaldehyde and Propionaldehyde

A tabular presentation of the effects of the three aldehydes is given on the following page. The rate constant, k, was based on the slower portion of the reaction for MA-Na and formaldehyde, and for the initial stage for propionaldehyde. The slower, second portion of the reaction for propionaldehyde nearly paralleled the control. Due to divergences from the origin for formaldehyde and MA-Na, and changes in reaction rates for propionaldehyde, determining a rate constant over the entire time period was not practicable.

From the table it can be seen that at 37°C, when lipase was reacted with the aldehydes for four hours at pH 6.0, followed by analysis at pH 9.0, MA-Na inhibited the enzyme more rapidly than formaldehyde or propionaldehyde. Based on percent of initial activity lost, formaldehyde was slightly more inhibitory than propionaldehyde.

Table I Relative Inhibition of Lipase by MA-Na, Formaldehyde and Propionaldehyde

aldehyde	conc. M	initial rapid reaction, deviation from origin, %	rate constant, k, hour^{-1}, for slower reaction	% activity lost after four hours contact
control	------	3	0.041	18.5
MA-Na	0.005	8	0.069	30
	0.0151	13	0.130	48
	0.0250	18	0.179	60
	0.0507	28	0.306	79
	0.0754	39	0.476	91
formal-dehyde	0.01	3	0.069	18.5
	0.025	11	0.073	33.5
	0.050	16	0.105	45
	0.075	18	0.106	46.5
	0.10	23	0.118	52.5
propion-aldehyde	0.025	0	0.082*	22.5
	0.050	0	0.131*	29
	0.075	0	0.171*	33
	0.10	0	0.260*	40.5

*The rate constant for propionaldehyde is based upon the initial reaction starting from the origin.

SUMMARY AND CONCLUSIONS

The effect of three aldehydes, malonaldehyde, formaldehyde, and propionaldehyde on the inhibition of lipase activity was compared. Malonaldehyde was selected because of its production during lipid oxidation and its possible in vitro and in vivo reactions with protein. Included for comparison were formaldehyde, an aldehyde known to be very reactive, and propionaldehyde, the monocarbonyl counterpart of malonaldehyde.

The lipase preparation used was found to have an activity optimum near pH 9.0. This pH was used for subsequent analyses. The relationship of the amount of enzyme present to the amount of fatty acids liberated was determined and found to be linear.

Maximum stability of the control enzyme appeared between pH 6.0 and 6.5, while maximum lability in the presence of MA-Na (the sodium salt of the enolic form of malonaldehyde) was also near pH 6.0. Formaldehyde and propionaldehyde, however, both exhibited a general non-pH-specific inhibition of lipase.

The effects of the various aldehydes on the lipase activity of a solution held at pH 6.0 and analyzed at pH 9.0 revealed that:

1. MA-Na was the most inhibitory at any given concentration. The reaction appeared to be two part. The first stage was rapid and

appeared to be a function of the MA-Na concentration. The second part was slower and was related linearly to the MA-Na concentration.

2. Methanol-free formaldehyde was a much less effective inhibitor of lipase than MA-Na. Low concentrations (0.01 M) of formaldehyde had no apparent effect on lipase activity, while increases in concentration above 0.05 did not produce commensurate changes in the degree of inhibition.

3. Propionaldehyde showed only slight inhibition of lipase activity. A break point in the reaction with time occurred at about one and a half to two hours. Following the break point the reaction nearly paralleled the thermal inactivation present in the control.

From the above it can be seen that MA-Na is capable of reactions of an inhibitory nature with an enzyme, lipase. It was more inhibitory at equal concentrations than either formaldehyde, considered a highly reactive aldehyde, or propionaldehyde. Other reactions of malonaldehyde in vitro or in vivo with other proteins could be analagous and of nutritional or physiological significance.

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