THERMAL DESTRUCTION OF FOLATE COMPOUNDS

by

A. P. MNKENI

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School of Food Science
McGill University
Montreal, P.Q.
Canada

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ABSTRACT

M.Sc. A. P. MNKENI Food Science

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First order rate constants (k) and activation energies (Ea) for the destruction of pteroylglutamic acid (PteGlu) and 5-methyltetrahydropteroylglutamic acid (5-CH_3-H_4PteGlu) were determined between 100 and 140°C in citrate buffer (pH 3-6) and in model food systems. For both compounds as pH increased from 3.0 to 6.0, the rate constants decreased. As temperature increased the rate constants also increased. Ea values for PteGlu destruction in the citrate buffer were 22.6, 19.5, 17.8 and 16.8 kcal/mole at pH 3, 4, 5 and 6 respectively. Ea values for 5-CH_3-H_4PteGlu were 19.0, 17.0, 19.7 and 19.8 kcal/mole at pH 3, 4, 5 and 6 respectively. When dissolved oxygen content was reduced to 1.7% of the saturation level, the stability of 5-CH_3-H_4PteGlu was increased substantially. Ea values ranged from 13.6 kcal/mole at pH 3 to 13.3 kcal/mole at pH 6.0.

PteGlu was more stable in apple juice (pH 3.4) than in citrate buffer. Ea values in apple juice and tomato juice (pH 4.3) were 20.0 and 19.7 kcal/mole respectively. 5-CH_3-H_4PteGlu was more stable in the buffer system than in the model food system.

RESUME

A. P. MNKENI

DESTRUCTION THERMIQUE DES COMPOSES DE L'ACIDE FOLIQUE

On a déterminé les constantes de vitesse (k) d'ordre 1 de même que les énergies d'activation (Ea) pour l'inactivation des acides ptéroyl glutamique (PteGlu) et 5-méthyltetrahydroptéroylglutamique (5-CH_3-H_4PteGlu). Les analyses furent faites entre 100 et 140°C, dans un tampon citrate (pH 3-6) ainsi que dans des systèmes "modèles" d'aliments. Pour chacun des acides une augmentation du pH de 3 à 6 correspond à une diminution des constantes de vitesse. Par contre, avec une élévation de température, on note une augmentation des constantes de vitesse. Les Ea pour la destruction de PteGlu dans le tampon citrate furent respectivement de 22.6, 19.5, 17.8 et 16.8 kcal/mole à pH 3, 4, 5 et 6. Les Ea pour 5-CH_3-H_4PteGlu furent respectivement de 19.0, 17.0, 19.7 et 19.8 kcal/mole à pH 3, 4, 5 et 6. Une réduction du contenu d'oxygène dissous à 1.7% de son niveau de saturation augmente considérablement la stabilité de 5-CH_3-H_4PteGlu. Les Ea varièrent entre 13.6 kcal/mole à pH 3 et 13.3 kcal/mole à pH 6.

On a noté une plus grande stabilité de PteGlu dans le jus de pomme (pH 3.4) que dans le tampon citrate. Les Ea dans le jus de pomme et dans le jus de tomate (pH 4.3) furent respectivement de 20.0 et 19.7 kcal/mole. 5-CH_3-H_4PteGlu s'est avéré plus stable dans le tampon que dans le "système-type" d'aliments.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure | Page

CHAPTER I

INTRODUCTION

The term folic acid is presently accepted to have two meanings. Generally, it covers the whole range of closely related pteroic acid compounds which include the monoglutamate, the polyglutamate forms and their reduced or substituted analogues. Specifically, it describes the non-reducing monoglutamic acid, i.e., pteroylglutamic acid. Pteroylglutamic acid is the basic structural unit in these compounds. According to the Commission on Biochemical Nomenclature (IUPAC-IUB Commission, 1966), the terms folic acid and folate may be used as general terms for any member of the family, and throughout this text the two terms are used interchangeably.

Investigation of the distribution of folic acid derivatives in nature has only recently become feasible with ready synthesis of polyglutamate derivatives and use of chromatographic procedures. Food materials of plant origin such as cabbage, lettuce, and orange juice have been shown to contain folic acid in the form of polyglutamates

mainly as 5-methyl-derivatives (Stokstad et al., 1973; Stokstad et al., 1977). The main derivatives in the living mammalian cell are the 5-methyl derivatives and 5-formyl derivatives (O'Broin et al., 1977).

Folic acid is an important co-enzyme factor responsible for much single carbon transfer and affects many synthetic biochemical pathways. It is involved in the formation of haemoglobin, production of purines and in reactions that synthesize thymine which is an essential component of DNA. Folate deficiency in man appears to be relatively common amongst premature infants, pregnant women, alcoholics and in tropical spure patients (Malin, 1975). Although the occurrence of deficiency is much higher in the economically poorer peoples of the world, it is also found in developed western societies. Chanarin (1969), in Britain, reported megaloblastic haemopoiesis in the marrow of 25% of pregnant women examined. The Nutrition Canada Survey reported folic acid deficiency in Canadians of all age groups (Nutrition Canada, 1974).

In 1968, the National Research Council set the Recommended Dietary Allowances (RDA) for different age groups. However, the amount of folic acid in food providing these recommended levels is variable because of uncertainty about how much is destroyed in cooking and processing. Vandermark and Wright (1972) reported that less than 10% of pregnant teenagers in the USA were meeting 1/3 of the RDA's and in nonpregnant teenagers less than 5% were meeting the RDA's. The data of Hoppner et al. (1977) suggest that it is difficult for adolescents and normal adults to obtain the recommended amounts of folic acid from an average composite diet. Consequently, folic acid

enrichment has been recommended by the National Research Council Committee on Food Standards and Fortification Policy in the U.S.A. Before any enrichment program is adopted, the nutrient added should be shown to be stable under customary conditions of processing, storage and use.

Several workers have generated data on folate levels and per cent destruction in foods given a certain heat treatment (Hoppner et al., 1977) but this kind of data does not lend itself to a quantitative description of the losses likely to occur under a variety of conditions, and besides heat, other factors have been shown to affect the stability of folates during processing. Among the factors which have been reported are source of heat (Cooper et al., 1978), pH (Blakley, 1969) and chemical environment (O'Broin et al., 1975; Paine-Wilson and Chen, 1979). The presence of oxygen has been reported as an important factor in degradation of folates during processing. Residual oxygen in milk affected significantly the stability of folacin during processing and storage (Rolls and Porter, 1973; Ford et al., 1969).

There is a general lack of quantitative data on the thermal degradation of folate compounds, particularly at temperatures above 100°C, which are temperatures commonly employed during industrial pasteurization and sterilization processes. Therefore, it appears there is need for kinetic data for thermal destruction of folic acid. The purpose of this study was:

1. To obtain destruction data (i.e., rate of destruction k at a reference temperature and the activation energy) for pteroylglutamic acid in buffer systems pH 3-6 at 100, 110, 121, 130 and 140°C.
2. To obtain destruction data for 5-methyl-tetrahydrofolate in the presence and absence of air in buffer systems pH 3-6 at 100, 110, 121, 130 and 140°C.
3. To obtain destruction data for pteroylglutamic acid and 5-methyl-tetrahydrofolate in model food systems.

These two forms of folate compounds were chosen because 5-methyl-tetrahydrofolate appears to be the major form of folate found naturally in plant tissues and pteroylglutamic acid is likely to be of considerable interest as a food fortifying agent.

CHAPTER II

LITERATURE REVIEW

a. Introduction

Folate is the term applied to a broad spectrum of substances which give rise to folacin in the body. Folacin and folic acid are respectively the more recent and original terms used to describe the various forms of the vitamin. The nutritional significance of folic acid as a vitamin was first shown in the early 1930's when Wills (1933) reported that autolyzed extracts of yeast or liver were effective in treating macrocytic anemia. During the same decade an unidentified factor in yeast, alfalfa and wheat bran was shown to promote growth of chicks maintained on purified diets. Liver and spinach were also found to contain essential nutrients for lactic acid bacteria. The isolation of a relatively pure compound was done by Hutchings et al. (1941) and Stokstad (1943). Its chemical structure was determined by the identification of its degradation products and the final proof of the structure of folic acid was obtained by its chemical synthesis.

b. Role of folic acid in metabolism

The role of folates in metabolism is associated with normal hematopoiesis and with the catalysis of chemical reactions, purine and pyrimidine metabolism leading to the synthesis of nucleic acids, particularly DNA (Henderson, 1969). As coenzymes, folates are involved in the transfer of one-carbon units such as methyl ($-CH_3$), hydroxymethyl ($-CH_2OH$), formyl (-CHO) and formimino (-CH=NH) groups from one compound to another. They also function in the conversion of ethanolamine to cholesterol, in the hydroxylation of phenylalanine to tyrosine, in the formation of porphyrin groups, in the serine-glycine conversion, in the differentiation of embryonic nervous system, the formation of active formate and methionine, choline synthesis and in niacin metabolism (Kutsky, 1973). Folic acid, together with vitamin B_{12}, plays an important role in nucleoprotein synthesis. Halsted (1975) suggested that the normal function of the small intestine may be partially regulated by dietary folates.

c. Folate deficiency

Folate deficiency is probably the most prevalent vitamin deficiency in man. In Canada, U.S.A. and U.K., low folate levels have been reported in association with such conditions of nutritional stress as pregnancy, infancy, alcoholism, and factors contributing to nutritional stress such as low family income and poor dietary habits (Keagy et al., 1975; Cook, 1977). Coleman et al. (1975) reported folate deficiency in the majority of South African rural population consuming

predominately maize diets. Factors other than improper nutrition can precipitate folate deficiency. Drugs such as oral contraceptives and anticonvulsants exert inhibitory activity on the intestinal conjugases resulting in folate deficiency (Attaway and Hill, 1972). Alcohol consumption, associated with folate and other vitamin deficiency states, probably causes these problems because of alcoholic dietary deficiencies, nutrient malabsorption, poor nutrient storage and liver damage, rather than simple ingestion of alcohol.

Folate deficiency disorders include various types of anemias: macrocytic, megaloblastic and pernicious. Attaway and Hill (1972) reported folate deficiency induced anemia to be second in frequency to iron deficiency anemias.

d. Recommended dietary allowances

The Food and Nutrition Board of the National Research Council established the Recommended Dietary Allowances (RDA's) for folacin at: 400 mcg for adults, 800 mcg for pregnant women, and 600 mcg for lactating women. For children, the RDA's are: 300 mcg for ages seven to ten years, 200 mcg for ages four to six, 100 mcg for ages one to three and 50 mcg for infants.

In many parts of the world suboptimal nutrition is responsible for a high incidence of folate deficiency in the community, particularly during periods of increased physiological demand such as pregnancy and lactation. Data of Hoppner et al. (1977) suggest that it is difficult for adolescents and normal adults to obtain these RDA's from an average

composite diet. Consequently, folacin enrichment of cereal products has been recommended by the National Research Council Committee on Food Standards and Fortification Policy. Folacin enrichment of staple foods has been suggested in other countries as well (Barker et al., 1974). Before any enrichment program is adopted, the nutrient added should be shown to be stable under customary conditions of processing, storage and use.

e. Folate nomenclature

According to the Commission on Biochemical Nomenclature (IUPAC-IUB Commission, 1966), folic acid and folate are used as general terms for any member of the family. Figure 1 shows the parent compound which is pteroic acid. Its salts and radicles are named pteroates and pteroyl. The numbering of the atoms is as shown in Figure 1 (Blakley, 1969) and numbering of the 9th and 10th atoms is necessary to assist the naming of derivatives of the vitamin.

When pteroic acid is conjugated with one molecule of L-glutamic acid the compound is called pteroylglutamic acid (Figure 3a). The compound formed when it is linked to two molecules of glutamic acid is called pteroyldiglutamic acid, etc. The link between second and subsequent glutamic acid molecules is through the γ-carboxyl group to the amino group as shown in Figure 2. Names of reduced compounds are preceded by "dihydro" or "tetrahydro" with numericals indicating the position of the hydrogen atoms if these are known. The common substituents and their abbreviated formulae are shown in Table 1.

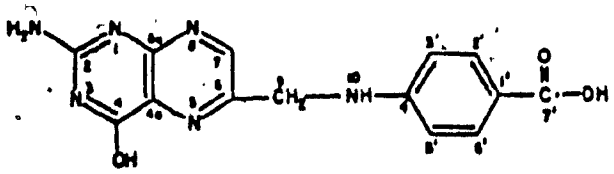

Figure 1. Pteroic acid.

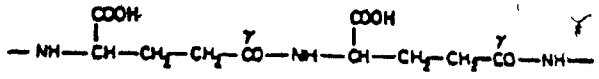

Figure 2. Manner of linking glutamic acid residues in polyglutamyl forms of folate.

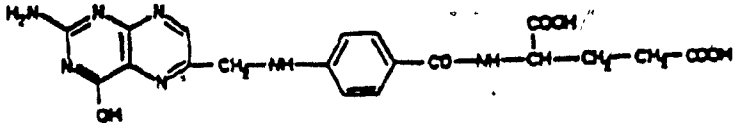

Figure 3a. Pteroylglutamic acid.

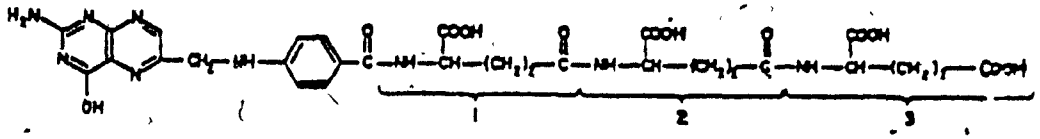

Figure 3b. Pteroyltriglutamic acid.

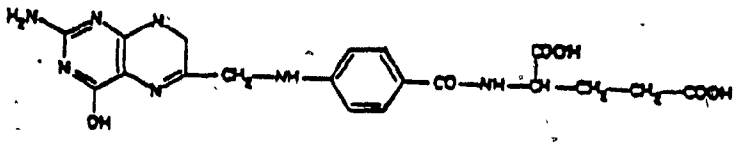

Figure 3c. 7,8-dihydropteroylglutamic acid.

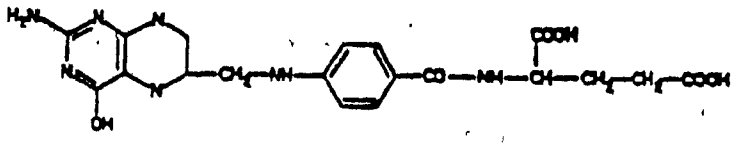

Figure 3d. 5,6,7,8-tetrahydropteroylglutamic acid.

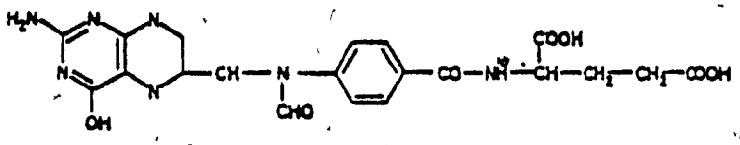

Figure 3e. 10-formyl,5,6,7,8-tetrahydropteroylglutamic acid.

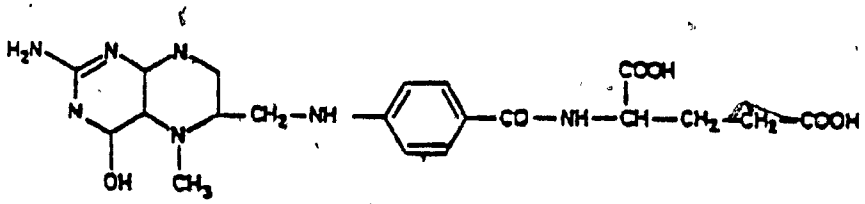

Figure 3f. 5-methyltetrahydropteroylglutamic acid.

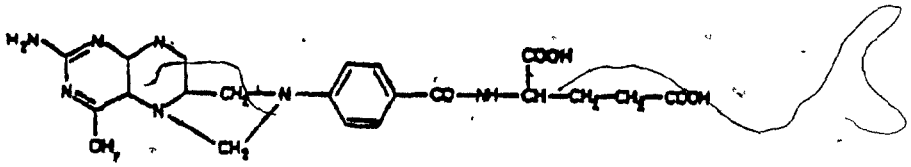

Figure 3g. 5,10-methylenetetrahydropteroylglutamic acid.

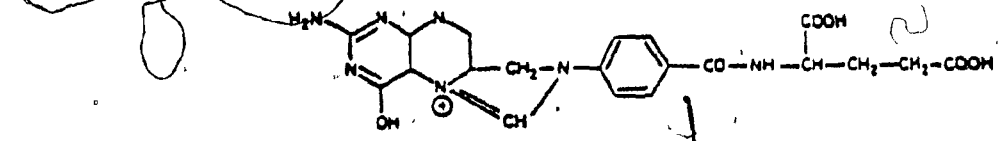

Figure 3h. 5,10-methenyltetrahydropteroylglutamic acid.

TABLE 1. Abbreviations of some folic acid compounds

Folate compound	Abbreviation
Pteroic acid or pteroate or pteroyl	Pte
Pteroylglutamic acid	PteGlu
Pteroyldiglutamic acid	$PteGlu_2$
Dihydropteroylglutamic acid	$7,8\text{-}H_2PteGlu$
Tetrahydropteroylglutamic acid	$5,6,7,8\text{-}H_4PteGlu$
10-formylpteroylglutamic acid	10-CHO-PteGlu
5-formyltetrahydropteroylglutamic acid	$5\text{-}HCO\text{-}H_4PteGlu$
5-methyltetrahydropteroylglutamic acid	$5\text{-}CH_3\text{-}H_4PteGlu$
5-formiminotetrahydropteroylglutamic acid	$5\text{-}CHNH\text{-}H_4PteGlu$
5,10-methenyltetrahydropteroylglutamic acid	$5\text{-}10\text{-}CH{=}H_4PteGlu$
5,10-methylenetetrahydropteroylglutamic acid	$5,10\text{-}CH_2H_4PteGlu$

The structures of some of these compounds are shown in Figures 3c-3h. The abbreviated formulae are the ones recognized by the Commission on Biochemical Nomenclature.

f. Properties of folic acid and its derivatives

Many of the chemical and physical properties of folic acid are predictable from the fact that it is a 6-alkylpterin. Like most pterins, folic acid is stable to alkali under anaerobic conditions, but alkaline hydrolysis under aerobic conditions cleaves the sidechain to yield p-aminobenzoylglutamic acid (p-ABG) and pterin-6-carboxylic acid (Stokstad et al., 1948). Interactions of folic acid with sulfite and nitrite which are chemicals involved in food processing has received some attention. Treatment with sulphurous acid leads to the cleavage of the side chain, with the liberation of a reduced pterin-6-carboxaldehyde and p-ABG (Blakley, 1969). Nitrous acid reacts in the cold with folic acid to give the 10-nitroso derivative (Cosulich et al., 1949). Polyglutamate derivatives of folic acid (conjugates) are hydrolysed by alkali under anaerobic conditions to yield folic acid and glutamic acid (Stokstad et al., 1948). Photodecomposition of folic acid solution by sunlight to yield p-ABG and an unidentified pterin was first reported by Stokstad et al. (1947). The unidentified pterin was later found to be pterin-6-carboxaldehyde (Blakley, 1969). Exposure to ultraviolet light results in rapid cleavage of the molecule at the C^9-N^{10} position to give a pteridine and a free aromatic amine.

Folic acid and many of its derivatives can easily be reduced by a variety of agents to the corresponding dihydro or tetrahydro derivative. Reduction of folic acid in acid solution, particularly if zinc is used as a catalyst, is followed by a split at the C^9-N^{10} bond. With platinum oxide as a catalyst in alkaline solution, folic acid is reduced to dihydrofolic acid (O'Dell et al., 1947) and in neutral solution it is further reduced to tetrahydrofolic acid.

The oxidative degradation of a few folate compounds has been studied under various conditions. Chippel and Scrimgeour (1970), studying the oxidative degradation of H_4PteGlu and H_2PteGlu under anaerobic conditions at pH 5.6 using ferricyanide, reported that the products were pterin, dihydroxanthopterin, folate and 6-formyldihydropterin. The pathway observed is shown in Figure 4. They suggested that the oxidation of tetrahydrofolate by ferricyanide occurs via two-one electron step to produce quinonoid-dihydrofolate (Figure 5). The unstable quinonoid dihydrofolate then undergoes acid-catalyzed breakdown to yield dihydropterin, p-ABG and formaldehyde. Blakley (1957), examining products of oxidation in air of both H_2PteGlu and H_4PteGlu, found small amounts of folic acid and large amounts of p-ABG, but could only account for a small proportion of pteridines as xanthopterin. On the other hand, Archer and Reed (1980), studying the oxidation of H_4PteGlu in air, reported that there was no folic acid seen as product. The products of oxidation of tetrahydrofolate in air, as identified by Archer and Reed (1980), are shown in Table 2.

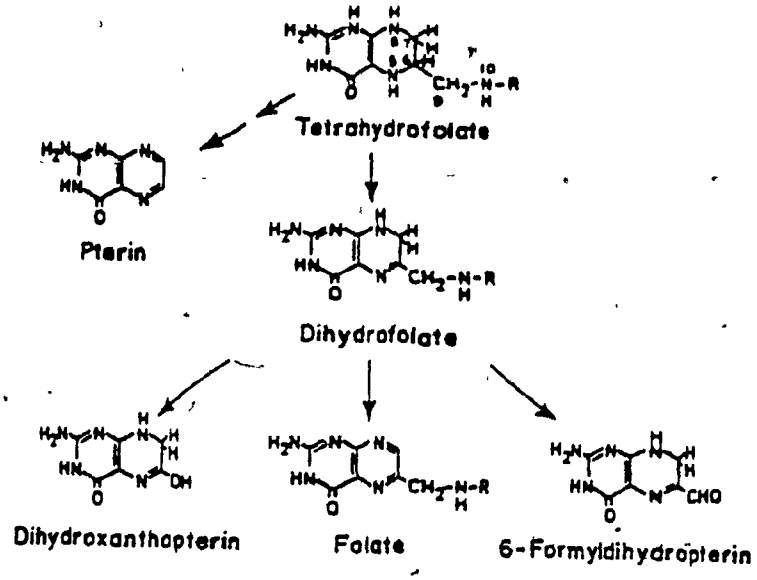

Figure 4. Summary of the pathways for degradation of tetrahydrofolate and 7,8-dihydrofolate.

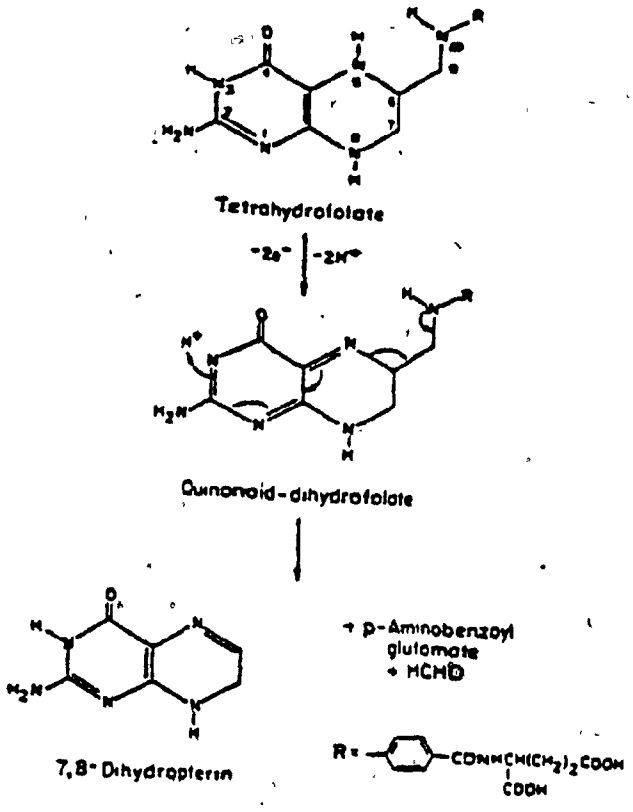

Figure 5. Mechanism of formation of dihydropterin from tetrahydrofolate.

TABLE 2. Yields of pterins in the oxidation of H_4PteGlu by air[1]

Product	pH 4 8 hours	pH 7 7 hours	pH 10 4.5 hours
6-formylpterin	18	57	78
xanthopterin	3	8	11
dihydropterins	30	16	3
pterin	49	19	8

[1]Results expressed as a percentage of total pterins produced.

Pterin was the major pteridine product at pH 4 but small amounts of pterin were produced at pH 7 and pH 10. 6-formylpterin was the major product at pH 7 and pH 10, while only a small amount of this compound was formed at pH 4. These results indicate that the mechanism of oxidation changes with pH. At pH 7 and 4, H_4PteGlu was degraded to p-ABG and pterin products, so under these conditions the vitamin activity will be completely lost.

The formation of 6-formylpterin at pH 7 was explained by a mechanism involving electron abstraction at N^{10} position (Figure 6). The N^{10} nitrenium ion formed by extraction of two electrons loses a proton from C^9 to yield the Schiff base. The Schiff base would then hydrolyze to yield p-ABG and 6-formyltetrahydropterin. Further oxidation would yield 6-formylpterin. At pH 10, loss of proton from N^3 position in the pterin ring (Kallen and Jencks, 1966) facilitates electron abstraction from the ring.

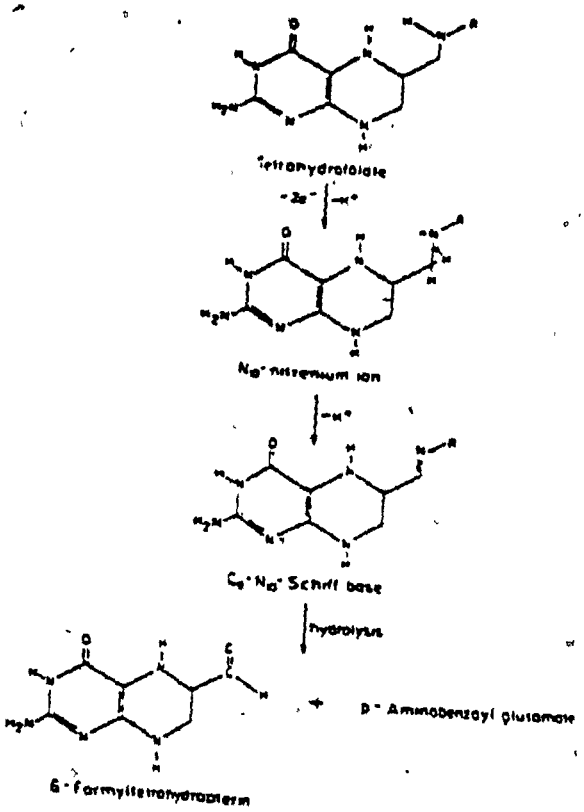

Figure 6. Mechanism of formation of 6-formyltetrahydropterin from tetrahydrofolate.

Lewis and Rowe (1978) reported that the oxidation by potassium permanganate effectively cleaved the C^9-N^{10} bond of PteGlu, H_2PteGlu, H_4PteGlu and 5-CHO-H_4PteGlu to yield p-ABG. 5-CH_3-H_4PteGlu was oxidized to 5-CH_3-H_2PteGlu. O'Broin et al. (1975) reported that at acidic pH the loss of microbiological activity for 5-CH_3-H_4PteGlu during heating could be due to formation of an unrecognized nutritionally inactive derivative rather than the oxidative cleavage at the C^9-N^{10} bond resulting in separation of the pteridine and p-aminobenzoyl ring. The 5-CH_3-H_2PteGlu has been found not effective in supporting the growth of L. casei (Blakley, 1969).

g. Methods of assaying for folates

Folate activity evaluation has required the development of both chemical and biological assay methods. In general, pure or pharmaceutical preparations contain sufficient folate to allow chemical assay; however, in biological materials having folate levels in the range of 100 µg/100 g material, chemical methods are unsuitable because of the low levels and interference from the complex chemical matrix of the biological material. In this latter case, fluorometric or more usually microbiological assays are employed. Recently the chromatographic technique of high pressure liquid chromatography has been attempted.

1. Chemical methods

The cleavage of pteroylglutamic acid (PteGlu) to produce p-aminobenzoic acid or other related moieties is the basis of several chemical methods to determine folic acid. They are used extensively to assay high potency pharmaceutical products. Reduction with titanous chloride, zinc or zinc amalgam forms p-aminobenzoyl glutamic acid. Oxidation with permanganate liberates p-aminobenzoic acid. Either species is then diazotized with nitrous acid and coupled with naphthyl-ethylenediamine and then the absorbance of the intensely coloured compound formed is determined. This method is unsuitable for natural materials since results are not dependable at low concentrations of folic acid (Blakley, 1969). Also, the determination is not specific as the colour is formed by all amines and consequently cannot be recommended for biological materials (Kuoblock and Cerna-Heyrovska, 1979).

2. Chromatographic methods

The multiplicity of naturally occurring chemical forms of folates and the lack of technology to measure these forms individually stimulated the application of chromatographic methods. Column, paper and thin layer chromatography have been used extensively for the separation and quantitative determination of folic acid derivatives. These time-consuming methods involve careful extraction from biological material at a suitable temperature in the presence of ascorbate to protect labile reduced derivatives (Noronha and Silverman, 1962). The extract is then fractionated on a column of DEAE-cellulose, elution

being effected by phosphate buffer which contains mercaptoethanol or ascorbate (Silverman et al., 1961). The eluate is collected in fractions which are then assayed by microbiological methods. Recently high pressure liquid chromatography (HPLC) has been used to identify naturally occurring folates. Although this technique has been shown to be sensitive and reproducible for routine analysis of naturally occurring folates (Clifford and Clifford, 1977), there are some difficulties of short column life, pH sensitivity and lengthy column regeneration times.

3. Fluorometric methods

Allfrey et al. (1949) developed a method of analysis whereby alkaline permanganate was utilized to oxidize folates to produce the strongly-fluorescent 2-amino-4-hydroxypteridine-6-carboxylic acid. The increment in intensity of fluorescence at 450 nm when excited with light of wavelength 365 nm is directly proportional to folate concentration. Various pigments may interfere, and are removed by chromatographic absorption. Duggan et al. (1957) reported that folate concentrations as low as 10 µg/ml may be assayed by their method. Kavanagh (1963) reported that high concentrations of tyrosine or tryptophan may give erroneously high results.

On the other hand, Rouseff (1979), using liquid chromatography and fluorescence detector, reported that folic acid does not fluoresce. Based on their findings, they suggested that fluorescence cannot be used directly to measure amounts of folic acid, but can be used to measure the amounts of oxidized folic acid.

4. Enzymatic methods

Various enzymatic methods for the determination of folate compounds have been suggested by Jaenicke (1971). These methods are applied when identification of folate derivatives is sought. These methods are more specific than chemical methods and are quicker than microbiological assays. The enzymes used are those which catalyze the interconversion of folate derivatives such as tetrahydrofolate formylase or methylenetetrahydrofolate hydrogenase used in determination of H_4PteGlu.

5. Animal methods

Chick assay methods of O'Dell and Hogan (1943) have been used to assay folates. Rats have also been used for the estimation of folate content in biological materials. The main disadvantage of animal assays is that they are expensive and time consuming. They are not sensitive enough to determine the total folate activity of foods but they can provide a comprehensive assessment of the nutritional value of a food as a source of folate.

6. Radioassay methods

The radioassay technique has been well described by Mincey et al. (1973). This method is faster, simpler and more accurate than microbiological method (Ruddick et al., 1978). The disadvantage is that it is only suitable for measuring singular forms of folate using standard curves constructed from data obtained using the same folate. Also, the method requires expensive instrumentation.

7. Microbiological methods

The growth promoting effect of folate derivatives for various bacteria played an important part in the discovery of folates, and bacteria have been used extensively for the assay of folate derivatives in natural materials. Three organisms have been commonly employed: Lactobacillus casei, Streptococcus faecalis and Pediococcus cerevisiae. These organisms do not respond in the same manner to the different forms of the vitamin. Cooperman (1967) emphasized that the choice of microorganism for any assay must take into consideration the type of folates likely to be present in the sample.

L. casei generally gives the highest results in assays and has thus been extensively used. It will respond to the mono, di and triglutamate forms of folic acid and to the reduced analogues. Herbert and Bertino (1967) pointed out that S. faecalis also grows well in the presence of pteroic acid and the failure to recognize this can lead to erroneously high results for folates in a food, since pteroic acid is metabolically inactive for man. Also, S. faecalis does not respond to methyl folate which is utilized by man. P. cerevisiae responds only to tetrahydroforms of folic acid. Table 3 summarizes the usual response of the three organisms to the more commonly occurring folates.

Folates often predominate in nature as polyglutamate conjugates (Dong and Oace, 1973). The organisms commonly used to determine folate derivatives are not capable of responding to those derivatives in which more than two glutamic acid residues are linked to folic acid (Table 3). Therefore it is necessary to remove the additional

TABLE 3. Relative activities of various folates as growth factors for 3 microorganisms

	P. cerevisiae	S. faecalis	L. casei
Reduced PteGlu derivatives except 5-CH_3H_4PteGlu	+	+	+
PteGlu	-	+	+
$PteGlu_2$	-	+	+
$PteGlu_3$	-	+	+
5-CH_3-H_4PteGlu	-	-	+
Pteroic acid	-	+	-

Blakley (1969)

glutamate moieties if these compounds are to be estimated microbiologically. Two enzymes termed conjugase are commonly used, namely carboxypeptidase found widely in nature, especially in hog kidney and rat liver; and γ-glutamic acid carboxypeptidase which is isolated from chicken pancrease. Hog kidney conjugase has a pH optimum at 4.0 to 4.5 (Bird et al., 1946) and the optimum temperature for its action is 45-48°C. It degrades heptaglutamate to monoglutamate. Chicken pancrease enzyme has a pH optimum at pH 7.8 and a temperature optimum at about 32°C. It degrades heptaglutamate to diglutamate. Hog kidney conjugase is more difficult to isolate (therefore expensive) than chicken pancrease, so in most cases chicken pancrease is commonly used.

In the folate literature the terms free and total folate are used quite often. Free folic acid represents the activity of L. casei prior to conjugase treatment. Total folate represents the activity

present after conjugase treatment. Substracting the free folic acid activity from the total folic acid activity, one obtains the conjugated folic acid activity.

Toennies et al. (1956) reported that ascorbic acid, when added to the extraction solvent, prevented the destruction of heat labile reduced forms of folic acid. Herbert (1961) confirmed that ascorbic acid was needed to protect the reduced folates during the assay of folates.

Presently, the accepted procedure for folacin assay is the L. casei assay. The AOAC (1980), however, still recommends S. faecalis as the appropriate microorganism. This has resulted in some confusion in the literature. The major advantage of the L. casei assay is its sensitivity to all folates which exhibit vitamin activity. There are no complicated extractions and separations required. While considerable time is required, it is not as time consuming as some of the other techniques.

h. Natural occurrence of folates

The distribution of folates in nature is variable to a great extent, not only in regard to substitution in the pterin ring, but also with regard to the identity and proportion of polyglutamates (Blakley, 1969). Difficulties are encountered in studying the naturally occurring folates due to the lability of many of these compounds, particularly the reduced derivatives. In the past there has been confusion concerning the natural occurrence of folates due to the lack of specificity of the microbiological assay procedures commonly employed

in attempts to identify and estimate various folate compounds, coupled with the fact that none of the test microorganisms responds directly to the folate derivatives which are now known to occur in largest amounts. Paper chromatography readily permits the separation of the folates which are present in extracts of biological materials but, again, the problem here is that many components usually resemble each other very closely in chemical properties, all being highly polar, polybasic acids (Blakley, 1969).

More recently, DEAE chromatography (Santini et al., 1964) and high performance liquid chromatography (Clifford and Clifford, 1977) have been used to identify the different forms of folates in food. Table 4 shows the percentage of different forms of folates found in a few foods. Dong and Oace (1973) observed that over 95% of the total folate activity in orange, grapefruit, lemonade and grape juice was methyl folate. Organ meats have been reported to be rich in folates; for example, pork liver contains 144.3 µg folic acid per 100 g (Malin, 1975), but the amounts of different folate derivatives have not been identified yet. The main type of folate derivative in chicken liver was found to be 5-CH_3-H_4PteGlu (Noronha and Silverman, 1962). Other derivatives found were 5-CHO-H_4PteGlu and 10-CHO-H_4PteGlu. In human milk the main folate form was found to be 5-CH_3-H_4PteGlu (Blakley, 1969).

Clifford and Clifford (1977), using high performance liquid chromatography, showed that the only folate in apple juice was 5-CH_3-H_4PteGlu. Folate profiles of almonds, wheat germ and raw peanuts

TABLE 4. Distribution of folates in foods

Food	Folate forms		Reference
Cabbage	51.7%	5-CH_3-H_4PteGlu$_7$	
	31.7%	5-CH_3-H_4PteGlu$_6$	Stokstad et al. (1977)
	7.4%	5-CH_3-H_4PteGlu$_8$	
	3.0%	5-CHO-H_4PteGlu$_7$	
Orange juice	40-50%	5-CH_3-H_4PteGlu$_5$	
	15-30%	5-CH_3-H_4PteGlu$_2$	"
	30-40%	5-CH_3-H_4PteGlu	
Milk	90-95%	5-CH_3-H_4PteGlu	
	2-3%	5-CHO-H_4PteGlu	"
	1-2%	10-CHO-H_4PteGlu	
Lettuce	34%	5-CH_3-H_4PteGlu$_5$	Chan et al. (1973)
	33%	5-CH_3-H_4PteGlu	
	22%	5-CH_3-H_4PteGlu$_2$	

showed that the predominant folates in these foods were 5-CH_3-H_4PteGlu and H_4PteGlu. This limited data would suggest that the major form of folates occurring naturally is 5-CH_3-H_4PteGlu$_{(n)}$.

i. Kinetics of vitamin destruction

Many investigators have shown how thermal processing inactivates nutrients (Bender, 1966; Lund, 1975). Although evidence would indicate that vitamins are very heat sensitive, few studies have been done to determine the kinetic parameters which describe reaction rate (k) and its dependence on temperature (Arrhenius activation energy, Ea).

Several mathematical procedures have been developed to aid in the prediction of nutrient retention in thermal processing (Ball and Olson, 1957; Hayakawa, 1969). Fortunately, a majority of reactions occurring in foods can be described by well established kinetic equations. The thermal destruction of microorganisms, most nutrients, quality factors such as colour and flavour, and enzymes generally can be described by first order reaction kinetics. Expressing the first order response mathematically gives

$$\frac{-dC}{dt} = kC \qquad \ldots (1)$$

where

$\frac{-dC}{dt}$ is the rate at which concentration decreases

C is the concentration of nutrient

k is the first order reaction rate constant.

Integrating equation (1) between limits C_o at time $t_1 = 0$ and C at time t results in

$$-\int_{C_o}^{C} \frac{dC}{C} = k\int_{t_1}^{t} dt$$

$$-\ln C + \ln C_o = k(t - t_1)$$

or

$$\log C = \log C_o - \frac{kt}{2.303}$$

$$kt = 2.303 \log \frac{C_o}{C} \qquad \ldots (2)$$

Equation (2) is the kinetic equation for a reaction of the first order. A plot of the log of the concentration ratio (or per cent retained) versus time (Figure 7) is a straight line of slope $\frac{-k}{2.303}$, thus permitting determination of the rate constant.

The slope of the line can also be expressed in terms of a D value. A D value is defined as the time required to destroy 90% of the nutrient originally present or the time required for the curve to traverse one log cycle (Figure 7).

$$\frac{\log a - \log b}{D} = \frac{-1}{D}$$

Since the slope is also $\frac{-k}{2.303}$, then

$$\frac{-k}{2.303} = \frac{-1}{D}$$

or

$$D = \frac{2.303}{k} \qquad \ldots (3)$$

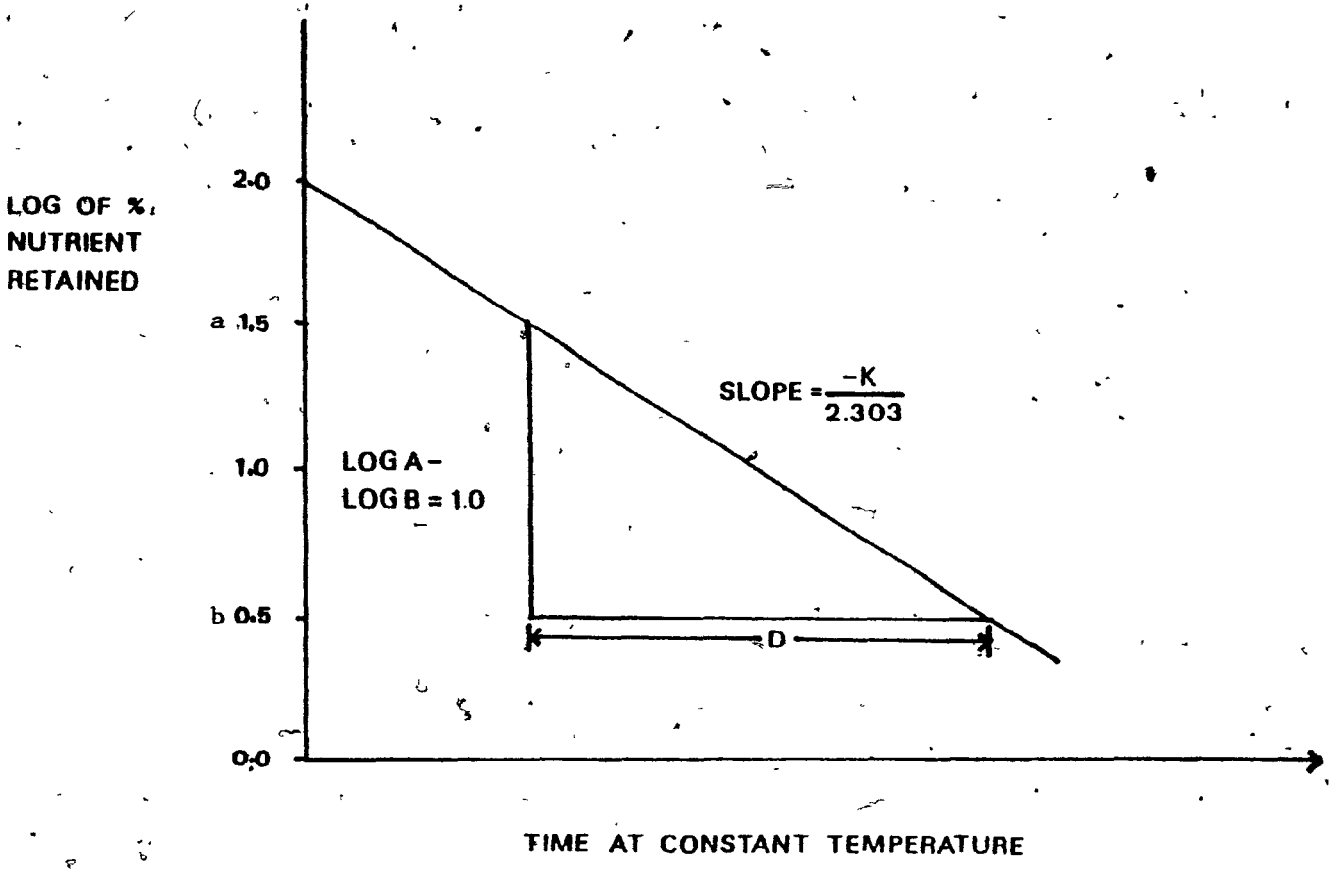

Figure 7. The logarithmic retention curve.

Many actual methods of process calculations utilize kinetic data in the form of a D value, which can be related to 1st order rate constant as shown in equation (3).

j. Effect of temperature on reaction rate

The rates of most chemical reactions increase as temperature increases. As a general and very approximate rule it is often stated that an increase in temperature of 10°C doubles the reaction rate. There are two principal methods of describing the temperature dependence of the reaction rate constant: (1) the Arrhenius equation and (2) thermal destruction time curves (Lund, 1975). The dependence of reaction rate constants on temperature as described by the Arrhenius equation is:

$$k = e^{-Ea/RT} \quad \ldots (4)$$

where

k = reaction rate constant

e = constant

Ea = activation energy

R = gas constant

T = absolute temperature

The activation energy is the energy required to get the molecules into active state. It may be determined by taking logarithms of both sides of equation (4).

$$\ln k = -Ea/RT$$

Therefore, a plot of ln k versus $\frac{1}{T}$ is a straight line of slope $-\frac{Ea}{R}$, and 'Y' intercept = ln e.

From D values obtained at different temperatures, it is possible to derive a thermal destruction time (TDT) curve. A curve of this type which is obtained by plotting the log of D values against corresponding temperatures defines the thermal destruction characteristics at given temperature as well as the sensitivity of the material to changes in temperature (Figure 8). The term Z employed in process calculation methods to account for the relative resistance of nutrients to different temperatures is equal numerically to the number of degrees Fahrenheit required for the TDT curve to traverse one log cycle.

$$\frac{\log D_2 - \log D_1}{Z} = \frac{-1}{Z}$$

Since the slope of the TDT curve (-1/Z) is used to characterize the dependence of the reaction rate constant on temperature it is related to Ea. Reactions that have small Z values are highly temperature dependent, whereas reactions with large Z values are less influenced by temperature.

k. Kinetic parameters of some vitamins

Although many authors have reported the percentage loss of nutrients in food product that was given a particular heat or cooking treatment, this kind of data is not complete enough to allow estimation of the kinetic parameters that can be used to calculate the response of nutrient to heat treatment (Lund, 1975). Among the vitamins kinetic data are available for thiamine, ascorbic acid (Labuza, 1972),

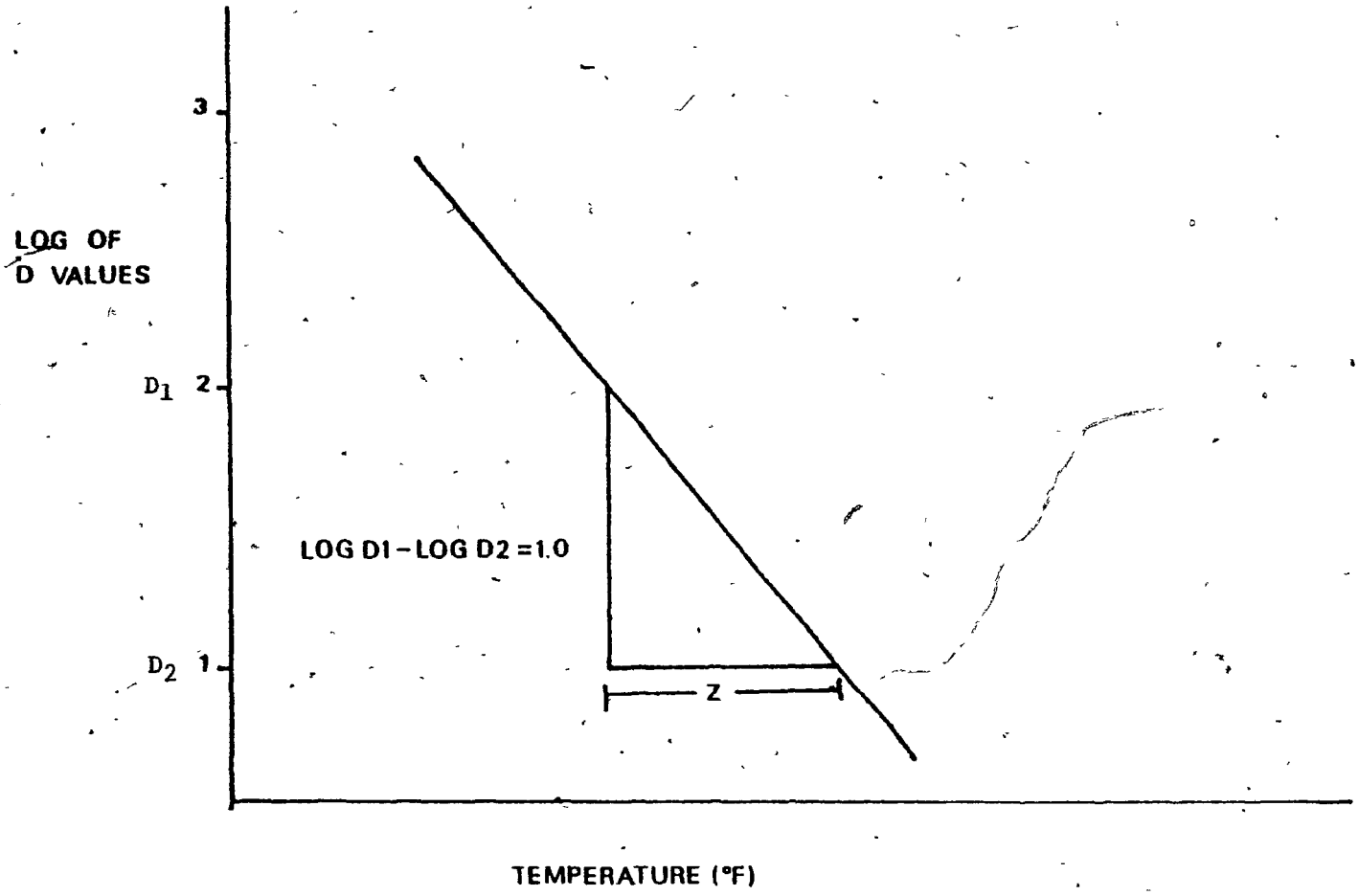

Figure 8. Thermal destruction time curve.

pantothenic acid (Hamm and Lund, 1978) and folic acid (Chen and Cooper, 1979). For these vitamins that have been studied most of the work has been limited to very few foodstuffs and to model systems.

Thiamine is probably the most heat sensitive of the B vitamins, especially in nonacid foods. Farrer (1955) made an extensive review of thiamine stability in foods for all work up to that time. He showed that in almost all cases the loss of thiamine due to heating could be predicted by first order reaction kinetics. He showed that thiamine loss is affected by the form of the molecule, i.e., free thiamine, thiamine incorporated to enzyme and protein-bound thiamine. The latter is the most stable, while the enzyme form has the least stability. As pH increased, the rate of destruction increased. Table 5 shows the destruction rate constants as calculated by Farrer (1955). Mulley et al. (1975) observed that when the pH of the phosphate buffer exceeded 6.0 the stability of the thiamine molecule dropped suddenly (Table 6). This is in agreement with Feliciotti and Esselen (1957), who observed that the most pronounced change in reaction rate occurred between pH 6.0 and 6.5.

Feliciotti (1955) hypothesized that the pH of the phosphate buffer influences the thiamine molecule by neutralization of the hydrochloride. The activation energy for the breakdown of the thiamine molecule in phosphate buffer was 29.4 kcal/mole (Mulley et al., 1975). Feliciotti and Esselen (1957) reported a value of 28.8 k cal/mole.

TABLE 5. Thiamine destruction rates in phosphate buffer at 100°C

pH	Rate constants (min^{-1})
3	0.0008
5	0.0015
7	0.0160
8	0.14

Data from Labuza (1972)

TABLE 6. Thiamine destruction rates in phosphate buffer at 129.4°C

pH	Rate constant (min^{-1})
4.5	0.0260
5.0	0.0236
5.5	0.0358
6.0	0.0831
6.5	0.1985

Data from Mulley et al. (1975)

Thiamine is more heat resistant in natural foods than in aqueous and buffered solutions. As shown in Table 7, in a food system with pH of 6.18 (pork or lamb) the rates of destruction are much lower compared with a buffer system of approximately the same pH (Table 6). Although the pH of the foods tested is quite close, except for the peas and spinach, the rates of destruction are different. For example, lamb and pork had the same pH but the rates of destruction of thiamine are different. This may be taken to suggest that the hydrogen ion concentration is not the only factor involved. McIntire and Frost (1944) showed that α and β amino acids and some of their derivatives have a marked stabilizing effect upon thiamine at pH 6. Proteins are known to protect thiamine even though the protective mechanism involved has not been completely elucidated (Mulley et al., 1975). Adsorption upon starch in foods may also play an important role in causing the retention of thiamine during heating. Activation energy of thiamine in food systems is shown in Table 7. The values are the same as those reported for buffer systems. This suggests that the mechanism of destruction in food and buffer system may be the same.

Very little work has been done in terms of providing kinetic data for the different derivatives of folic acid. Garret (1956) studied degradation of PteGlu in a liquid multivitamin preparation at pH 3.2 in the temperature range of 50-70°C. He reported that the thermal degradation was initially pseudo zero order and subsequently first order. The extent of the former increased with decreasing temperature. The first order rate constants and Ea obtained by Garret

TABLE 7. Rates of destruction of thiamine in food systems

Author	Food system	Rates of destruction (min^{-1})				Activation energy k cal/mole
		108.9°C	118.9°C	128.9°C	138.9°C	
Feliciotti and Esselen (1957)	carrots (pH 6.13)	0.0049	0.0120	0.0285	0.0711	
	green beans	0.0049	0.0122	0.0311	0.0717	
	peas (pH 6.75)	0.0051	0.0114	0.0276	0.0708	
	spinach (pH 6.70)	0.0067	0.0143	0.0336	0.0825	27.0
	beef heart (pH 6.10)	0.0068	0.0157	0.0392	0.0461	
	beef liver (pH 6.07)	0.0067	0.0147	0.0364	0.0892	
	lamb (pH 6.18)	0.0062	0.0138	0.0377	0.0814	
	pork (pH 6.18)	0.0055	0.0129	0.0288	0.0717	
		121.0°C		126.6°C	132.2°C	
Mulley et al. (1975)	pea puree	0.0093		0.0116	0.0221	27.5
	beef puree	0.0091		0.0144	0.0251	27.4
	peas in brine puree	0.0102		0.0158	0.0303	27.0

TABLE 8. Kinetic parameters for PteGlu and 5-CH_3-H_4PteGlu

Folate derivative	System	pH	Temperature °C	Rate constants (hr^{-1})	Ea k cal/mole	Reference
PteGlu	Multivitamin preparation	3.2	50	0.0004	16.8	Garret (1956)
			60	0.0010		
			70	0.0020		
5-CH_3-H_4PteGlu	Aqueous solution	7.0	49	0.0043	9.5	Chen and Cooper (1979)
			65	0.0085		
			78	0.0145		
			100	0.0323		

(1956) are shown in Table 8. Chen and Cooper (1979) reported that the destruction of 5-CH_3-H_4PteGlu could be described by first order kinetics and the rates of destruction obtained at pH 7 are shown in Table 8. For both PteGlu and 5-CH_3-H_4PteGlu the rate constants increased with temperature. The Ea for 5-CH_3-H_4PteGlu is lower than that of PteGlu, indicating that thermal degradation of PteGlu is more temperature dependent than the thermal degradation of 5-CH_3-H_4-PteGlu.

Paine-Wilson and Chen (1979) undertook a study to determine the effect of pH and buffer ions on the thermal stability of folate derivatives. Their results show that buffer ions have no effect on the rate of destruction of PteGlu (at pH 3). This is in agreement with Dick et al. (1948), who reported that the destruction of PteGlu is dependent on pH only and does not depend on buffer ions. The thermal stability of 5-CH_3-H_4PteGlu is affected by buffer ions. O'Broin et al. (1975) found variation in rate of destruction of 5-CH_3-H_4PteGlu as a function of buffer ions. The effect of pH on two folate compounds is shown in Figure 9. For 5-CH_3-H_4PteGlu, the greatest stability was displayed at pH 7, while the stability of H_4PteGlu decreased as the pH increased.

Ascorbic acid is highly sensitive to various modes of degradation. Factors which can influence the nature of the degradative mechanism include temperature, salt and sugar concentration, pH, oxygen, enzymes, metal catalysts, initial concentration of ascorbate

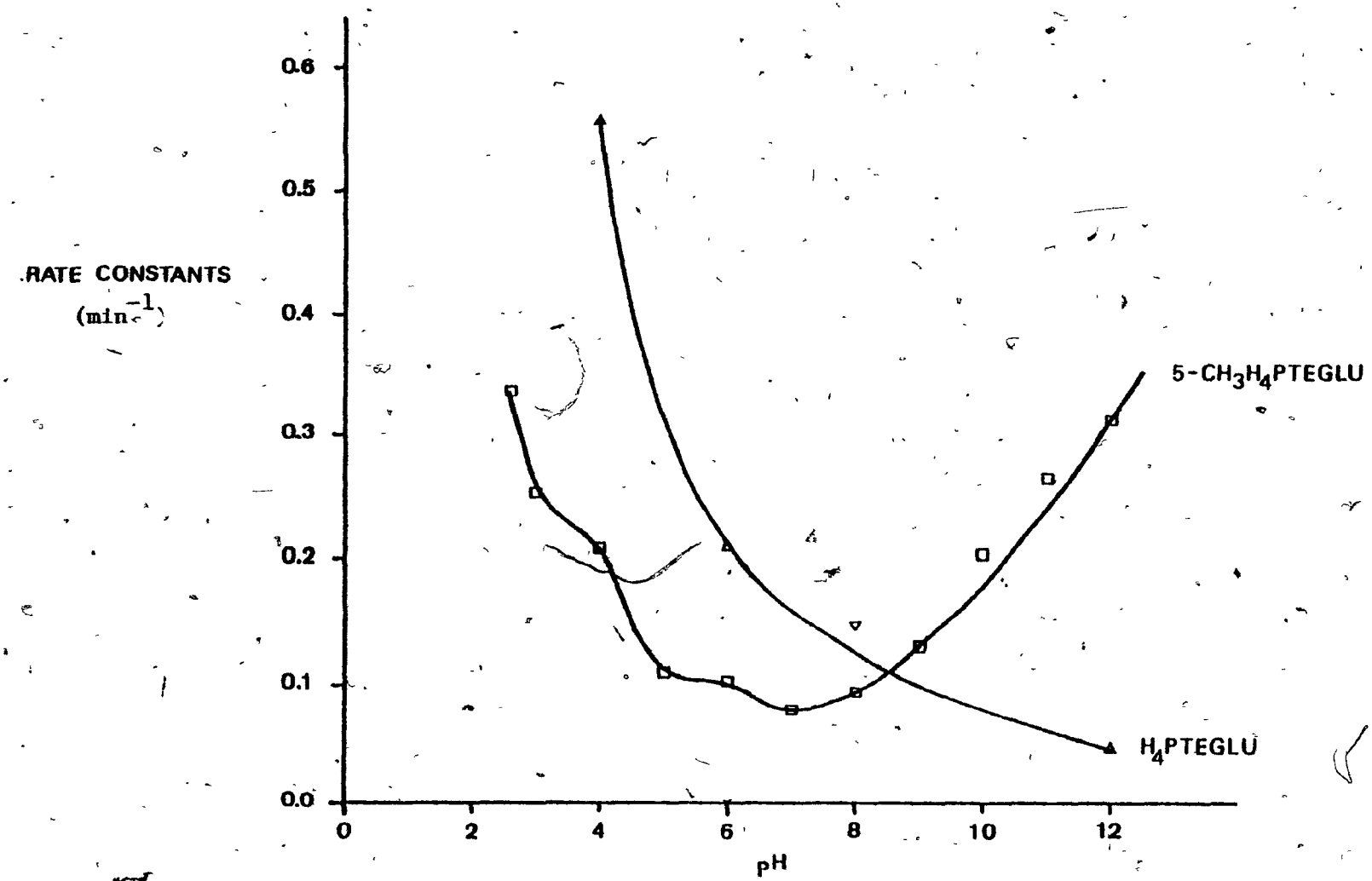

Figure 9. Effect of pH on rates of destruction of folate derivatives in universal buffer. (Data from Paine-Wilson and Chen, 1979.)

and the ratio of ascorbic acid to dehydroascorbic acid (Archer and Tannenbaum, 1979). Since so many factors can influence the nature of the ascorbic acid degradation, it is not feasible to construct clearly defined precursor-product relationships for the products in the reaction pathway.

In the presence of oxygen, ascorbic acid is degraded to dehydroascorbic acid. The rate of formation of dehydroascorbic acid can be described by first order reaction kinetics (Archer and Tannenbaum, 1979). Barron et al. (1936) found that the rate of ascorbic acid oxidation under aerobic conditions is very slow in acid and neutral solutions in the absence of metal catalysts. Finholt et al. (1963) reported that the rate of ascorbic acid destruction in acidic medium reaches a maximum near the pKa, of ascorbic acid, which is 4. Lee et al. (1977) reported that the destruction rate constants changed with pH, reaching a maximum at pH 4.06 (Table 9), which is near pKa of ascorbic acid. This is in agreement with Huelin (1953), who reported that under anaerobic conditions in citrate buffer the reaction proceeded most rapidly at pH 3-4.

The activation energy for anaerobic destruction of ascorbic acid in tomato juice changed with pH (Table 10). The change of Ea with pH could be due to the fact that destruction of the complex form of ascorbic acid requires less energy than required for the dissociated and undissociated forms of ascorbic acid, and the amount of the complex formed could reach a maximum near pKa, of ascorbic acid (Lee et al., 1977).

TABLE 9. The first order rate constants of ascorbic acid as a function of pH at 37.8°C

pH	Rate constant (10^{-1} day^{-1})
3.53	1.8408
3.78	2.2793
4.06	2.4788
4.36	2.2749

From Lee et al. (1977)

TABLE 10. Activation energy of ascorbic acid in tomato juice at different pH values

pH	Ea (kcal/mole)
3.53	4.493
3.78	4.015
4.06	3.295
4.36	4.847

The comparison of Ea values of ascorbic acid in model and in buffer system indicate that kinetic data obtained from model systems may not be very useful in food systems. Table 11 shows a selection of the values reported in the literature. Within the food systems some Ea values are very low (tomato juice), while in canned peas the Ea is quite high. These differences within foods and between foods and model systems could be due to different destruction mechanisms occurring in each system.

The physiological activity of pantothenic acid is destroyed by hydrolysis of the molecule into pantoic acid and β-alanine (Frost, 1943). This hydrolysis is a function of temperature, pH, moisture content and buffers. Hamm and Lund (1978) reported that the destruction of pantothenic acid in model and in food systems at temperatures between 118 and 143°C could be described by first order kinetic equations. This is in agreement with Garret (1956), who stated that the destruction of pantothenic acid at pH 3.2 in a multivitamin preparation could be described by first order kinetics. He reported an Ea value of 21 k cal/mole. Table 12 shows the results obtained by Hamm and Lund (1978). Their results show that as the pH of pantothenic acid system increases the Ea also increased. This was taken to suggest that the mechanism of thermal inactivation changed as the pH changed.

TABLE 11. Activation energy of ascorbic acid in food and model systems

Reference	Food/Model system	Ea (k cal/mole)
Labuza (1972)	Wheat flour Aw 0.65	22.3
Labuza (1972)	Corn-soya milk	36.5
Labuza (1972)	Dehydrofrozen peas Aw 0.9	45.0
Lee et al. (1977)	Canned tomato juice	3.3
Lathrop et al. (1980)	Canned peas	41
Huelin (1953)	Phosphate buffer pH 5.6	18
Blaug and Hajratwala (1972)	Phosphate buffer pH 6	22.4
Dennison and Kirk (1978)	Dehydrated model food system Aw 0.65	18.3

TABLE 12. Activation energy of total pantothenic acid in model and food systems

Food/model system	Ea (k cal/mole)
Phthalate buffer pH 4	20
Phthalate buffer pH 5	22
Phthalate buffer pH 6	27
Meat puree pH 5.4	25
Pea puree pH 7.0	36

Data from Hamm and Lund (1978)

1. Folate destruction during processing

The folacin content of foods is greatly affected by conditions associated with processing, storage and preparation. Several workers have generated data on folate levels (Hoppner et al., 1977) and per cent destruction of folates in foods (Table 13), but this kind of data does not lend itself to a quantitative description of the losses likely to occur under a variety of conditions.

Direct comparison of the data available in the literature on folate losses in foods (Table 13) is not possible since in most cases, time, methods of cooking, temperatures, as well as the amounts of water used were not reported. For example, in Hurdle's (1968) experiments the cooking water was not included in the assay and they reported a loss of 89% folate activity in broccoli. However, Fennema (1975) reported that 8-10 minutes cooking gave losses of only 25-35% in broccoli and 100% of this was recovered in the cooking water. Klein et al. (1979) reported high values of retention in vegetables (Table 13). Although they stated the amount of cooking water used, the assay of folates in the cooking water was not reported. The differences reported on the same food under almost the same process condition could be due to differences in assay techniques. Few studies are reported where ascorbate protection of labile forms of folate was used along with conjugase treatment to release the conjugated forms of the vitamin. This suggests that it is difficult to estimate accurately nutrient content of diets from tabulated values.

TABLE 13. Reported observations of folate losses in foods subjected to various processes

Food product	Process method	Loss of folate activity %	References
Beef liver	fried 10-15 min	15	Cheldelin et al. (1943)
Halibut	fried 7-10 min	46	"
Cauliflower	steamed 30 min	88	"
Carrots	steamed 30 min	97	"
Potatoes	boiled 30 min	93	"
Cabbage	steamed 30 min	92	"
Egg	scrambled 5 min	31	"
Egg	fried 5 min	21	Hanning & Mitts (1949)
"	scrambled 7 min	18-48	"
"	poached	33-39	"
Cabbage	boiled 5 min	98	Hurdle et al. (1968)
Potato	boiled	90	"
"	fried	89	"
Broccoli	boiled	89	"
Egg yolk	boiled	70	"
"	fried	29	"
Spinach	boiled 10 min	12.4	Klein et al. (1979)
Green peas	boiled 6 min	18	"
Green beans	boiled 9 min	None	"
Broccoli	boiled 5 min	51-59	"
Meat/vegetable	canning & storage for 1 ½ years	negligible	Hallendoorn et al. (1971)
Flour	milling	20-80	Schroeder (1971)

Folate derivatives have been found to vary greatly in thermal stability. The differences in stability of various folacin derivatives may account for the wide variability in data concerning folate loss during the cooking of foods; therefore, it is worthwhile to characterize all naturally occurring forms in terms of stability during processing and storage.

1. Pteroylglutamic acid

PteGlu is the folate used therapeutically and in food supplementation. Thermal stability of PteGlu has been studied under various conditions. It is stable to boiling in aqueous solution up to 2 hours (Coleman et al., 1975). Exposure to higher temperatures results in some losses of this form of the vitamin. Keagy et al. (1975) observed an average of 11% loss of PteGlu in baking bread fortified with 5 mcg PteGlu/gram flour. Cooper et al. (1978) showed that PteGlu is quite stable to heat at 100°C in neutral solutions. Dick et al. (1948) undertook a study to determine the thermal stability of PteGlu. Their results showed that PteGlu is stable up to one hour when heated at 100°C in solution of pH above 5. It has been shown that buffer ions affect the destruction of folates. O'Broin et al. (1975) compared stability of PteGlu in different buffer systems and found that in the presence of phosphate buffer at pH 6 and 8.0 PteGlu underwent more thermal destruction as compared with the other buffer systems tested at the same pH. Dick et al. (1948) found that the variation of thermal destruction of PteGlu does not depend on the constituents of

the buffer solution. Their findings are in agreement with those of Paine-Wilson and Chen (1979), who reported that at pH 3 the rates of thermal destruction of PteGlu were similar using four buffer systems. Phosphate buffer was not included in their study.

2. Naturally occurring folates

The naturally occurring reduced forms of folate are unstable and considerable loss of the vitamin activity occurs during the course of food processing and storage (Archer and Tannenbaum, 1979). Although various studies have shown that the primary inactivation process is oxidative (Burton et al., 1970; Ford et al., 1969), the extent and mechanism of loss of these forms in foods have not been elucidated. Cooper et al. (1978) reported that the most stable reduced folate was 5-CHO-H_4PteGlu which had stability comparable to PteGlu. There was no significant difference in stability of 5-CHO-H_4PteGlu when heated at pH 3.0 in various buffer systems. Rabinowitz (1960) reported that at pH 7, 5 CHO-H_4PteGlu was relatively stable in maleate buffer but was rapidly hydrolyzed in the presence of phosphate, pyrophosphate and arsanate buffers. However, O'Broin et al. (1975) did not observe this effect of phosphate buffer on 5-CHO-H_4PteGlu at room temperature. Paine-Wilson and Chen (1979) reported that H_4PteGlu is highly unstable as compared with other folates and the lability was greatly influenced by pH and buffer ions. They reported that H_4PteGlu was very susceptible to oxidation by atmospheric oxygen which made it difficult to handle except under solutions containing reducing agents.

3. 5-methyltetrahydropteroylglutamic acid

As stated earlier, the limited data that are available indicate that, particularly plant derived materials contain 5-CH_3-H_4PteGlu$_{(n)}$ forms as the major constituent. Cooper et al. (1978) observed that over 90% degradation of 5-CH_3-H_4PteGlu occurred within 65 minutes of heating at 100°C. The half life of this derivative was 21 minutes in aqueous solution at pH 7 (Chen and Cooper, 1979). Paine-Wilson and Chen (1979) observed that the greatest thermal stability was displayed at pH 7 at 100°C, while O'Broin et al. (1975) reported that at room temperature the compound had maximum stability at pH 9.0 and was relatively stable in alkaline conditions.

m. Effect of oxygen on destruction of folates

The presence of oxygen has been shown to affect the degradation of folates during heating. Ford et al. (1969) reported that the variation in the stability of folates in milk was directly related to the presence of oxygen in the milk. Rolls and Porter (1973) showed that the percentage of folacin destroyed during heat processing of milk is greatly affected by the level of residual oxygen in the milk. Chen and Cooper (1979) observed that both 5-CH_3-H_4PteGlu and H_4PteGlu were considerably stabilized when heated at 100°C under nitrogen atmosphere. 5-CH_3-H_4PteGlu lost little activity through one hour of heating under nitrogen. The stability afforded by the nitrogen atmosphere was comparable to the stability afforded by a level of 0.1% ascorbate.

CHAPTER III

MATERIALS AND METHODS

Water used in all the experiments was double distilled water.

a. Sample preparation

1. Pteroylglutamic acid

PteGlu (1 gram) was dissolved in 100 ml of 0.1 N NaOH and the concentration was checked by using Beckman A25 spectrophotometer. According to Uyeda and Rabinowitz (1963), a 1% solution of PteGlu in 0.1 N NaOH shows an absorption maxima at 256, 282 and 365 nm, with values of 585, 570 and 206, using a 1 cm light path. These values were used to check the concentration of the prepared solution. From this solution a stock solution (1 ml diluted to 1 litre) was prepared. The stock solution was frozen in 5 ml aliquotes and stored in the dark at -20°C. New solutions were prepared every two weeks. When needed, 1 ml of the stock solution was diluted to 10 ml with citrate buffer of the desired pH and ionic strength. The final concentration was 0.89 µg/ml. This solution was made fresh each day.

2. 5-Methyltetrahydrofolic acid

DL-N-5-CH_3H_4PteGlu (sodium salt, 90% purity) was obtained from Sigma Chemical Company, St. Louis, Mo, and stored in a freezer at -20°C. When needed, 11.1 mg was dissolved in 5 ml of cold water (4°C) and then 1 ml of this solution was diluted to 100 ml. This solution was stored in 5 ml aliquotes in a freezer at -20°C in the dark. When needed, 1 ml of the stock solution was diluted to 10 ml with citrate buffer of desired pH. The final concentration was 1.998 µg/ml. This solution was made fresh each day.

b. Preparation of buffer solutions

Citric acid monohydrate powder was used to prepare the buffers. The amounts of citric acid used to give the desired pH and ionic strength were calculated by using the Henderson-Hasselbalch equation which states that

$$pH = pKa + \log_{10} \frac{[salt]}{[acid]}$$

and

$$I = \frac{1}{2} \Sigma^{i} \; c_i z_i^2$$

where I = ionic strength of solution

c_i = molar concentration of the ionic type i

z_i = valency

The pH, ionic strength and weight of citric acid used are shown in Table 14. 1.0 N NaOH was used to adjust the pH to the appropriate value.

TABLE 14. Amounts of citric acid used to prepare buffer solutions

pH	Ionic strength	Moles of citric acid per litre
3	0.1	0.249
	0.15	0.374
	0.2	0.639
	0.4	0.998
4	0.1	0.095
5	0.1	0.047
6	0.1	0.029

c. Preparation of food samples

1. Preparation of bacto-chicken pancrease (conjugase)

Bacto-chicken pancrease (1 gram) was added to 30 ml of 0.1 M phosphate buffer pH 7.0, covered with toluene and incubated at 37°C overnight. After incubation the toluene was carefully poured off and the mixture was centrifuged for 1 hour at 2000 rpm. Dowex 1 X 8 (chloride, 10% w/v) was added to the supernatant and stirred for 1 hour in a cold room at 4°C. The mixture was centrifuged and the supernatant was stored in 5 ml aliquotes in a freezer at -20°C until ready to use.

The phosphate buffer was prepared by mixing equal volumes of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 and the pH adjusted to 7.0 by adding 0.1 M Na_2HPO_4.

2. Removal of native folates from tomato juice

Tomato juice purchased locally was used. Tomato juice (400 ml, pH 4.3) was diluted with 100 ml of water and the pH adjusted to 6.1 with 5% ammonium hydroxide. This was then heated in a boiling water bath for 5 minutes. After cooling to room temperature, 5 ml of chicken pancrease preparation was added; the mixture incubated at 37°C for 18 hours, then heated for 15 minutes in a boiling water bath then cooled to room temperature. Dowex 1 X 8 (chloride 40% w/v) was added and the mixture was stirred in an ice bath in a cold room (4°C) for 8 hours. The mixture was centrifuged for 20 minutes at 2000 rpm

and another 40% (w/v) Dowex 1 X 8 (chloride) was added to the supernatant and stirred in a cold room for 12 hours (supernatant included the solids from tomato juice). This was repeated for another 12 hours and then a proportion of the supernatant obtained was assayed for folates using the standard L. casei procedure to check if all folates had been removed. The folate free supernatant was then freeze dried and kept in a freezer at -20°C. When needed, a weighed amount of solids was rehydrated to 93.6% moisture and the pH adjusted to 4.3 using 1.0 N citric acid. This prepared tomato juice (9 ml) was mixed with 1 ml of PteGlu or 5-CH_3-H_4PteGlu and put into capillary tubes and sealed as described for destruction testing, which will be described later.

3. Removal of native folates from apple juice

Apple juice, pH 3.4, purchased locally, was used. The same procedure was followed as for tomato juice except that there was no dilution done and after adding Dowex 1 X 8 (chloride) stirring was done for 6 hours and then centrifuged and repeated for another six hours. The folate free supernatant obtained was adjusted to pH 3.4 with malic acid and kept in 10 ml aliquotes in a freezer at -20°C until ready to use. When needed, 9 ml of the thawed juice was used to dilute 1 ml of PteGlu or 5-CH_3-H_4PteGlu solution and then put into destruction tubes.

d. Thermal destruction of folates

The method of Stumbo (1965) was used. Approximately 50 mg of a buffered solution containing 0.89 µg/ml PteGlu or 1.998 µg/ml 5-CH_3-H_4PteGlu was placed in a capillary tube (1.6 x 100 mm) by capillary action and the ends of the tube sealed in a small flame. The weight of the solution in the tube was determined by weighing the tube before and after filling and its volume computed by assuming a density of 1.0 g/cc. This procedure allowed trapping of the sample in the approximate centre of the capillary tube to reduce or eliminate end effects which can occur if the sample contacts the end of an irregularly sealed capillary tube. The tubes were placed in a specially constructed stainless steel rack. All capillary tubes were set up in triplicate.

Heating was done in a Braun Thermomix 1480 stainless steel bath filled with stabilized high temperature oil. Individual capillary tubes were removed at specified time periods and placed in a second especially constructed rack immersed in an ice bath. Individual capillary tubes were removed from the cooling bath, dried, dipped in ethanol and flamed prior to immersion in 5 ml sterile water, previously pipetted asceptically into sterile, capped plastic 15 ml centrifuge tubes. The capillary tube was then crushed with a sterile glass rod and the tube contents thoroughly mixed with a vortex mixer. The tubes were then centrifuged for 2 minutes to ensure that all glass particles were separated. One hundred µl of the supernatant was added to 9.9 ml sterile assay media and assayed for folates.

e. Anaerobic destruction of 5-CH_3-H_4PteGlu

One ml of the prepared stock 5-CH_3H_4PteGlu was diluted with 9 ml of citrate buffer of desired pH and nitrogen was bubbled through the solution for 30 minutes in a glove box (Fisher Scientific) which had previously been flushed with nitrogen. The dissolved oxygen in the solution after flushing for 30 minutes was determined by using a dissolved oxygen analyzer (New Brunswick Scientific, N.J., U.S.A.). The amount of dissolved oxygen after flushing was 1.7%. Filling and sealing the capillary tubes was done in the glove box. The destruction was then done as described previously.

f. Assay for folates

1. Maintenance of stock culture

Pure lyophilized culture of L. casei ATCC 7469 was obtained from American Type Culture Collection and stored at 4°C until ready to use. One week before the preliminary assays were started, the vial which contained the culture was opened asceptically and 0.4 ml of rehydrated MRS broth (Difco) was added. The culture was shaken in order to achieve uniform suspension and then transferred asceptically to a 16 x 150 mm test tube containing 10 ml of MRS broth. The test tube was incubated at 37°C for 24 hours. After the incubation period, the culture was allowed to cool to room temperature, then inoculated into 16 x 150 mm test tube containing sterilized Bacto-Lactobacilli Agar (Difco) and incubated at 37°C for 24 hours. Stab cultures were

then obtained from these cultures and stored in a cold room at 4°C. The scheme for maintenance of stock culture in agar stabs was according to Cooperman et al. (1960) as shown in Figure 10. Transfers were made once every month. Prior to each test the culture was transferred for two consecutive days on Bacto-Lactobacilli Agar (Difco) slants to revive the microorganism.

2. Preparation of inoculum

Inoculum for assay was prepared by subculturing from the agar slant into a tube containing 10 ml prepared MRS broth. After incubation for 18 hours at 37°C the tube was centrifuged, the supernatant discarded and the cells resuspended in 10 ml sterile 0.85% saline solution. The culture was recentrifuged and twice resuspended in fresh sterile saline. The culture was then diluted (1 ml to 100 ml) with sterile saline solution and one drop of this was used to inoculate the assay tubes.

3. Microbiological assay

The method of Waters and Mollin (1961) with modification by Tamura et al. (1972) was used. Rehydrated Bacto-Folic Acid Casei media (5 ml) containing 0.05% ascorbic acid was dispersed into test tubes and 4.9 ml of water was added to each tube to make the volume 9.9 ml. All the tubes were autoclaved for 10 minutes at 15 psi (121°C) and then cooled to room temperature. Sample solution (100 µl) and a drop of the diluted L. casei cell suspension were added to each tube and vortexed. The tubes were incubated at 37°C in an agitated Magni Whirl

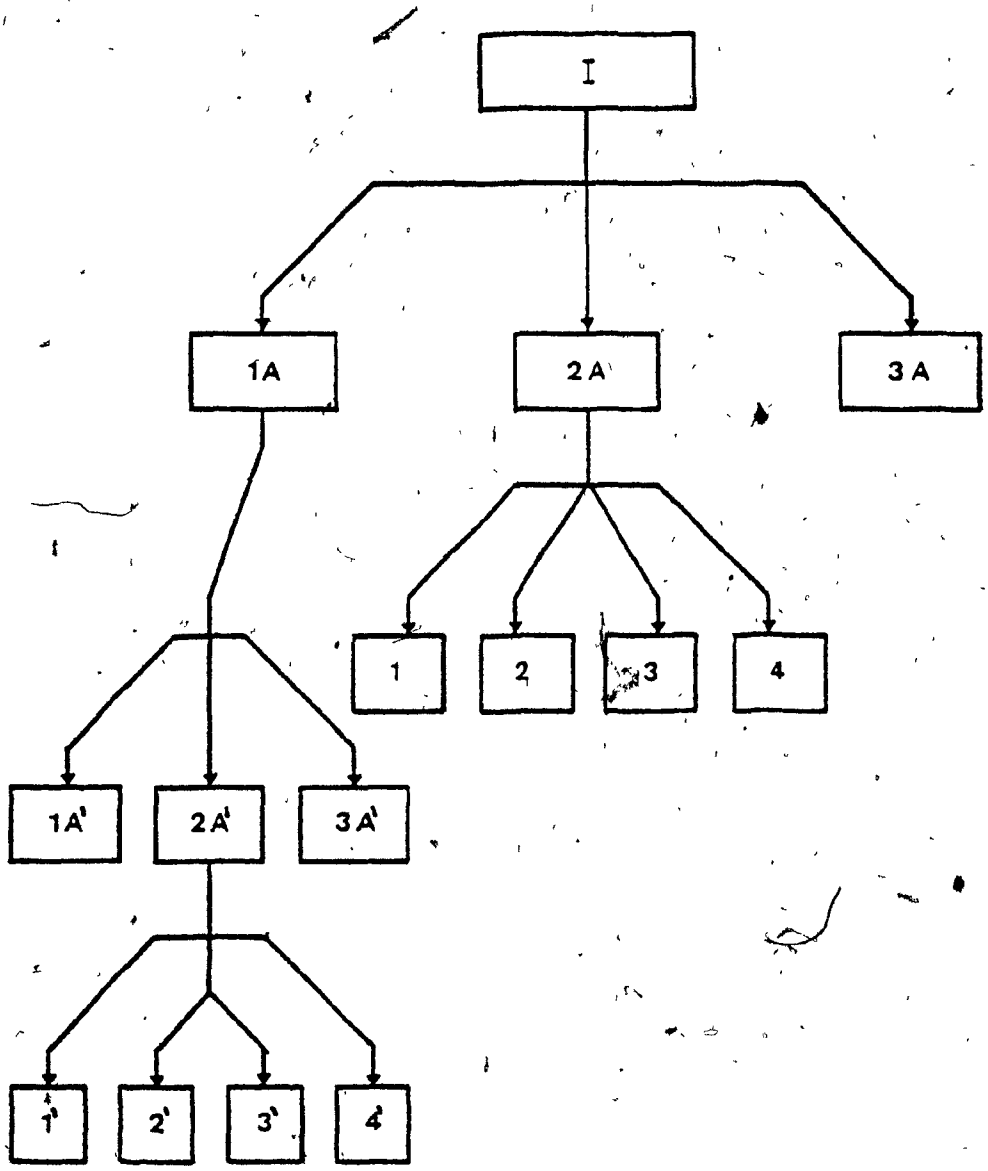

Figure 10. Scheme for the maintenance of stock culture in agar stabs.

I - stock culture
1A and 1A' for next month
2A and 2A' for use
3A and 3A' for spare

1 and 1' for first week
2 and 2' for second week
3 and 3' for third week
4 and 4' for fourth week

constant temperature bath (Blue M, Electrical Company, Illinois, U.S.A.). After 18 hours incubation, the tubes were removed and autoclaved for 15 minutes at 121°C. After thorough mixing on a vortex mixer, the turbidity was measured at 660 nm on a Beckman A25 spectrophotometer equipped with the sipper accessory. The amount of folate left was determined by using a standard curve obtained as described below.

g. Preparation of standard curves

1. PteGlu standard curve

i) Stock solution of folic acid

PteGlu (1 gram) was dissolved in 100 ml of 0.1 N NaOH and the concentration was checked on the Beckman A25 spectrophotometer as described before. From this solution, 1 ml was taken and diluted to 100 ml with 0.01 N NaOH with 20% ethanol. The solution was then covered with toluene and stored in a dark glass bottle (ground glass stopper) in a refrigerator at 4°C. New solution was prepared every two weeks.

ii) Working standard solution

The stock solution (1 ml) was diluted to 1 litre in a volumetric flask and 10 ml of this solution was further diluted to 500 ml. This solution was made fresh each day.

iii) Preparation of standard tubes

To triplicate 16 x 150 mm tubes, 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml of the working standard folic acid was added. Water was added to each tube to bring the volume to 5 ml. To each tube 5 ml of folic acid casei media which contained 0.05% ascorbic acid was added. The tubes were autoclaved for 5 minutes at 121°C. The tubes were then cooled to room temperature before inoculation.

iv) Inoculation and incubation

All the tubes were inoculated asceptically with one drop of the inoculum, mixed for one minute on a vortex mixer and then incubated at 37°C in an agitated constant temperature water bath. A new standard curve was prepared for each set of assay.

2. 5-CH_3-H_4PteGlu standard curve

i) Stock solution of 5-CH_3-H_4PteGlu

5-CH_3-H_4PteGlu (11.1 mg) was dissolved in 10 ml of water and 1 ml of this solution was diluted to 1 litre and stored in 5 ml aliquotes in a freezer at -20°C. New solution was prepared each week.

ii) Working standard solution

The stock solution of 5-CH_3-H_4PteGlu (3 ml) was diluted to 1 litre in a volumetric flask. This solution was made fresh each day. The solution was sterilized by filtering through a millipore filter paper type HA 0.45 µm.

iii) Preparation of standard tubes

To triplicate tubes, 5.0, 4.9, 4.8, 4.7, 4.6, 4.5, 4.4, 4.3, 4.2, 4.1 and 4.0 ml of water was added. To each tube 5.0 ml of rehydrated folic acid casei media (Difco) which contained 0.05% ascorbic acid was added. The tubes were autoclaved for 10 minutes at 121°C and then cooled in a cold water bath. After cooling, 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml of the sterilized 5-CH_3-H_4PteGlu solution was added asceptically to the tubes to bring the volume in each tube to 10 ml.

iv) Inoculation and incubation

All the tubes were inoculated asceptically with one drop of the inoculum, mixed for 1 minute and then incubated at 37°C in a water bath for 18 hours. After incubation, the tubes were autoclaved, cooled and after thorough mixing, the absorbance was measured at 660 nm on a Beckman A25 spectrophotometer. Distilled water was used to zero the instrument.

h. Calculations

Reaction rate constants were determined using the first order reaction equation

$$kt = 2.303 \log \frac{co}{c}$$

where

k = rate constant

t = time

co = concentration at 0 time

c = concentration at time t

Plotting $\log_{10}$ of per cent folic acid retained versus time of heating at constant temperature, a straight line was obtained. The slope of the line was obtained by linear regression analysis (Snedecor and Cochran, 1967) and the difference between slopes of lines tested by a covariance technique described by Ostle (1975). The rate constant was then obtained by multiplying the slope of the line by 2.303.

The relationship between folate destruction and temperature was obtained by using Arrhenius equation which states that

$$\ln k = \ln e - E/RT$$

where

e is a constant

E = activation energy

R = gas constant

T = absolute temperature

The natural logarithm of the rate constant was plotted versus the reciprocal of the absolute temperature and the slope of the line was obtained by linear regression analysis. The activation energy was obtained by multiplying the slope by the gas constant R. Covariance analysis (Ostle, 1975) was used to test whether the slopes of the Arrhenius plots were significantly different.

CHAPTER IV

RESULTS AND DISCUSSION

a. Thermal destruction of PteGlu

1. PteGlu standard curve

Figure 11 shows three standard curves obtained on three different occasions. The standard curves were in good agreement with that obtained by Waters and Mollin (1961). The differences observed between lines obtained on different occasions were judged sufficient to require a unique standard curve run with each folate determination and so a unique standard curve was run on each occasion.

2. Destruction of PteGlu in citrate buffers

The thermal destruction curves for PteGlu obtained at pH 3, 4, 5 and 6 in citrate buffer at 121°C are shown in Figure 12. By using linear regression techniques, a model based on first order kinetic was fitted to the data. The correlation coefficients of these plots ranged from 0.98 to 0.99 and were highly significant ($p \geq 0.01$). Similar results were obtained at 100, 110, 130 and 140°C (Table 15).

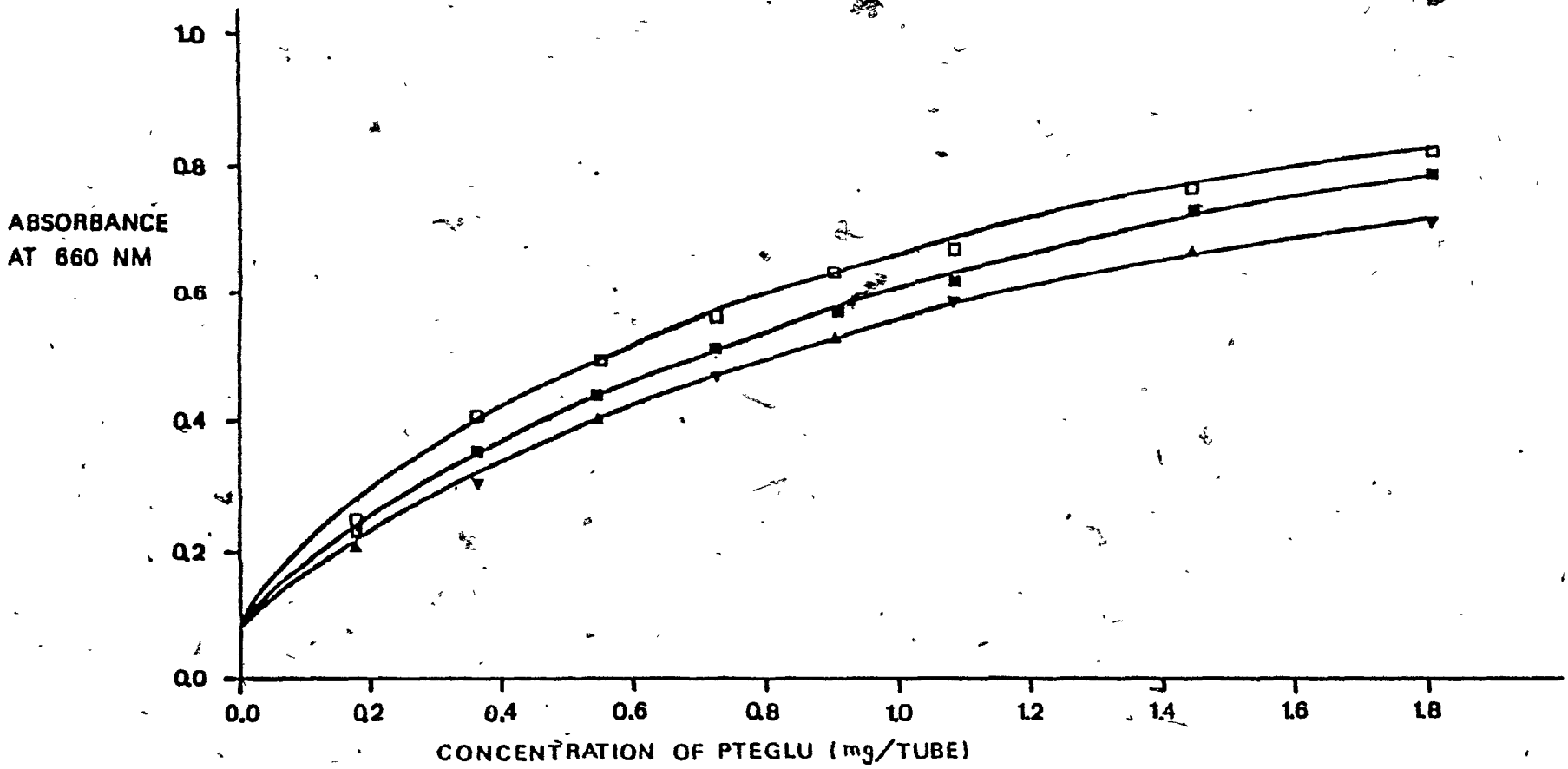

Figure 11. Response of Lactobacillus casei ATCC 7469 to PteGlu on incubation for 18 hours at 37°C.

- □ Data obtained on June 16, 1979
- ■ Data obtained on June 24, 1979
- ▼ Data obtained on June 19, 1979

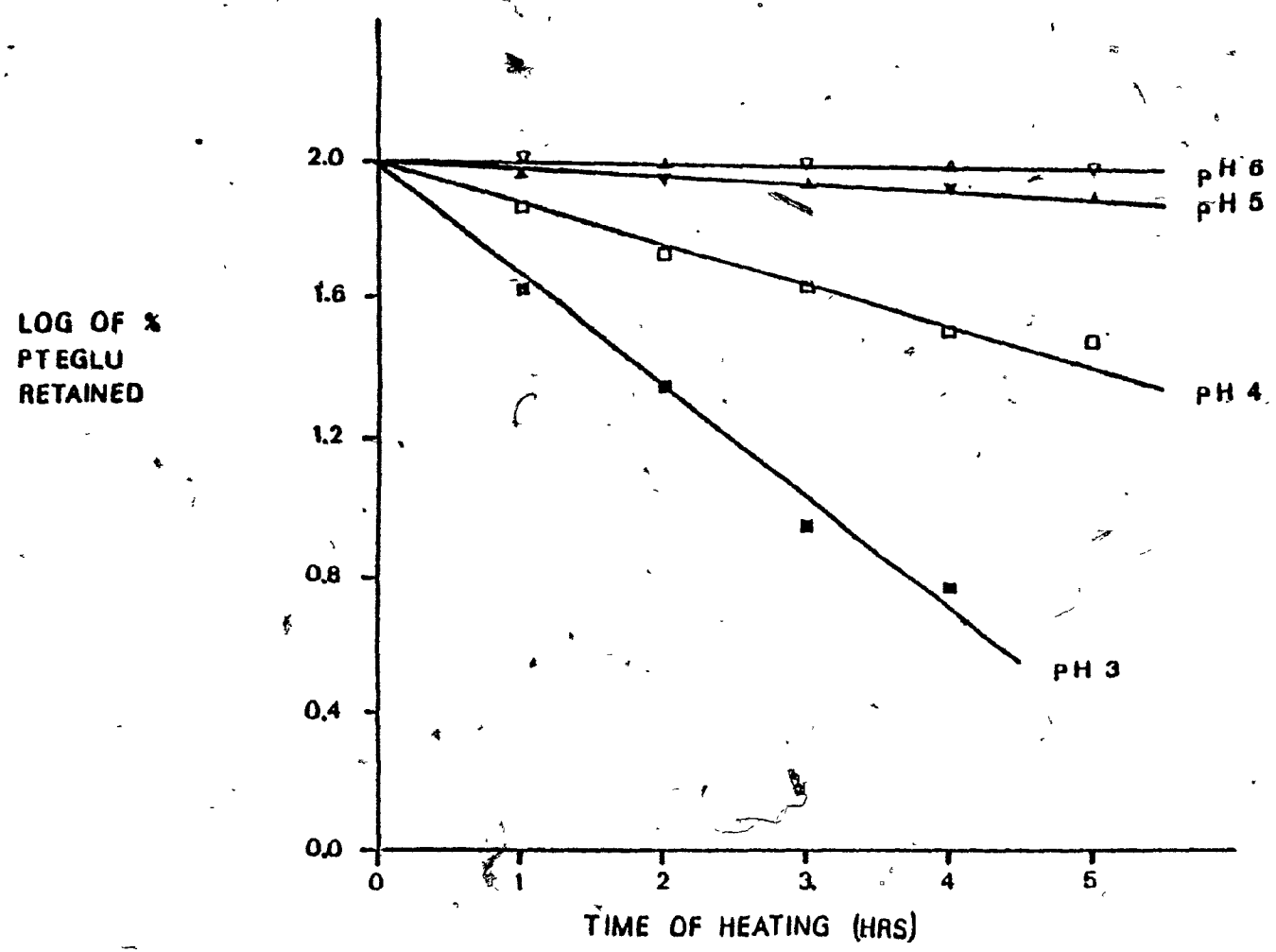

Figure 12. Rate of destruction curves for PteGlu in citrate buffer at 121°C, pH values as shown. Correlation coefficients, 0.99 at pH 6, 0.98 at pH 5, 0.98 at pH 4 and 0.98 at pH 3 are significant ($p \geqslant 0.01$).

TABLE 15. Effect of temperature and pH on the rates of destruction of PteGlu in citrate buffer

pH	Rates of destruction* (hr^{-1})				
	100°C	110°C	121°C	130°C	140°C
3.0	0.145 (0.97)[a]	0.293 (0.98)	0.744 (0.99)	1.66 (0.98)	2.36 (0.99)
4.0	0.074 (0.98)	0.128 (0.98)	0.234 (0.98)	0.580 (0.98)	0.840 (0.99)
5.0	0.021 (0.95)[b]	0.031 (0.96)	0.053 (0.93)	0.112 (0.95)	0.207 (0.97)
6.0	0.015 (0.94)	0.026 (0.97)	0.049 (0.99)	0.076 (0.96)	0.133 (0.99)

*Rate of destruction = slope x 2.303

[a]The correlation coefficients (in parenthesis) are significant ($p \geq 0.01$)

[b]Rate constants bracketed by the same line are not significantly different ($p \geq 0.05$)

Considering the inherent error in microbiological assays, the extremely good fit of the lines to the experimental points is strong evidence that the thermal destruction of PteGlu in citrate buffer can be described by first order reaction kinetics at 100-140°C. This agrees well with the findings of Garrett (1956) for PteGlu destruction at pH 3.2 and 50-70°C and those of Paine-Wilson and Chen (1979) for PteGlu inactivation at 100°C at pH values ranging from 3.0 to 6.0 in several buffer systems.

The possibility that folate destruction rates could be affected by ionic strength has not been addressed in the literature. However, it was determined (Table 16) that ionic strength did not affect destruction rates ($p \geq 0.05$) between ionic strengths of 0.1 and 0.4 at pH 3. The value obtained at ionic strength of 0.40 is quite high in view of the other values, in spite of being not significantly different ($p \geq 0.05$).

3. Effect of pH and temperature on destruction of PteGlu

The effect of pH on the rate constants is shown in Table 15. At all temperatures, as the pH increased from 3 to 6 the rates of destruction decreased. At 100, 110, 121 and 130°C the rates of destruction of PteGlu at pH 5 were not different from the rates of destruction at pH 6 ($p \geq 0.05$). At 140°C the rate constants were all statistically different at all pH levels ($p \leq 0.05$).

Comparison of the data obtained in the present study at 100°C with available literature values is shown in Table 17. The rate constant reported by O'Broin et al. (1975) at pH 3 at room temperature

TABLE 16. Effect of ionic strength at pH 3.0 on the rate of destruction of PteGlu at 100°C

Ionic strength	Rate constant (hr^{-1})	Correlation coefficient
0.10	0.143	0.92*
0.15	0.148	0.93**
0.20	0.149	0.96**
0.40	0.185	0.97**

* Significant at 5% level

** Significant at 1% level

| Rate constants bracketed by the same line are not statistically different ($p \geq 0.05$)

TABLE 17. Comparison of data obtained in the present study and literature values

pH	System	Temperature °C	Rate constant hr^{-1}	Reference
3	Citrate buffer	100	0.145	Present study
4	Citrate buffer	100	0.074	Present study
5	Citrate buffer	100	0.021	Present study
6	Citrate buffer	100	0.015	Present study
3	Citrate buffer	100	0.071	Paine-Wilson and Chen (1979)
4	Universal buffer	100	0.031	Paine-Wilson and Chen (1979)
5	Universal buffer	100	0.01	Paine-Wilson and Chen (1979)
6	Universal buffer	100	0.006	Paine-Wilson and Chen (1979)
3	0.05 M citrate/ phosphate buffer	Room temperature	0.029	O'Broin et al. (1975)
6	0.05 M citrate/ phosphate buffer	Room temperature	0.001	O'Broin et al. (1975)
3.2	Multivitamin preparation	50	0.004	Garret (1956)
3.2	Multivitamin preparation	60	0.001	Garret (1956)
3.2	Multivitamin preparation	70	0.002	Garret (1956)

(possibly 20°C) is much higher compared with the values reported by Garrett (1956) at pH 3.2 at temperatures of up to 70°C. The differences in these values could be due to the different systems in which the destruction was taking place.

There is an apparent disagreement between the rate constants obtained at 100°C in this study and those reported by Paine-Wilson and Chen (1979). The reasons for the differences are not known, but if the values obtained in the present study are divided by 2.303, the factor converting $\log_{10}$ to $\log_e$, excellent agreement is obtained. It might be that the data of Paine-Wilson and Chen (1979) are in error by this factor, but it is impossible to tell from the published information.

The temperature dependence of first order rate constants can be described by the Arrhenius activation energy (Glasstone, 1946) derived from plots such as shown in Figure 13. The activation energies (Ea) were obtained from the slopes of the lines derived by simple linear regression. The slope of the line for pH 3 was significantly different from the slope of the line for pH 6 ($p \leq 0.05$). The remaining slopes were not different from each other. The Ea values obtained are shown in Figure 14. In spite of lack of significant differences, the figure shows that the Ea values for thermal degradation of PteGlu decreases with pH in a regular manner.

The decrease in Ea values with increasing pH could be taken to indicate that the mechanism of thermal inactivation changes with pH. However, the change in the mechanism must be a gradual transition since there is no sharp break in the curve such as might be expected if a

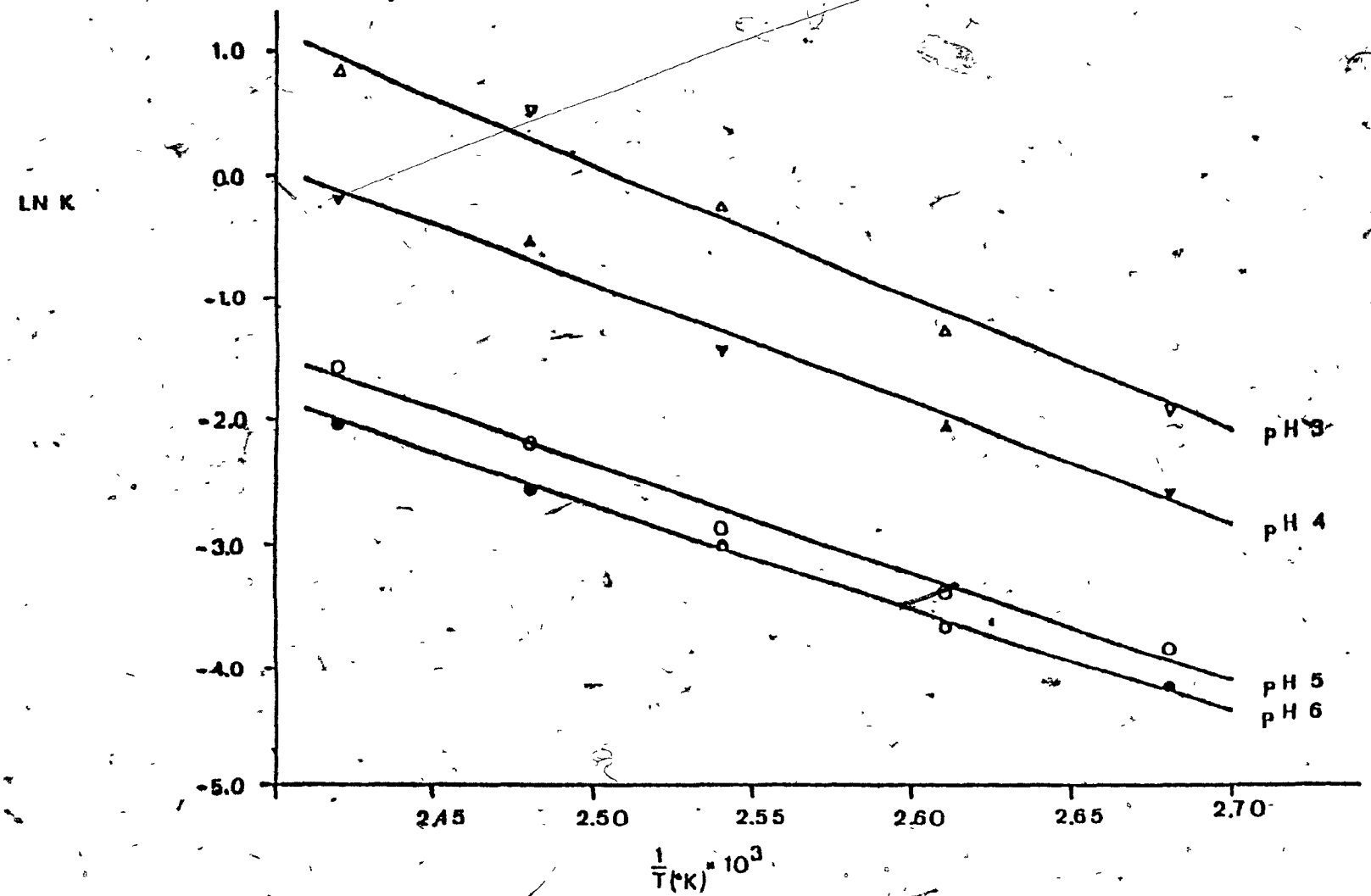

Figure 13. Arrhenius plot for thermal degradation of PteGlu in citrate buffer. Correlation coefficients, 0.99 at pH 3, 0.99 at pH 4, 0.98 at pH 5 and 0.99 at pH 6 are significant ($p \geq 0.01$). The slope of the line for pH 3 is different from that of pH 6 ($p \leq 0.05$). The rest of the lines are not different.

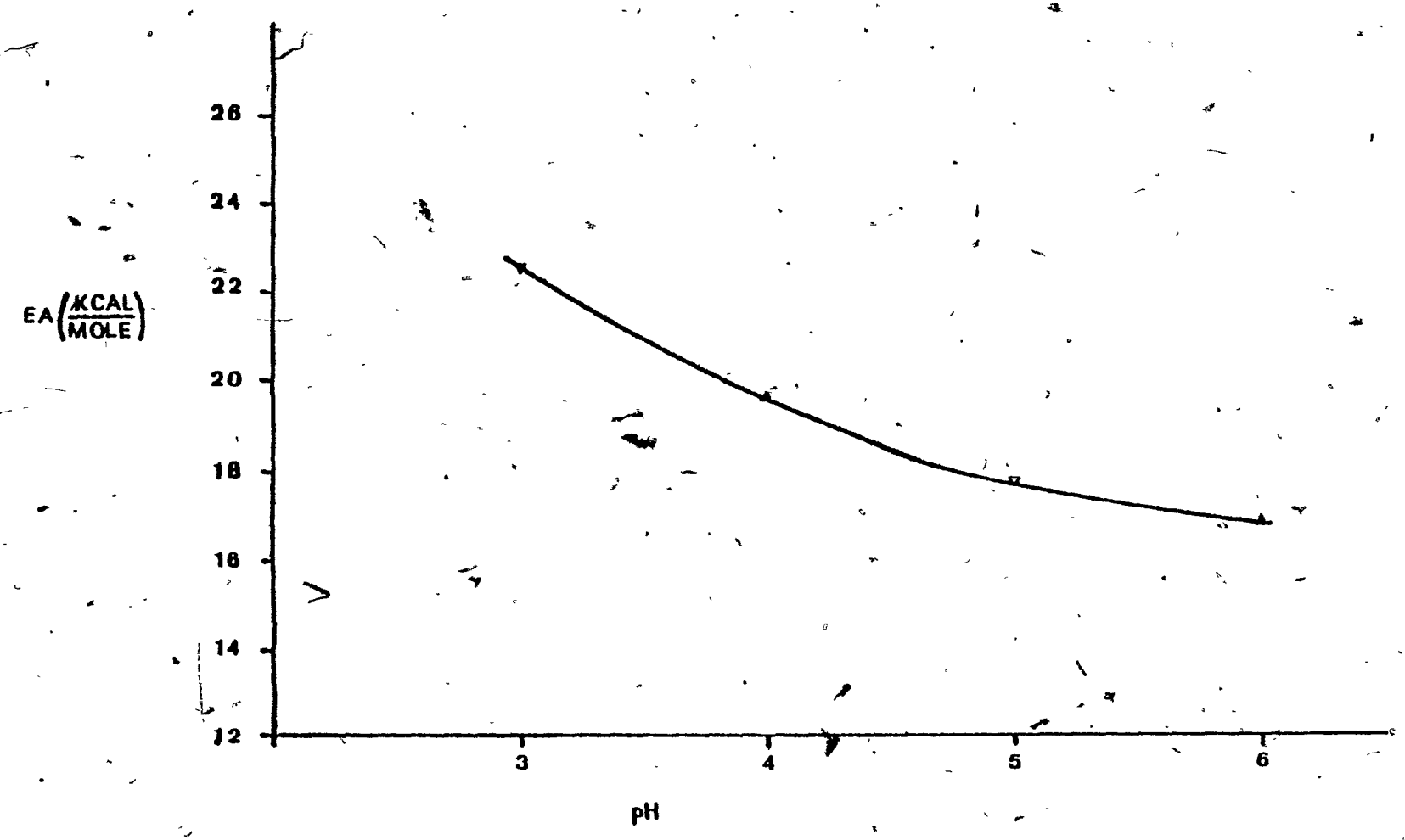

Figure 14. Effect of pH on the activation energy for destruction of PteGlu in citrate buffer. The Ea values (kcal/mole) are 22.6 at pH3, 19.6 at pH 4, 18.7 at pH 5 and 16.8 at pH 6. The only Ea values different are those at pH 3 and pH 6 ($p \leq 0.05$).

sharp change in mechanism were observed. Archer and Reed (1980) demonstrated that tetrahydrofolate undergoes a variety of degradation pathways in air and both the rate of reaction and nature of the products depended on the pH of the system.

The activation energies for the destruction of other vitamins such as ascorbic acid and pantothenic acid have been shown to change with the pH of the system (Lee et al., 1977; Hamm and Lund, 1978). The pattern with which the Ea values of these vitamins changes with the pH is shown in Table 18. For pantothenic acid Ea values increase with pH while in the case of ascorbic acid the Ea values decrease with increasing pH to a minimum at pH 4.06 and then increase with increasing pH.

The Ea values obtained for thermal inactivation of PteGlu in this study are comparable to Ea values of other vitamins in similar temperature range. The reported Ea values for thiamine at temperatures of 108.9 to 138.9°C range from 27 to 29 kcal/mole (Hamm and Lund, 1978). The only Ea value available in the literature for inactivation of PteGlu is 16.8 kcal/mole reported by Garret (1956) at pH 3.2 in a multivitamin preparation at temperatures between 50 and 70°C.

TABLE 18. Effect of pH on the activation energy (Ea) for thermal inactivation of ascorbic acid and pantothenic acid

Vitamin	System	pH	Ea	Reference
Ascorbic acid	Tomato juice	3.53	4.49	Lee et al. (1977)
		3.78	4.02	
		4.06	3.30	
		4.36	4.85	
Pantothenic acid	Phthalate buffer	4	20	Hamn and Lund (1978)
		5	22	
		6	27	

b. Thermal destruction of 5-CH_3-H_4PteGlu

1. Standard curve for 5-CH_3-H_4PteGlu

Figure 15 shows two standard curves obtained on two different occasions. The figure shows that there is a variation between curves obtained on different occasions, so a unique standard curve was run with each assay as with PteGlu.

2. Destruction of 5-CH_3-H_4PteGlu in citrate buffers

The plot of the logarithm of per cent 5-CH_3-H_4PteGlu retained versus time of heating at 100°C yielded straight lines (Figure 16). Straight lines were also obtained at 110, 121 and 130°C. The linearity of the curves indicated that the reaction of 5-CH_3-H_4PteGlu degradation in citrate buffer could be described by first order reaction kinetics, in agreement with Chen and Cooper (1979).

3. Effect of pH and temperature on the destruction of 5-CH_3-H_4PteGlu

Table 19 shows the effect of pH on the rates of destruction for 5-CH_3-H_4PteGlu. At 100, 121 and 130°C the rate constants at pH 5 were not significantly different from those at pH 6 ($p \geq 0.05$). This indicates that at lower pH values the rates of destruction are much faster compared with high pH values. No data were collected for pH 3 and 4 at 130°C because destruction was essentially complete within 2 minutes. The half lives for the degradation of 5-CH_3-H_4PteGlu at 100°C obtained in this study are in good agreement with values reported in the literature (Table 20). Chen and Cooper (1979) reported a half life of 21.4 min

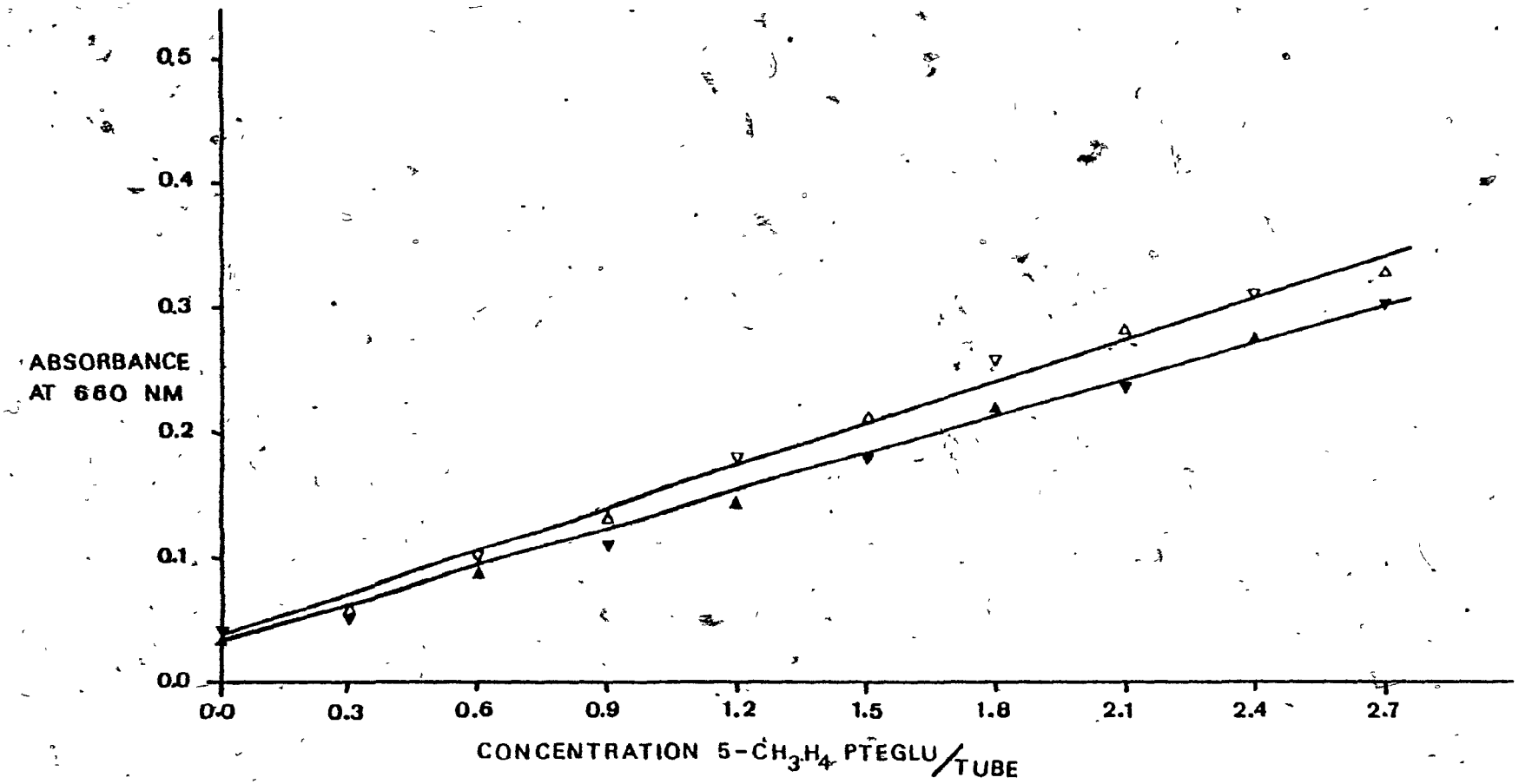

Figure 15. Response of Lactobacillus casei ATCC 7469 to 5-CH_3-H_4PteGlu on incubation for 18 hours at 37°C.

△ – Data obtained on 18th May 1980

▲ – Data obtained on 22nd May 1980

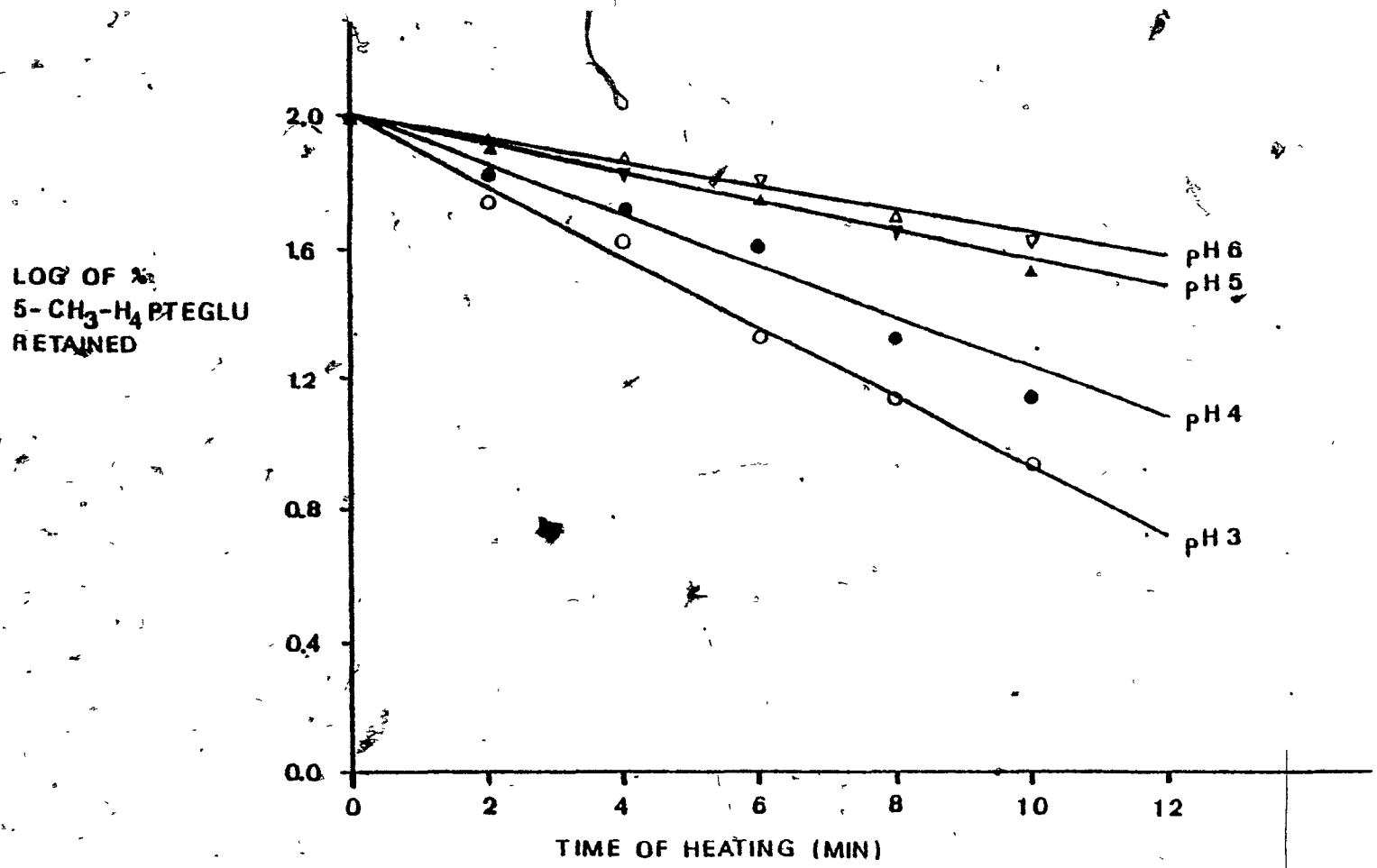

Figure 16. Rate of destruction curves for 5-CH_3-H_4PteGlu in citrate buffer at 100°C. Correlation coefficients, 0.94 at pH 6, 0.99 at pH 5, 0.96 at pH 4 and 0.98 at pH 3 are significant ($p \geq 0.01$).

TABLE 19. Effect of temperature and pH on the rates of destruction of 5-CH_3-H_4PteGlu in citrate buffer

	Rates of destruction* (min^{-1})			
	100°C	110°C	121°C	130°C
3.0	0.243 (0.98)	0.448 (0.96)	0.927 (0.98)	– [b]
4.0	0.192 (0.96)	0.318 (0.94)	0.635 (0.97)	–
5.0	0.110 (0.99) [c]	0.215 (0.97)	0.432 (0.98)	0.808 (0.94)
6.0	0.104 (0.94)	0.188 (0.98)	0.366 (0.93)	0.783 (0.96)

*Rate of destruction = slope x 2.303

[a]The correlation coefficients (in parenthesis) are signif[illegible] ($p \geq 0.01$)

[b]No data collected

[c]Rate constants bracketed by the same line are not sig[illegible]icantly different at the 5% level

TABLE 20. The effect of pH on half lives for the destruction of 5-CH_3-H_4PteGlu

pH	Temperature	System	Half life (min)	Reference
3	100	citrate buffer	2.85	present study
6	100	citrate buffer	6.66	present study
3	100	universal buffer	2.73	Paine-Wilson and Chen (1979)
6	100	universal buffer	6.73	Paine-Wilson and Chen (1979)
7.3	100	phosphate buffer	6.40	Ruddick et al. (1980)
7.0	100	aqueous solution	21.4	Chen and Cooper (1979)

in aqueous solution at pH 7.0, while Ruddick et al. (1980) reported a half life of 6.4 minutes in phosphate buffer at pH 7.3. The difference between the two values could be due to the effect of buffer ions. Thermal stability of 5-CH_3-H_4PteGlu has been shown to be affected by buffer ions (Paine-Wilson and Chen, 1979; O'Brpin et al., 1975).

The effect of temperature on the rates of destruction is shown in Table 19. The rates of destruction increased with temperature. In evaluating the dependence of the rate constants on temperature, the natural logarithm of the rate constant was plotted versus the reciprocal of the absolute temperature in Figure 17. Activation energies obtained as described previously were 19.0, 17.0, 19.7 and 19.8 kcal/mole at pH 3, 4, 5 and 6. The slopes of the lines in Figure 17 are not significantly different ($p \geq 0.05$), indicating that the activation energies are not significantly different. This suggests that the mechanism of destruction does not change over the pH range 3-6 in contrast to PteGlu. The Ea values obtained in this study are higher than values reported in the literature. Chen and Cooper (1979) reported an Ea value of 9.5 kcal/mole at temperatures ranging from 49 to 100°C. Ruddick et al. (1980), working at the same temperature range reported an Ea value of 7.1 kcal/mole. The differences between the values obtained in the present study and the literature values could be due to the different temperature ranges employed since an Ea value of 9.58 kcal/mole (Table 26) was obtained at pH 3.4 over the temperature range of 50-70°C.

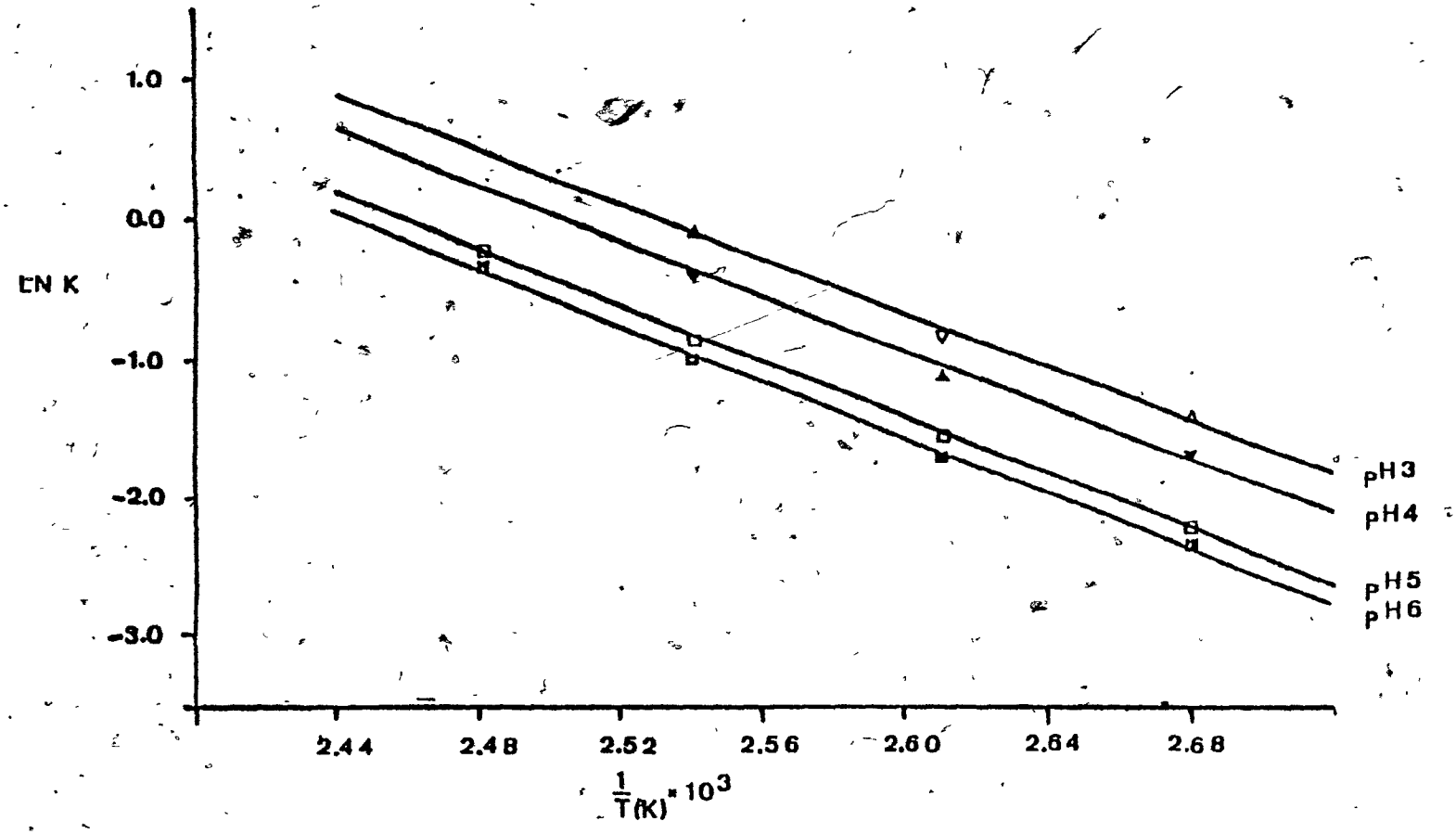

Figure 17. Arrhenius plot for thermal degradation of 5-CH_3-H_4PteGlu in citrate buffer. Correlation coefficients, 0.99 at pH 3, 0.97 at pH 4, 0.99 at pH 5 and 0.98 at pH 6 are significant ($p \geq 0.01$). The slopes of the lines are not different ($p \geq 0.05$).

It was reported by Larrabee et al. (1961) and confirmed by Gupta and Huennekens (1967) that when 5-CH_3-H_4PteGlu is shaken in air or oxygen at pH 8.7 it is converted to the dihydro-compound 5-CH_3-H_2PteGlu. This compound is not effective in supporting growth of L. casei (Blakley, 1969).

4. Effect of nitrogen atmosphere on degradation of 5-CH_3-H_4PteGlu

The presence of oxygen has been implicated as a possible factor in the degradation of folates during heating. Ford et al. (1969), investigating the effects of UHT processing and subsequent storage of milk on folic acid content, concluded that variation in the stability of folates was related directly to the presence of oxygen in the milk, the de-aeration of milk before processing effectively reducing the vitamin loss. Chen and Cooper (1979) observed that 5-CH_3-H_4PteGlu was considerably stabilized when heated at 100°C under a nitrogen atmosphere.

The destruction rate curves for 5-CH_3-H_4PteGlu at 100°C in citrate buffer with limited oxygen are shown in Figure 18. The correlation coefficients obtained by applying first order reaction equation to the data (Table 21) were quite high so the degradation reaction for 5-CH_3-H_4PteGlu could be described by first order reaction kinetics.

Table 22 shows a comparison of the rates of destruction of 5-CH_3-H_4PteGlu at 100°C in limited and unlimited oxygen. At all pH levels tested the rates of destruction are lower in limited oxygen,

TABLE 21. Effect of temperature and pH on rates of destruction of 5-CH_3-H_4PteGlu in citrate buffer with limited oxygen (1.7% of saturation level)

	Rates of destruction * (min^{-1})				
	100°C	110°C	121°C	130°C	140°C
3.0	0.106 (0.95)[a]	0.133 (0.96)	0.215 (0.97)	0.420 (0.95)	–[b]
4.0	0.092 (0.96) [c]	0.128 (0.97)	0.180 (0.98)	0.313 (0.96)	–
5.0	0.072 (0.97)	0.094 (0.95)	0.152 (0.95)	0.286 (0.97)	0.357 (0.96)
6.0	0.059 (0.97)	0.081 (0.98)	0.137 (0.95)	0.223 (0.97)	0.312 (0.94)

*Rate of destruction = slope x 2.303

[a]Correlation coefficients (in parenthesis) are significant ($p \geq 0.01$)

[b]No data collected

[c]Rate constants bracketed by the same line are not significantly different (5% level)

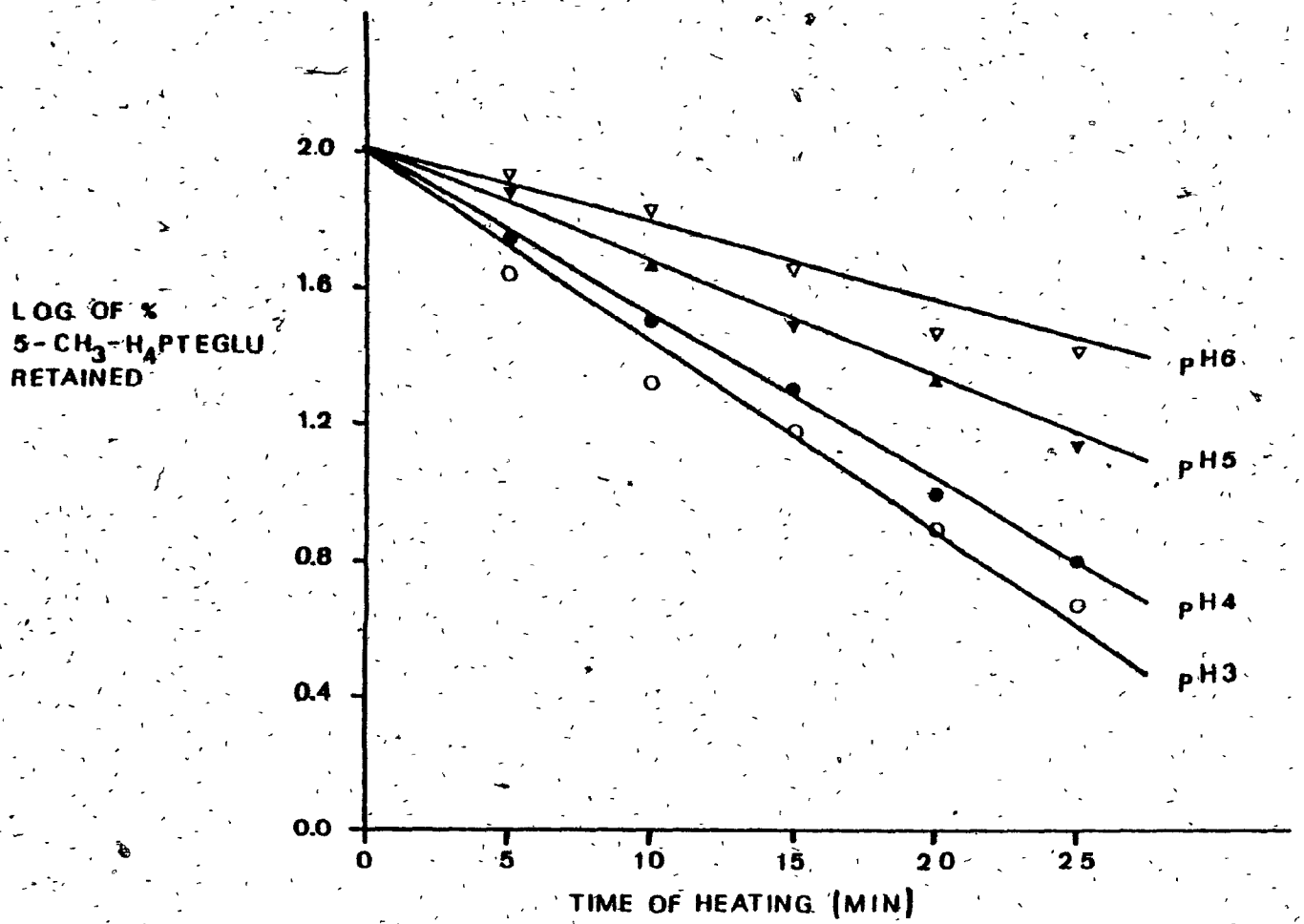

Figure 18. Rate of destruction curves of 5-CH_3-H_4PteGlu in buffer with limited oxygen at 100°C. Correlation coefficients, 0.97 at pH 6, 0.97 at pH 5, 0.96 at pH 4 and 0.95 at pH 3 are significant ($p \geqslant 0.01$).

TABLE 22. Rates of destruction of 5-CH_3-H_4PteGlu at 100°C in citrate buffers in unlimited and limited oxygen

pH	Rate constants k (min^{-1})[a]	
	Unlimited O_2	Limited O_2
3	0.243	0.106
4	0.192	0.092
5	0.110	0.072
6	0.104	0.059

[a]The data in the table are repeated here to facilitate comparison.

indicating that 5-CH_3-H_4PteGlu destruction is related to the oxygen content of the system. This is in agreement with Chen and Cooper (1979), who reported that at pH 7 in aqueous solution, 5-CH_3-H_4PteGlu lost little activity through one hour of heating at 100°C under nitrogen. Ruddick et al. (1980) reported that the degradation of 5-CH_3-H_4PteGlu depended on the concentration of dissolved oxygen. Monitoring the amount of oxygen in the system during degradation of 5-CH_3-H_4PteGlu in limited oxygen at 20°C, they reported that the amount of oxygen in the system decreased with time. This was taken to indicate that an oxidation reaction was taking place.

The Ea values obtained for the degradation of 5-CH_3-H_4PteGlu in limited and unlimited oxygen are shown in Table 23. The Ea values at limited oxygen were significantly lower ($p \leq 0.05$) than the values obtained for the degradation in unlimited oxygen. These values suggest that the mechanism of the degradation reaction is not the same in the two cases. The mechanism and products of degradation of 5-CH_3-H_4PteGlu in limited oxygen have not been identified yet so further speculation on this point would be premature at this time.

In spite of the lack of statistical significance it is curious that at pH 4.0, lower values than at any other pH were obtained both in limited and unlimited oxygen. The significance of this dip is not known, and maybe more accurate or precise future work may discern a significant difference here. The activation energy of ascorbic acid has been shown to decrease to a minimum at pH 4.06 and then increasing with pH (Lee et al., 1977).

TABLE 23. Activation energies for the degradation of 5-CH_3-H_4PteGlu in unlimited and limited oxygen

pH	Activation energy (kcal/mole)	
	Unlimited O_2	Limited O_2
3	19.0 [a]	13.6
4	17.0	11.8
5	19.7	13.2
6	19.8	13.3

[a] Activation energy values bracketed by the same line are not significantly different ($p \geq 0.05$).

c. Destruction of PteGlu and 5-CH_3-H_4PteGlu in model food systems

Most workers reporting kinetic data on vitamin destruction in food systems have suggested that the data obtained from model systems may not be applicable to food systems. A good example is ascorbic acid. In buffer systems the activation energy for destruction of ascorbic acid varies from 18 to 22.4 kcal/mole (Huelin, 1953; Blaug and Hajratwala, 1972), while in food systems the Ea varies from 3.3 kcal/mole in tomato juice (Lee et al., 1977) to 45 kcal/mole in peas (Labuza, 1972). Thiamine (Mulley et al., 1975) has also been shown to be more stable in food systems than in model systems.

To study the destruction of the two forms of folic acid in food systems the native folates must be removed from the food systems. In solid foods usually water extraction is used to get rid of native folates (Herbert, 1963). Use of heat in acid solution, irradiation or treatment with sulfite has been tried as means of removing folates from natural sources such as liver extracts and tomato juice serum, but these methods have been shown not to be very effective (Daniel and Kline, 1947). Some adsorbing agents such as activated charcoals have been shown to be effective in removing folic acid from tomato juice (Daniel and Kline, 1947) but this could not be used for this study because the juice pigments would also be changed and this was not desired. Dowex 1 X 8 (Cl^-) has been used to remove folates from conjugase enzymes commonly used in standard procedures for assaying for folates (Keagy et al., 1980; Sotiriadis, 1979).

Using the procedure described earlier, it was possible to reduce the folate content in the tomato juice from 0.107 µg/ml to 0.01 µg/ml. This amount of folic acid left in the tomato juice was considered negligible compared with the amount of folic acid added to the system. In apple juice it was possible to reduce the folic acid concentration from 0.01 µg/ml to undetectable levels. The possible reason the folic acid in apple juice could be reduced to undetectable levels but not the tomato juice could be due to the presence of different forms of folate in each system and also the tomato juice contained approximately ten times more folic acid.

1. Destruction of PteGlu in apple juice and tomato juice

The rate of destruction curves for PteGlu in apple juice and in tomato juice is shown in Figure 19 and Figure 20. The correlation coefficients at all temperatures tested ranged from 0.92 to 0.98 for the apple juice and from 0.89 to 0.98 in tomato juice. Linearity of these curves indicates that thermal destruction of PteGlu in apple juice and tomato juice could be described by first order reaction kinetics. The rate constants increased with temperature.

The temperature dependence for PteGlu destruction was further analyzed according to Arrhenius equation. Conformity of the temperature data to the Arrhenius equation is shown graphically in Figure 21. The linearity of the plots supports the use of the Arrhenius equation for expressing the temperature dependence of the reaction rate constants for PteGlu in model food systems.

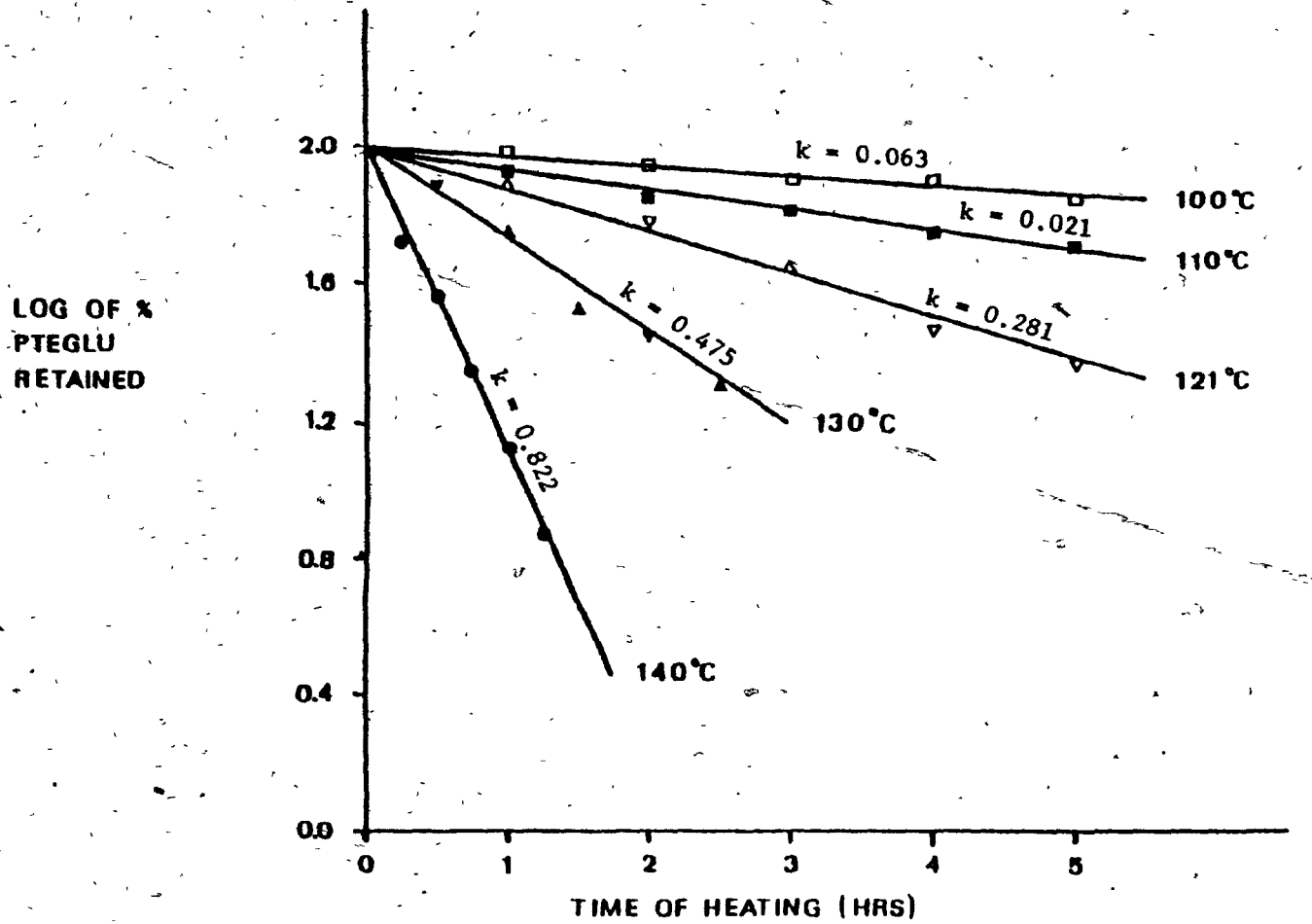

Figure 19. Rate of destruction curves for PteGlu in apple juice, pH 3.4. Correlation coefficients, 0.97 at 100°C, 0.98 at 110°C, 0.96 at 121°C and 0.98 at 140°C are significant ($p \geq 0.01$). Correlation coefficient 0.92 at 130°C is significant ($p \geq 0.05$). The slopes of the lines at 100 and 110°C are not different ($p \geq 0.05$).

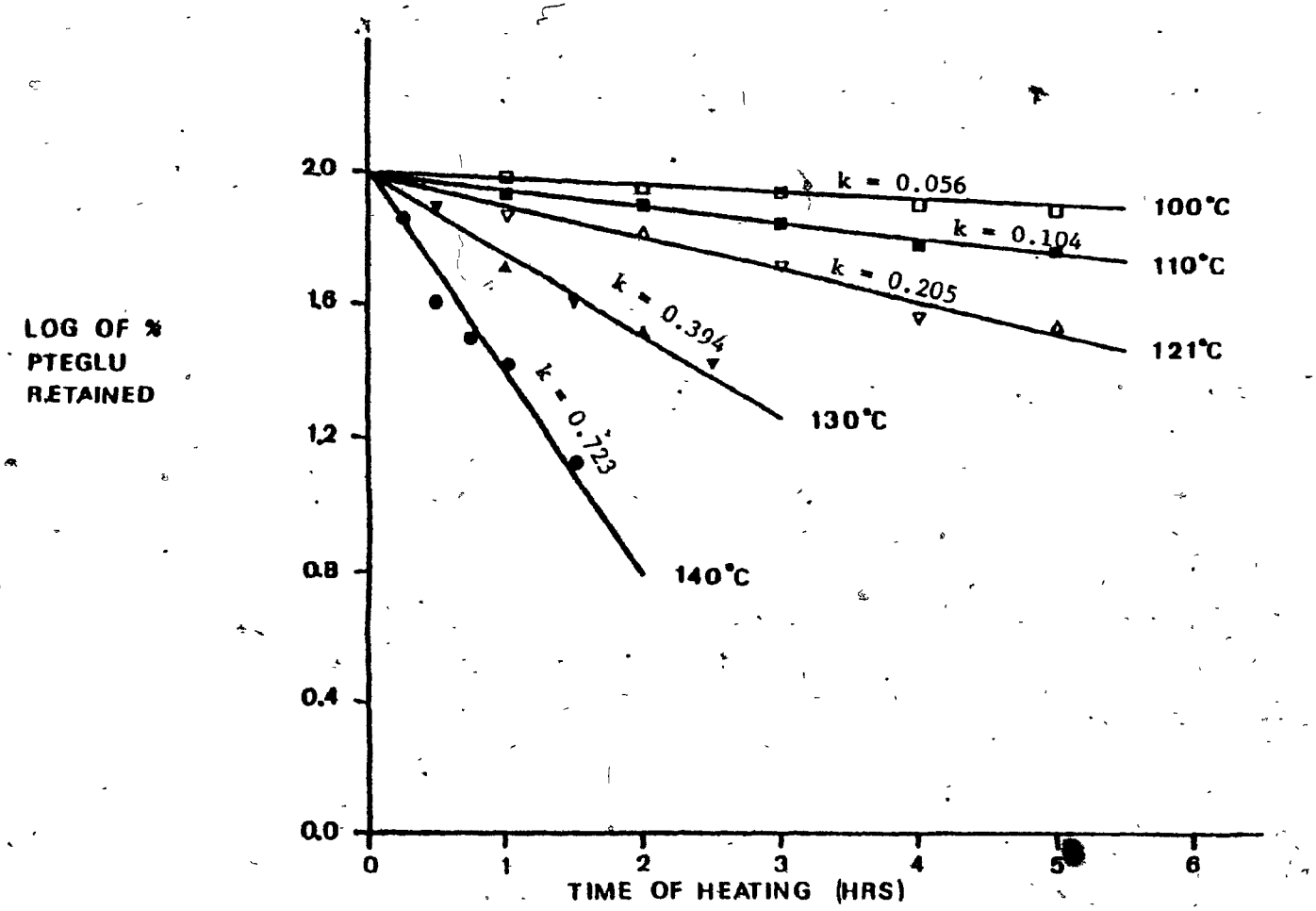

Figure 20. Rate of destruction curves for PteGlu in tomato juice pH 4.3. Correlation coefficients, 0.96 at 100°C, 0.98 at 110°C and 0.96 at 121°C are significant ($p > 0.01$). Correlation coefficients, 0.89 at 130°C and 0.92 at 140°C are significant ($p > 0.05$). The slopes of the lines at 100 and 110°C are not significantly different ($p > 0.05$).

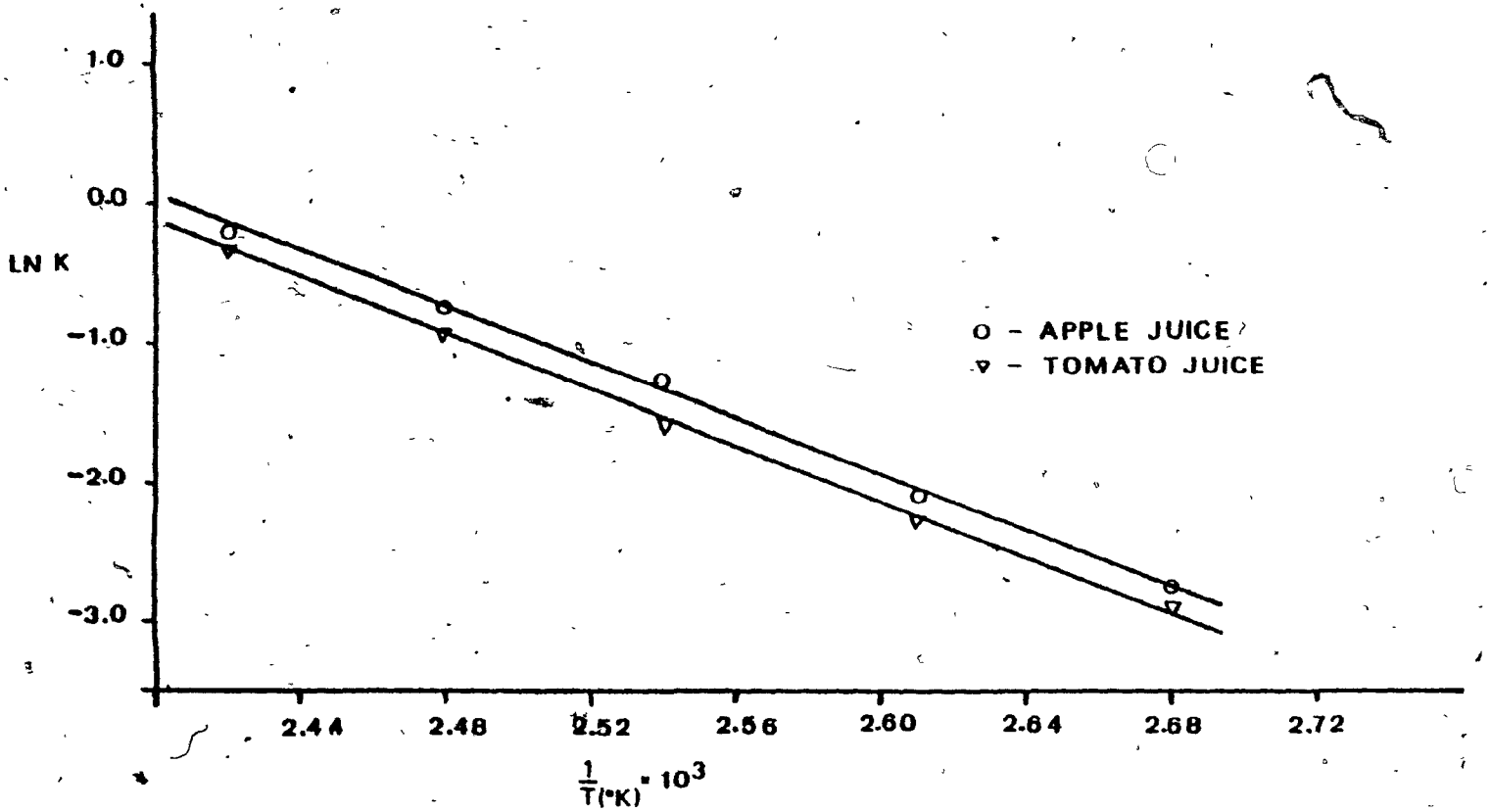

Figure 21. Arrhenius plot for thermal degradation of PteGlu in tomato juice and apple juice. The correlation coefficients, 0.98 in apple juice and 0.97 in tomato juice are significant ($p \geq 0.01$). The slopes of the two lines are not different ($p \geq 0.05$).

The activation energies as calculated from the regression lines in Figure 21 were 20.0 kcal/mole in apple juice and 19.7 kcal/mole in tomato juice. These two values are not significantly different ($P \geq 0.05$), which indicates that the mechanism of destruction in the two food systems could be the same. The Ea values are also similar to the Ea values for PteGlu destruction in buffer systems at pH 3-4.

Comparing the rate constants in the food systems with those in buffered systems at 100°C indicates that the rate of destruction of PteGlu in apple juice (pH 3.4) is significantly lower ($p \leq 0.05$) than the rates of destruction in citrate buffer at pH 3 (Table 24). This was also true for the rest of the temperatures tested. At 100°C the half life of PteGlu in citrate buffer at pH 3 was 4.78 hours while the half life in apple juice pH 3.4 was 11.0 hours. At pH 4 in buffer system, the rate of destruction is still higher although not statistically different ($p \geq 0.05$).

The rates of destruction for PteGlu in the apple juice have to be viewed with some caution because the canned apple juice contained ascorbic acid added during processing (35 mg/100 ml). Chen and Cooper (1979) reported that ascorbic acid has a protective effect on folate compounds but then the procedure used in preparing the apple juice sample probably was enough to destroy most of the ascorbic acid present before spiking with folic acid. So it can be stated that the stability of PteGlu is higher in apple juice than in buffered systems. This indicates the existence of factors other than heat and pH that can modify the degradation reaction. Factors such as amino acids, proteins and starch have been reported to have a protective effect on

TABLE 24. Rates of destruction for PteGlu in citrate buffers, pH 3, 4 and 5 and in tomato juice, pH 4.3 and apple juice, pH 3.4 at 100°C

System	Rates of destruction k (h^{-1})
Citrate buffer, pH 3	0.145
Apple juice, pH 3.4	0.063
Citrate buffer, pH 4	0.074
Tomato juice, pH 4.3	0.056
Citrate buffer, pH 5	0.021

The data for pH 3, 4 and 5 were obtained in this study. They are repeated here to facilitate comparison.

thiamine (McIntire and Frost, 1944). According to Smock and Neubert (1950) canned apple juice contains very little amounts of protein (0.1%) and no starch or amino acids; so such factors may not play an important role in stabilizing the PteGlu. Hence, further work is required to quantify the variation in kinetics as a function of other factors such as product composition.

In tomato juice, pH 4.3, the rate of destruction of PteGlu was not significantly different from that at pH 4.0 in buffer systems, but it was significantly lower than that at pH 5.0 in buffer systems ($p \leq 0.05$). This indicates that there is no difference between the rates of destruction in the buffer system and in the tomato juice. In the case of tomato juice data obtained from the buffer system could be used to estimate destruction rates of PteGlu in tomato juice.

Comparing the two systems at 100°C (Table 24), the rate of destruction in apple juice at pH 3.4 is not significantly different from the rate of destruction in tomato juice, pH 4.3. This suggests that in spite of the differences in composition of the two systems (Gould, 1974; Smock and Neubert, 1950), PteGlu does not appear to react with the food components.

2. Destruction of 5-CH_3-H_4PteGlu in apple juice and tomato juice

The destruction rate curves for 5-CH_3-H_4PteGlu in apple juice and tomato juice are shown in Figures 22 and 23 respectively. The high correlation coefficients (Tables 25 and 26) indicate that over the temperature range studied the kinetics of 5-CH_3-H_4PteGlu

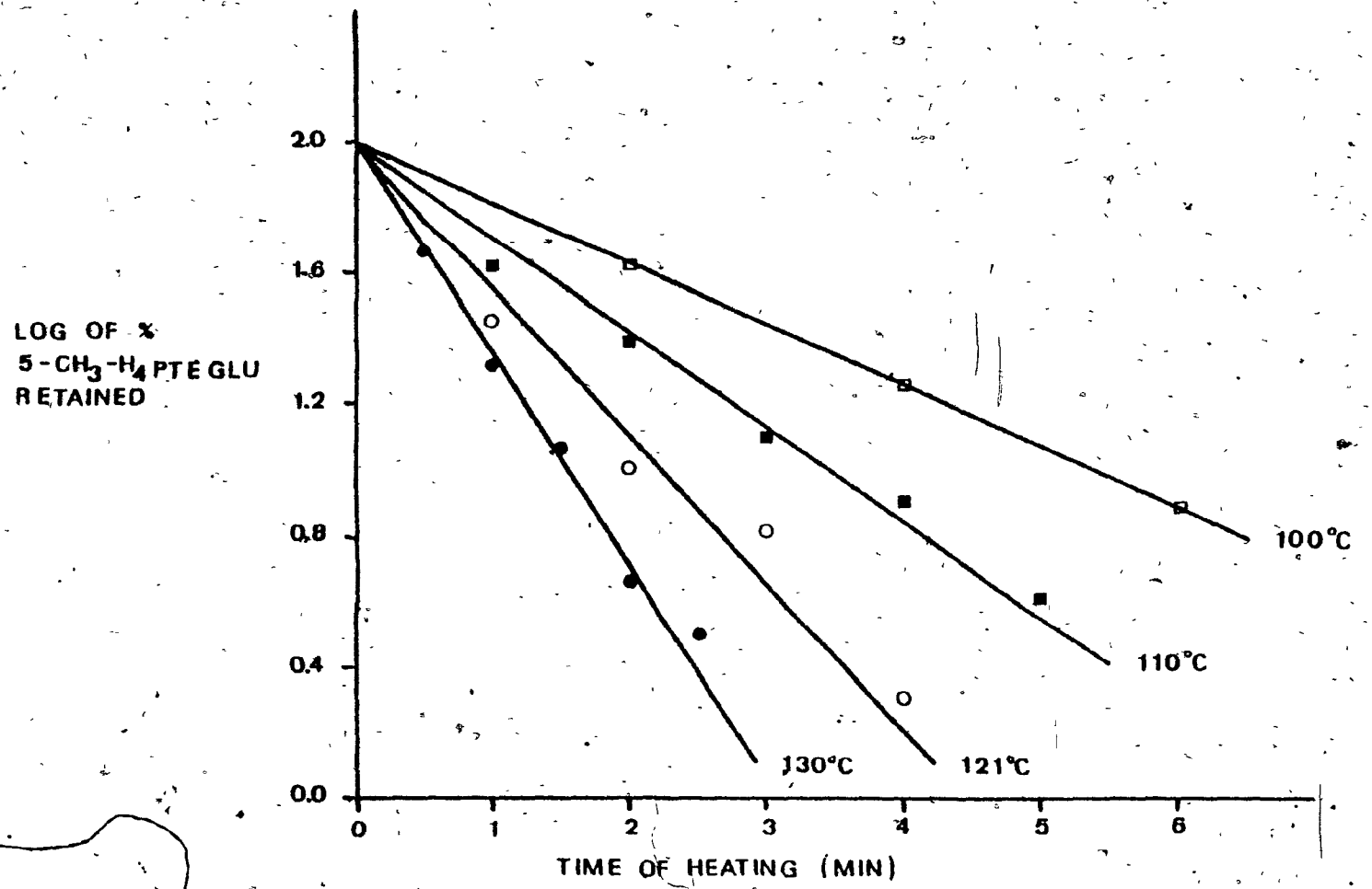

Figure 22. Rate of destruction curves for 5-CH_3-H_4PteGlu in tomato juice, pH 4.3. Correlation coefficients, 0.99 at 100°C, 0.95 at 110°C, and 0.93 at 130°C are significant ($p \geq 0.01$) and the correlation coefficient 0.92 at 121°C is significant ($p \geq 0.05$). The slopes of the lines at 100 and 110°, 110 and 121° are not different ($p \geq 0.05$).

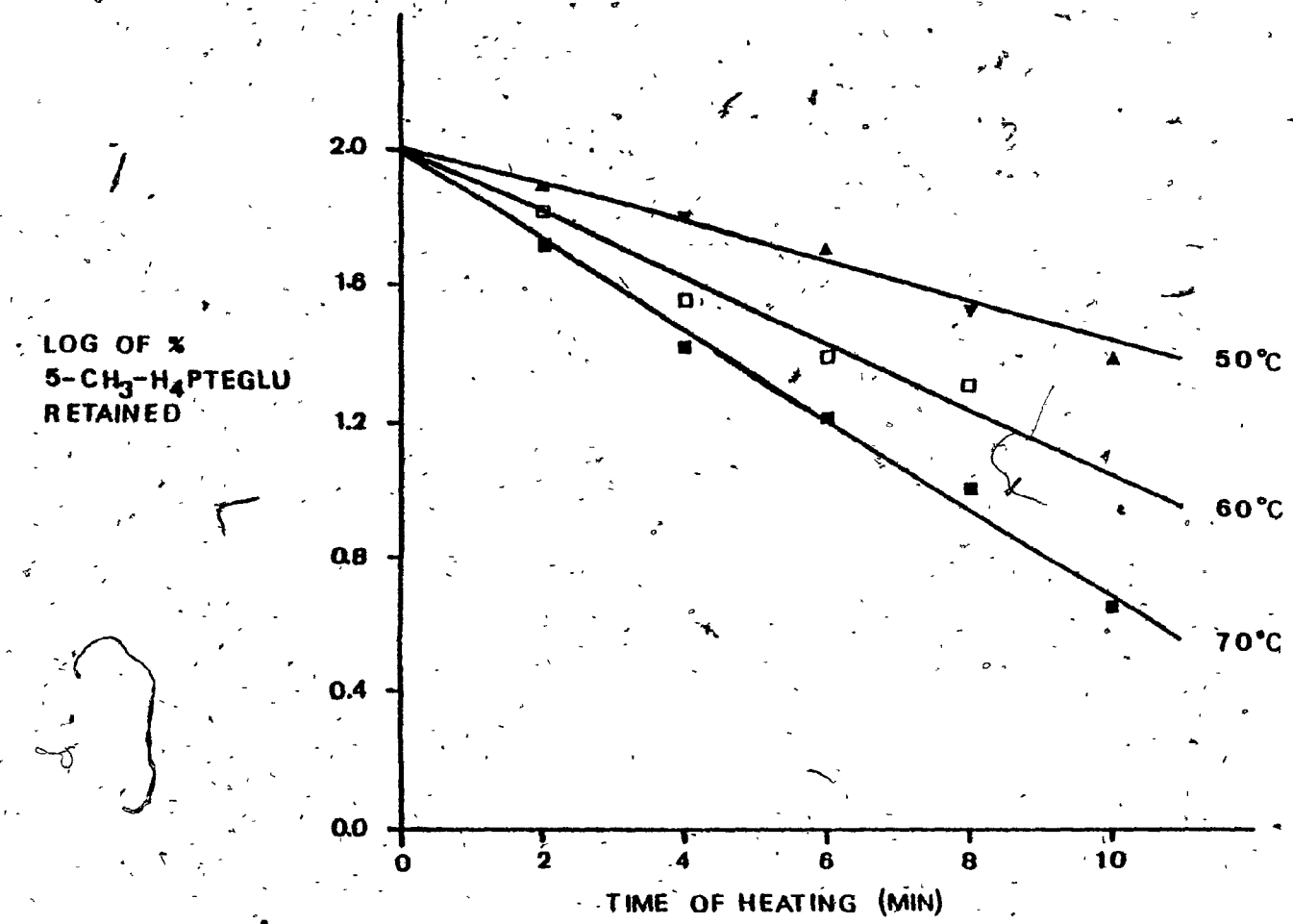

Figure 23. Rate of destruction curves for 5-CH_3-H_4PteGlu in apple juice, pH 3.4. The correlation coefficients, 0.96 at 50°C, 0.95 at 60°C and 0.97 at 70°C are significant ($p \geq 0.01$).

TABLE 25. Rates of destruction of 5-CH_3-H_4PteGlu in tomato juice, pH 4.3, and in citrate buffers, pH 4 and 5

System	Rate constants k (min^{-1})			
	100°C	110°C	121°C	130°C
Citrate buffer pH 4.0	0.192	0.318	0.635	
Tomato juice pH 4.3	0.374	0.508	0.792	1.065
Citrate buffer pH 5.0	0.110	0.215	0.432	0.808

The rate constants in citrate buffers pH 4 and 5 are repeated here to facilitate comparison.

TABLE 26. Rates of destruction of 5-CH_3-H_4PteGlu in buffer (pH 3.4) and apple juice (pH 3.4) (min^{-1})

	50°C	60°C	70°C	Ea (kcal/mole)[b]
Citrate buffer pH 3.4	0.0148 (0.98)[a]	0.022 (0.96)	0.036 (0.97)	9.58
Apple juice pH 3.4	0.123 (0.96)	0.193 (0.95)	0.249 (0.97)	7.85

[a]Correlation coefficients (in parentheses are significant ($p \geq 0.01$)).

[b]Activation energies are not significantly different ($p \geq 0.05$).

destruction in apple juice and tomato juice were consistent with first order kinetics.

The destruction of 5-CH_3-H_4PteGlu in the apple juice could not be done at temperatures of 100°C and above because the destruction was too fast to be measured by the present technique. Essentially 100% destruction occurred after 2 minutes.

In both apple juice and tomato juice the rates of destruction of 5-CH_3-H_4PteGlu increased over that obtained in comparable simple buffer systems (Tables 25 and 26). The high rates of destruction could be due to unknown reactions between the 5-CH_3-H_4PteGlu and some of the components of the food systems which could have accelerated the destruction of 5-CH_3-H_4PteGlu. Apple juice contains fructose, glucose and sucrose. On heating the apple juice one would expect the sugars to be degraded, and the degradation was actually observed, especially at high temperatures, by browning of the solutions. The high rates of destruction in apple juice could then be due to reactions between the degradation products of the sugars and the 5-CH_3-H_4PteGlu.

There might also be some reaction between the NH_2 group of the 5-CH_3-H_4PteGlu and the reducing sugars, but the rate of such reaction would be expected to be very low since the system was quite acidic (Fennema, 1976). Another reason could be that an important component in the food system which could have stabilized the 5-CH_3-H_4PteGlu might have been removed with the native folates during the Dowex treatment. More research is required on this subject to be able to determine as to why the rates of destruction were so high in the food systems.

Activation energies determined from the Arrhenius plot (Figure 24) in apple juice and citrate buffer were 7.85 and 9.58 kcal/mole respectively. In tomato juice the Ea value (Figure 25) was 10.6 kcal/mole. These three values were not significantly different ($p \geq 0.05$). These values obtained for the destruction of $5\text{-}CH_3\text{-}H_4PteGlu$ in the three systems suggest that the mechanism of destruction is not significantly different.

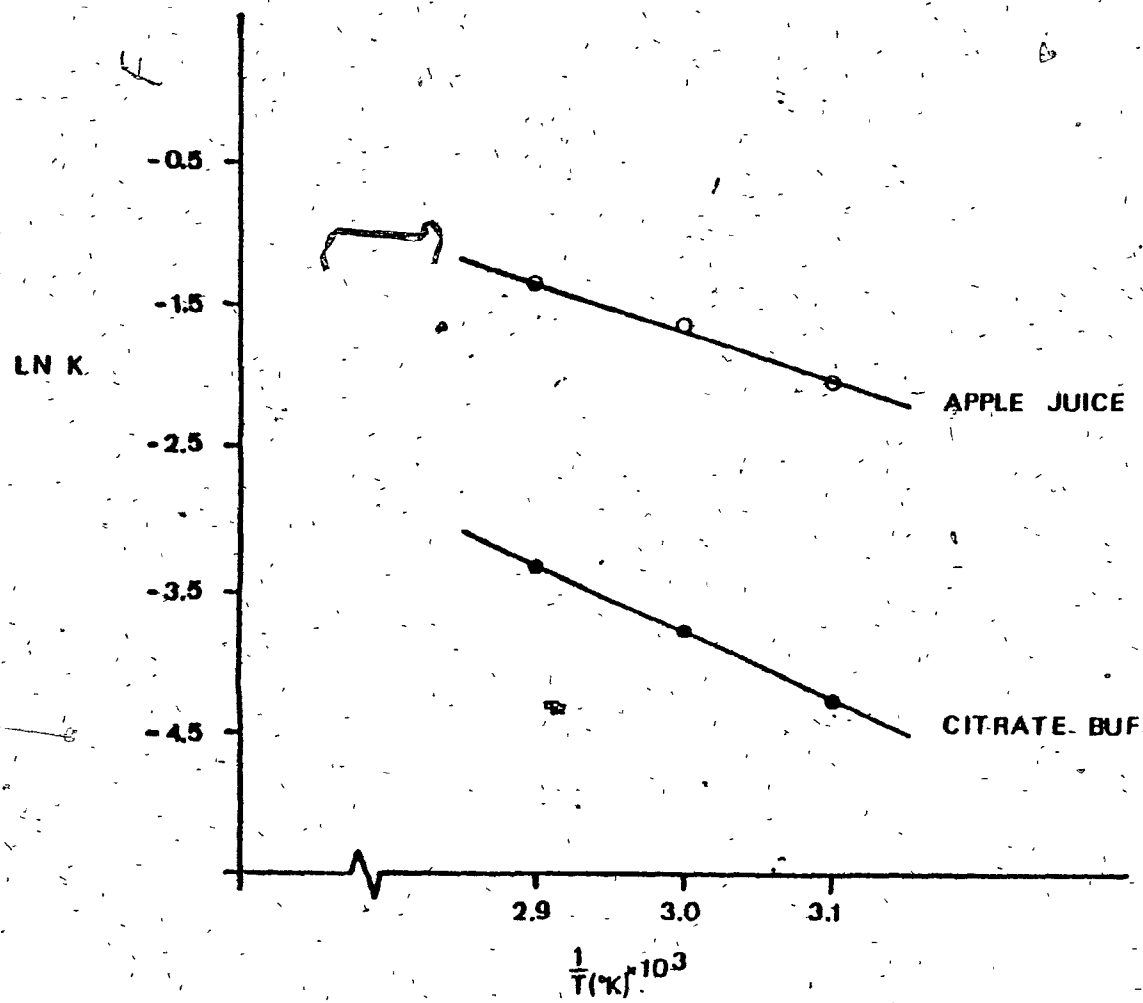

Figure 24. Arrhenius plot for thermal degradation of 5-CH_3-H_4PteGlu in apple juice and citrate buffer. Correlation coefficients, 0.99 in apple juice and 0.99 in citrate buffer are significant ($p \geq 0.01$). The slopes of the lines are not different ($p > 0.01$).

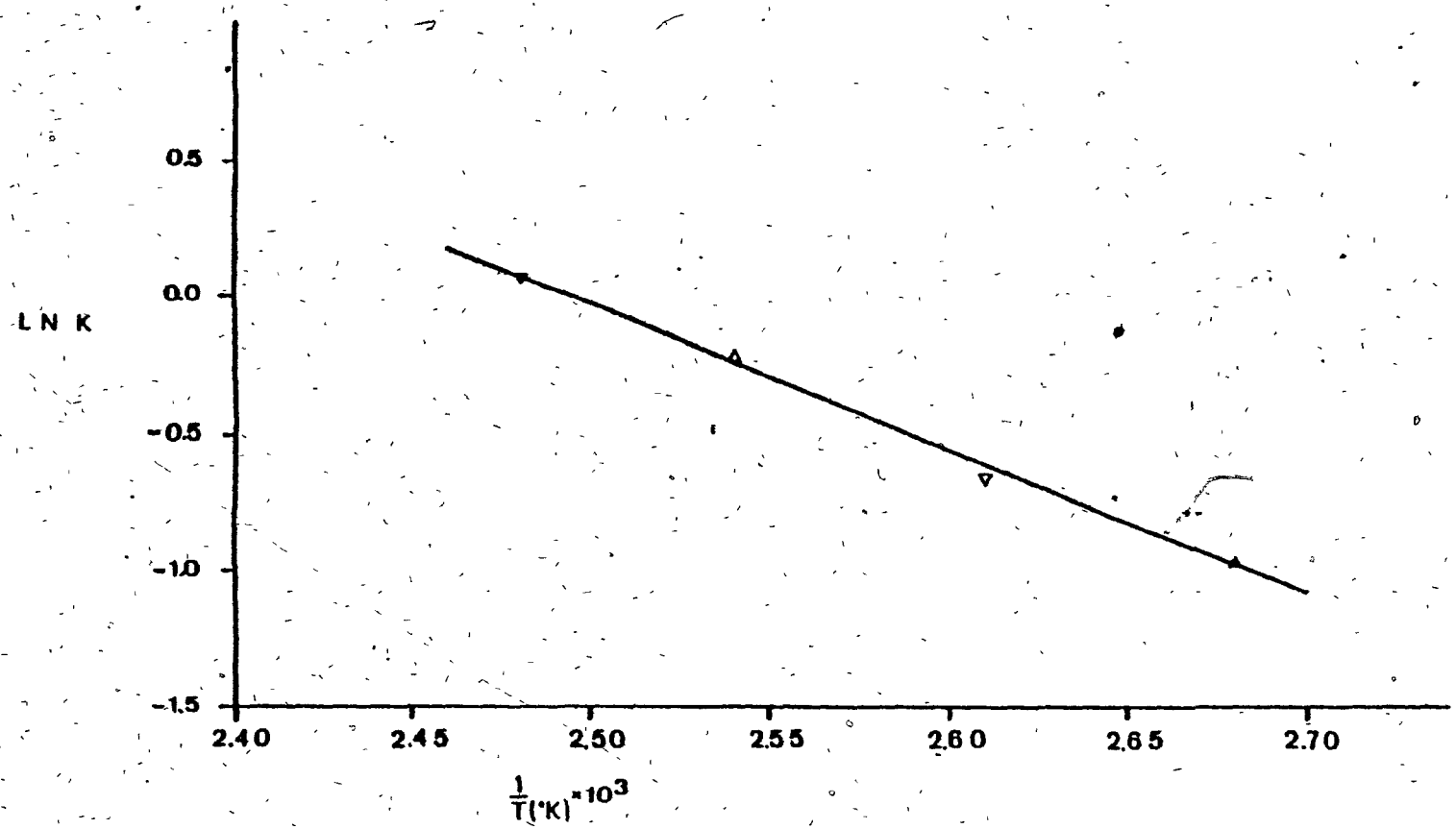

Figure 25. Arrhenius plot for thermal degradation of 5-CH_3-H_4PteGlu in tomato juice, pH 4.3. Correlation coefficient was 0.99 and was significant ($p \geq 0.01$).

CHAPTER V

SUMMARY

Thermal degradation of PteGlu and 5-CH_3-H_4PteGlu in buffer systems and model food systems could be described by first order reaction kinetics. The rate constants increased with temperature and the relationship between rate constants and temperature could be described by the Arrhenius equation. Therefore, kinetic data for thermal destruction of both PteGlu and 5-CH_3-H_4PteGlu were obtained in buffer and model food systems.

The activation energy for thermal degradation of PteGlu in buffer systems decreased with increasing pH, indicating that the mechanism of inactivation may be different depending on pH.

The stability of 5-CH_3-H_4PteGlu was increased in nitrogen atmosphere. This indicates that the degradation of 5-CH_3-H_4PteGlu at temperatures studied is due to an oxidative process requiring the presence of oxygen.

The stability of PteGlu was increased in the apple juice. This suggests the existence of factors other than heat and pH which can affect the stability of PteGlu. There was no significant difference between the rates of destruction of PteGlu in buffer system and in tomato juice, therefore the data obtained from buffer systems can be used to estimate rates of destruction of PteGlu in tomato juice. There was no significant difference between the destruction rates of PteGlu in tomato juice and in apple juice, suggesting that PteGlu destruction rates were not affected by the different components of the two systems.

The rates of destruction of 5-CH_3-H_4PteGlu in the model food system were higher compared with buffered systems but the activation energies were not significantly different, suggesting that the mechanism of destruction may not be significantly different.

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