A STUDY OF THE MEASUREMENT AND DEGRADATION OF FOLIC ACID

by

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ABSTRACT

Folic acid is present in foods in many different forms. Concentrations of N^5-methyltetrahydrofolic acid (one form of folate) were determined by a radioassay and the Lactobacillus casei microbiological assay methods. The coefficient of determination for samples analyzed by both methods was 0.86 but the radioassay method was found to be faster, simpler and more accurate than the microbiological method. The milk folate binder and L. casei were found to react differently to pteroylglutamic acid (another form of folate) compared to N^5-methyltetrahydrofolic acid. The two methods of assay were found to be suitable only for measuring the form of folate used to construct the standard curve for the assay. It was found that neither method could be used to quantify folate levels in foods accurately. Thus, at best, presently available data on food folate levels can only be compared to other values obtained by an identical method. Also folate concentration values presently available may not necessarily be a reflection of their nutritional significance to man.

The radioassay method was used for the measurement of N^5-methyltetrahydrofolic acid degradation under various conditions. It was found that N^5-methyltetrahydrofolic acid degradation in the presence of an unlimited oxygen supply could be described as a pseudo first order reaction. Rate constants for the reaction were found to increase as the temperature of the reaction increased and were described in terms of the Arrhenius equation.

The overall reaction appeared to be second order in the presence of a limited oxygen supply. The presence of mercaptoethanol in the assay system delayed the beginning of the N^5-methyltetrahydrofolic acid degradation reaction. The presence of ascorbic acid in the N^5-methyltetrahydrofolic acid/buffer solution also delayed the start of the reaction. The reaction rate constants were not altered by the presence of the two reducing agents, however.

The degradation of N^5-methyltetrahydrofolic acid was therefore concluded to be an oxidation reaction. The degradation product was identified as N^5-methyldihydrofolic acid by ultraviolet spectroscopy.

The results of this research implicate the importance of reducing agents in foods containing folate which are subjected to heat processing. Experiments with food materials containing ascorbic acid and exposed to high temperatures indicated that the degradation of free folate was delayed by the presence of the reducing agent.

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INTRODUCTION

The folates are a group of water soluble compounds within the vitamin B complex. They are physiologically and biochemically important especially to higher animals and to man, playing an important role in amino acid metabolism and in the synthesis of purine and pyrimidine nucleotides. A deficiency of this vitamin due to dietary inadequacy, failure of alimentary absorption or elevated metabolic requirement produces a characteristic change in the blood known as megaloblastic anemia. Folate deficiency is probably the most prevalent vitamin deficiency in man and is common the world over.

It has been suggested that one of the major causes of folate deficiency is the destruction of folates during the processing and cooking of food (Herbert, 1968b). Because of their essential role in nutrition it becomes important to attempt to ensure maximum retention of folates during processing. Such measures can only be achieved as a result of a better understanding of the respective resistance to destructive processes of the various folate compounds.

A search of the literature indicates that while some studies have been conducted on losses of folate within food materials (Cheldin et al., 1943; Schweigert et al., 1946; Hanning and Mitts, 1949; Ghitis, 1966; Herbert, 1967; 1968b; Ford et al., 1968; Schroeder, 1971), there is a complete lack of information pertaining to the kinetics of folate breakdown within a simple system. (This may be due to both the

complexity of the vitamin and the lack of a rapid, accurate and simple assay method). Kinetic information such as this is the first step to correlating losses of the vitamin to food composition and to various processes and storage techniques.

Since the folate vitamin is so complex, the form chosen for the kinetic studies was N^5-methyltetrahydrofolic acid ($N^5CH_3FH_4$). The structure of this form of folate is illustrated in Figure 1. The effect of heating $N^5CH_3FH_4$ in phosphate buffer to various temperatures and at various oxygen concentrations was evaluated. The effect of various reducing agents on the rate of degradation of $N^5CH_3FH_4$ was also studied. Following initial comparative studies with the Lactobacillus casei microbiological assay method, a radioassay system was chosen for measuring $N^5CH_3FH_4$ levels.

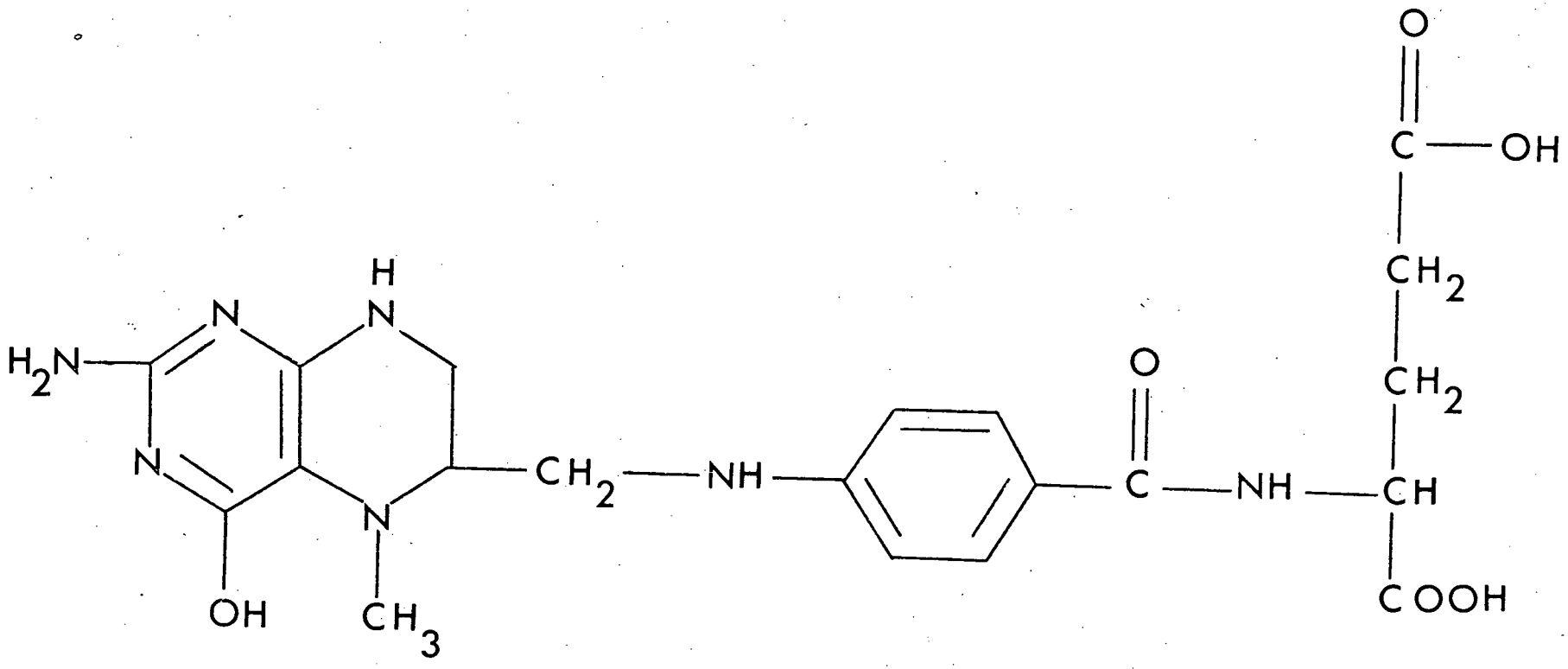


Figure 1. Structure of N^5 methyltetrahydrofolic acid.

CHAPTER 1. REVIEW OF LITERATURE

DEFINITION

Folic acid has been the subject of many investigations involving several species of animals and microorganisms. Hence, the vitamin had a confusing roster of names before it was learned that the same or similar substances were being studied.

As early as 1935, a nutritional deficiency in monkeys, which eventually led to anemia, now known to respond to folic acid, was described. This factor was called "Vitamin M" (Day *et al.*, 1935). In 1939, Hogan and Parrott described an anemia in chicks due to a deficiency of an unknown factor which they termed "Vitamin B". In 1943, Stokstad reported a factor necessary in the nutrition of *Lactobacillus casei*, which he named "the *L. casei* factor" and Mitchell *et al.* (1941) isolated a crystalline compound from spinach which they called "folic acid", from the Latin term for leaf (folium). These and several other factors were shown to belong to the same nutritionally and chemically related family of compounds, folic acid.

The term "folic acid" is presently accepted to have two meanings (Malin, 1975). Generically, it covers a broad range of closely related pteroic acid compounds which include the monoglutamate, the polyglutamate forms and their reduced or substituted analogues. Specifically, it is used to describe the compound, pteroylglutamic acid. It has been proposed by the IUPAC-IUB commission (Stokstad and Thenen, 1972) that the terms "folic acid" and "folate" be used in the general sense for any member of the family of compounds, or a mixture of these, with the basic structure shown in Figure 2, either free or conjugated with two or more glutamic

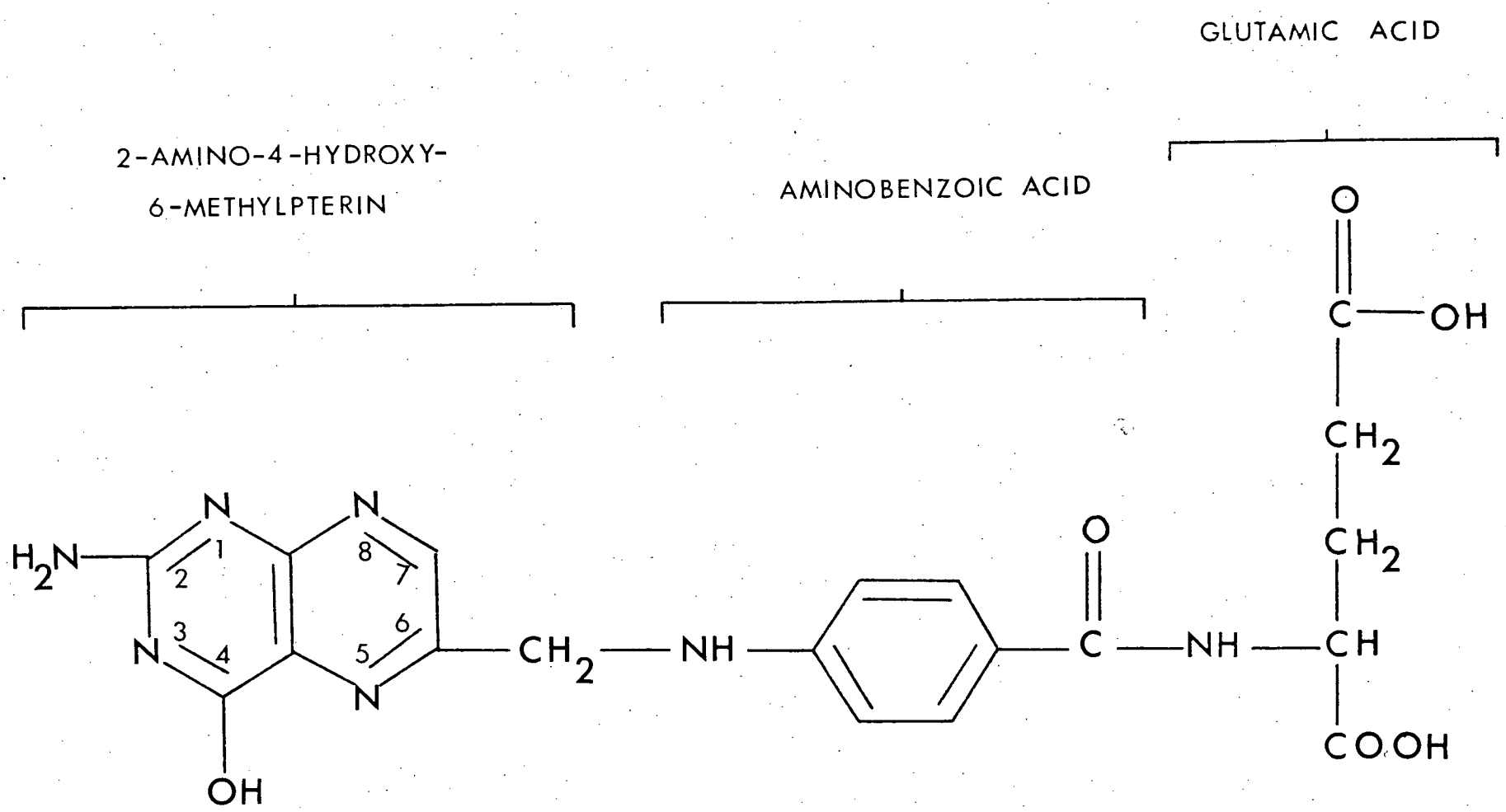


Figure 2. Basic structure of all folate compounds-pteroylglutamic acid.

acid residues.

BIOCHEMICALLY ACTIVE FOLATES - THEIR DISTRIBUTION AND AVAILABILITY IN FOODS

The forms of folic acid that are biochemically active differ from the structure shown in Figure 2 in various ways. Firstly, the biochemically active forms are reduced - i.e. they have additional hydrogens attached to the pteridine ring. Secondly, the folate molecule may hold an additional carbon attached to the pteridine ring or to the ρ-amino benzoic acid as a formyl or a methyl group or as a bridge between these two positions as methylene or methenyl. The third important biochemical change is that some naturally occurring forms are conjugated in peptide-like linkage through their gamma carboxyl groups to one or more glutamic acid residues.

Malin (1975) has stated that there are more biologically active forms of folic acid than of any known vitamin. Attempts have been made to separate the many natural folic acid derivatives by various chromatographic methods (Silverman et al., 1961; Shin et al., 1972a, 1972b; Kas and Cerna, 1976). By employing such procedures, the distribution of folic acid has been determined for certain representative foods and tissues. Results show a wide distribution of general types of folate derivatives. Rat kidney and liver folates are approximately 40 percent methyl derivatives - mainly pentaglutamates - whereas red cells contain only the methyl form present as both penta-and hexaglutamate (Shin et al., 1972a, 1974). Milk also contains mainly methyl derivatives, 60 percent

of which are present as the monoglutamate (Shin, et al., 1975). Plant material varies in its distribution of folate derivatives. Lettuce cabbage and orange juice contain mainly methyl derivatives (Batra et al., 1973; Chan et al., 1973, Tamura et al., 1976) but soybeans contain only 15 percent of this form, the rest being either the 5- or 10- formyl derivative (Shin et al., 1975).

Much work has been published on the availability and absorption of the folic acid derivatives in food (Swendseid et al., 1947; Spray, 1952; Jandl and Lear, 1956; Streiff and Rosenberg, 1967; Herbert, 1968a; Butterworth et al., 1969; Rosenberg and Godwin, 1971; Tamura and Stokstad, 1973). It is known with certainty that prior to absorption, the γ-glutamyl peptide conjugates are acted upon by intestinal enzymes (γ-glutamyl carboxypeptidase - "conjugase") releasing the monoglutamic folate. In the absence of certain conjugase inhibitors known to be present in various food materials (Stokstad et al., 1977), there is little difference in the availability of the conjugate and the monoglutamate form of folate. An estimate based on their studies with tritium labelled folate monoglutamate suggests that 80% may be absorbed.

METHODS OF FOLATE MEASUREMENT

Methods for determining folic acid levels may be grouped into three categories:

1) Biological methods

2) Microbiological methods

3) Biochemical, chemical and instrumental methods

Biological Methods

O'Dell and Hogan (1943) and Campbell *et al.* (1944) developed the chick assay method of folate measurement. Recovery from deficiency symptoms when fed crystaline folic acid and foods of unknown potency form the basis of this method. Rats have also been used for biological assays (Ransome and Elvehjem, 1943, Asenjo, 1948). The main disadvantages of animal assays are their expense and the time factor involved. Freed (1966) however, advocated the use of animal assays to supplement microbiological data.

Microbiological Methods

Teply and Elvehjem (1945) developed a method of folate analysis using the titrimetric measurement of growth of *Lactobacillus casei*. This organism has since become the most popular although the following are, or have been, employed:- *Bacillus coagulans* (Baker *et al.*, 1955) *Pedicoccus cerevisiae* (Kavanagh, 1963) *Streptococci* species (Luckey *et al.*, 1944). The ciliated protozoan, *Tetrahymena geleii* (Jukes, 1955) and the flagellate, *Crithidia fasciculata* (Nathan *et al.*, 1958).

Cooperman (1967) has emphasised that the choice of microorganism for any assay must take into consideration the type of folates likely to be present in the sample. For example, *L. casei* is the only organism responding well to N^5 methyl folates - (as previously stated, such folates are very common in food materials). *S. faecalis* (AOAC recommended assay method) does not respond to N^5 methyl folates. However,

it does grow well in the presence of pteroic acid which is metabolically inactive for man.

Folates are frequently found in nature to be present in the polyglutamate form. Thus, it was found necessary by many workers to liberate free folates prior to microbiological assay. Conjugase has been employed for this purpose (Bird et al., 1945, Dabrowska et al.,1949).

Because reduced forms of folic acid have been shown to be heat labile, Toennies et al. (1956) employed ascorbic acid in the extraction solvent to prevent folic acid destruction. The ascorbate was later shown by Herbert (1961) to act as a growth stimulant to Lactobacillus casei. Thus he deemed it necessary to include ascorbate in the standards to obtain a true assessment of growth response.

Microbiological methods less time for preparation than animal assays and are particularly well adapted to simultaneous assays of several samples. However, different microorganisms show varying growth responses to the various folate compounds. In addition, their growth may be affected by the presence or absence of several growth factors. This is particularly important when measuring folate levels in complex food materials.

Biochemical, Chemical and Instrumental Methods

Most biochemical, chemical and instrumental methods are suitable only for pharmaceutical preparations of folates since their sensitivity is inadequate to detect the low concentrations of folates occurring in foodstuffs. Details of much of this methodology will therefore not be discussed here.

A relatively new method, however, developed for the quantitative estimation of serum folate levels, has been shown to be sensitive to low levels of folate, similar to those commonly found in foods.

The reported presence of a folate binding fraction in milk (Ghitis, 1967) and the later association of this folate binding fraction with the lactoglobulin fraction of milk protein (Ford et al., 1969) has been shown to provide the basis for this rapid and sensitive assay method. A radioligand competitive inhibition assay was first reported by Metz et al. (1968). Waxman et al. (1971) described a sensitive, direct competitive assay using tritiated 5-methyl tetrahydrofolic acid ($^{3}H\ N^{5}CH_{3}FH_{4}$). However, the authors recommended that tritiated pteroylglutamic acid (^{3}H PteGlu) be substituted for the $^{3}H\ N^{5}CH_{3}FH_{4}$ in a sequential assay system. Archibald et al. (1972), Rothenberg et al. (1972) and Tajuddin and Gardyna (1973) employed the recommended procedure with slight modifications. Waxman and Schreiber (1973) employed a crystalline bovine beta lactoglobulin as a source of protein binder while binders other than bovine milk have been utilized by Kamen and Caston (1974).

At the present time, the radioassay method has not been used for the assay of folate levels in food materials although the sensitivity of the method - 0.62 to 50 ng/ml of sample (for $N^{5}CH_{3}FH_{4}$) - appears to be adequate for food folate assay.

LOSSES OF FOLIC ACID IN FOOD PROCESSING

The majority of publications pertaining to folic acid research involve studies of its biochemical and clinical aspects. Very little data are available concerning the effect of food processing on vitamin content.

Early work has demonstrated that folates are very susceptible to destruction. Cheldin et al. (1943) carried out a survey on the cooking of foods and concluded that of all vitamins studied, folates were the most susceptible to breakdown. Schweigert et al. (1946) reported losses of folic acid in meat upon cooking. Hanning and Mitts (1949) investigated the effect of frying, boiling and scrambling of eggs on folic acid content and found losses of 18-48%. Herbert (1967) described how thrice boiling of foods destroyed the majority of folate activity. In 1968b, he also estimated that as much as 95% of the initial folates in foods could be lost by oxidative heating processes and that the losses were aggravated when the food was finely divided and cooked in water for long periods. Ghitis (1966) and Ford et al. (1968) showed that variable losses of folate in milk occurred during ultra-high-temperature processing, the losses being directly related to the presence of oxygen in the milk. These authors also found that the presence of ascorbate in milk was necessary to stabilise folate retention.

Losses of folate have also been reported to be due to the action of ultraviolet light (Stokstad et al., 1947). Schroeder (1971) reported a 20-80% loss of folate during the milling of flour. More recently, slight increases in the total folate content of Brussels

sprouts treated with various processing methods have been reported by Malin (1977).

CHAPTER II. A COMPARISON OF MICROBIOLOGICAL ASSAY AND RADIOASSAY METHODS FOR MEASURING FOLATE

INTRODUCTION

The difficulties and limitations of assay methods for naturally occurring monoglutamate and polyglutamate folates are reflected in the variability of reported levels for this vitamin in foodstuffs.

Until recently, microbiological assays have been the most frequently used method for quantitatively measuring folate content of food, serum and other materials. The reported presence of a folate-binding fraction in milk (Ghitis, 1967) and the later association of this folate-binding activity with the lactoglobulin fraction of milk protein (Ford, et al., 1969) has been shown to provide the basis for a rapid and sensitive test for folates (Waxman et al., 1971; Archibald et al., 1972; Rothenberg et al., 1972; Tajuddin and Gardyna, 1973).

Because of the increasing use of the radioassay method for the quantitative measurement of folate and the frequently contradictory reports on the correlation of results obtained by this method with those obtained by microbiological assays (Waxman et al., 1971; Rothenberg et al., 1972; Mincey et al., 1973; Dunn & Foster, 1973; Tajuddin and Gardyna, 1973; Shaw et al., 1974; Kamen and Caston, 1974; Waddell et al., 1976; Rudzki et al., 1976) it was of interest to determine both the reproducibility of standard curves and the correlation of results obtained by both methods using buffered solutions of $N^5CH_3FH_4$ as the standard and as the unknown.

Food materials were then analyzed by both methods of assay and values for free and total folate activities (i.e. the folate activities before and after treatment of the food extract with conjugase (γ glutamyl peptidase)) obtained by each method were compared.

In an attempt to explain the differences in food folate levels obtained by the two methods and the variation in previously reported results, standard curves obtained using pteroylglutamic acid (PteGlu) (Fig. 2) were then compared with those obtained using $N^5CH_3FH_4$.

Microbiological methods for the determination of folate have been well documented (Teply and Elvehjem, 1945; Herbert, 1966; Cooperman, 1967; Herbert and Bertino, 1967). L. casei was utilized in these experiments since this organism exhibits a growth response to $N^5CH_3FH_4$. The radioassay technique used was a modification of that described by Rothenberg et al. (1972) and utilized a two-step sequential addition technique.

MATERIALS AND METHODS

Preparation of N^5-Methyltetrahydrofolic Acid Standards

Samples of $N^5CH_3FH_4$ (Clinical Assays, Inc. Massachusetts) were diluted with 0.1M phosphate buffer (pH 7.3) containing 0.3% mercaptoethanol to give an initial $N^5CH_3FH_4$ concentration of 50 ng/ml. This stock solution was then serially diluted to give the concentrations shown in Table I. Additional dilutions to give concentrations of 5.0, 7.5, 10.0,

Table I. $N^5CH_3FH_4$ concentrations used in preparation of standard curves.

Dilution	$N^5CH_3FH_4$ / 100 microlitres (ng)	Sample Equivalent (ng/ml)
1/1	5.000	50.00
1/3	1.667	16.67
1/9	0.556	5.56
1/27	0.185	1.85
1/81	0.062	0.62

15.0 and 20.0 ng/ml $N^5CH_3FH_4$ were later prepared in order to increase the precision of the microbiological assay standard curve. Because of the reported sensitivity of $N^5CH_3FH_4$ to air oxidation and to the action of ultraviolet light, assay tubes were kept in diffuse light and a fresh standard vial, reconstituted immediately prior to use, was utilized for the preparation of each set of standard curves.

Preparation of Unknown Concentrations of $N^5CH_3FH_4$

A series of unknown concentrations of $N^5CH_3FH_4$ in phosphate buffer (0.3% mercaptoethanol) was made up such that all samples were within the concentration range of the standard curves.

Preparation of Food Extracts

Three samples of strained baby foods were analyzed for 'free' and 'total' folate. The contents of each jar were mixed thoroughly before the preparation of the extracts.

The test extracts were prepared as follows: to approximately 2g strained food sample in a 15 ml centrifuge tube was added 5 ml 0.1M phosphate buffer (pH 7.3, 1% ascorbate). The extracts were mixed thoroughly using a vortex mixer. The prepared tubes were then treated in a boiling water bath for 10 minutes and cooled in cold water.

Desiccated chicken pancreas (Difco) was suspended in water (5 mg/ml), thoroughly mixed with a glass rod and incubated at 37°C for 45 minutes. After filtration of the slurry through Whatman No. 1 paper, the clear filtrate was used immediately.

For the determination of 'total folate activity' 1 ml of the filtrate was added to each tube. Toluene (as a preservative) was added and the sample tubes and enzyme blanks (5 ml buffer plus 1 ml enzyme preparation) were incubated at 37°C for 16 hours. The contents of the flask were then adjusted to 10 ml with phosphate - ascorbate buffer. After further mixing, centrifugation and appropriate dilution, the extracts were assayed by the methods described below.

For the determination of 'free folate' activity, the conjugase treatment was omitted and the contents of the tubes were adjusted directly to 10 ml, remixed, centrifuged and after appropriate dilution, assayed.

Preparation of Pteroylglutamic Acid Standards

Samples of PteGlu (Nutritional Biochemicals Corp., Ohio) were diluted in 0.1 M phosphate buffer (pH 7.3) containing 0.3% mercaptoethanol to give concentrations of 50.00, 16.67, 5.56, 1.85 and 0.62 ng/ml PteGlu.

Maintenance of Stock Culture and Preparation of Inoculum

Lactobacillus casei ATCC 7469 was maintained as a stab culture in tubes of prepared Bacto-Lactobacilli Agar. Cultures were incubated

at 35-37°C for 18-24 hours and then stored in a refrigerator at 4°C. Transfers were made at monthly intervals. Inoculum for the assay was made by subculturing from the stock culture into a tube containing 10 ml prepared Bacto-Lactobacilli Broth. After incubation for 16-18 hours at 37°C the tubes were centrifuged, the supernatant was discarded and cells were resuspended in 10 ml 0.85% sterile saline. The culture was recentrifuged and suspended in fresh saline twice more. An aliquot was diluted 100-fold in a sterile container with sterile saline and one drop of this was used to inoculate the assay tubes.

Microbiological Assay Procedure

5 ml of rehydrated Bacto-Folic Acid Casei Medium (Difco) were dispensed into test tubes. 0.1 ml of the $N^5CH_3FH_4$ dilution, (or PteGlu dilution) was added to each tube and the volume made up to 10 ml with 0.1 M phosphate buffer containing 1% ascorbate. When the strained baby foods were assayed, dilutions (within the range of the assay) of untreated extract, conjugase-treated extract or conjugase blanks were added to duplicate tubes containing 5 ml rehydrated medium. Volume was made up to 10 ml with 0.1 M phosphate buffer containing 1% ascorbate. All tubes were autoclaved for 5 minutes at 15 p.s.i.g. (121°C) and then cooled to room temperature. One drop of the diluted L.casei cell suspension was added to each tube. Tubes were incubated for 18 hours at 37°C. After thoroughly mixing, turbidity was measured. This was expressed in terms of absorbance which was read at a wavelength of 660 nm.

Radioassay Procedure

The procedure for radiometric assay was that outlined in the Clinical Assays ^{3}H Folic Acid Radioassay Kit (Clinical Assays, Massachusetts, 1975). All solutions used in the radioassay were made up and the assay procedure followed exactly as described in the kit with the exception that 0.1 M phosphate buffer pH 7.3 was used in place of tris-NaCl buffer (Appendix A). Duplicate samples of $N^{5}CH_{3}FH_{4}$, PteGlu, or food extract dilutions were incubated with milk folate binder for 20 minutes at room temperature. After addition of ^{3}H PteGlu there was a further 20 minute, room temperature incubation. The protein-bound and the free ^{3}H PteGlu were then separated by adsorption of the latter on dextran coated charcoal. Supernatant and charcoal were separated by centrifugation in a refrigerated centrifuge (Sorvall Model RC2-B) at $4^{o}C$, 2,000 x g for 20 minutes. The supernatant was decanted into vials and 12 ml scintillation fluid (Scintiverse-Fisher Universal LSC Cocktail) were added. After cooling, sample radioactivity was counted for 10 minutes in a Nuclear Chicago Isocap 300 liquid scintillation counter using the tritium program for low quench samples. Correction for counting efficiency (quench correction), was carried out at each assay using the channels ratio technique (Appendix B).

A maximum binding control was run in duplicate with every assay to determine the amount of radioactive material which was able to compete with cold $N^{5}CH_{3}FH_{4}$ for milk protein binding sites. In addition, background control samples containing buffer and ^{3}H PteGlu were run in

duplicate with each assay to determine levels of radioactivity not removed by dextran coated charcoal.

When food extracts were assayed, duplicate background samples containing buffer, extract and ^{3}H PteGlu were analyzed for each extract in order to correct for the food's non-specific protein binding capacity. This precaution was recommended by Tajuddin and Gardyna (1973) who, when measuring human serum folate levels, added a serum blank to correct for non-specific binding of ^{3}H PteGlu. When extract background count rates were found to be greater than 20% of extract samples the total count rate for ^{3}H PteGlu was also measured.

Experimental Procedure

Microbiological and radioassays were carried out concurrently using duplicate samples of the freshly prepared $N^{5}CH_{3}FH_{4}$. This ensured that any variation in the standard curves due to differing concentrations of $N^{5}CH_{3}FH_{4}$ between batches was taken into account by both methods.

Twenty-five unknown dilutions of $N^{5}CH_{3}FH_{4}$ were assayed by both methods. Concentrations were determined from $N^{5}CH_{3}FH_{4}$ standard curves prepared at the same time and under conditions identical to the unknown samples.

Five dilutions of each food extract (both total and free folate extracts) were prepared in duplicate for each assay method. $N^{5}CH_{3}FH_{4}$ standard curves were prepared simultaneously by each assay method.

Five PteGlu standard curves were prepared by each method using duplicate samples of each dilution. PteGlu standard curves were prepared at the same time as an additional five $N^5CH_3FH_4$ standard curves in order to ensure that conditions of assay remained as similar as possible for each standard used.

$N^5CH_3FH_4$ Recovery Study

A powered milk sample (3g) was mixed with 5 ml 0.1 M phosphate buffer (pH 7.3, 1% ascorbate) in a 15 ml centrifuge tube. The tube was then heated in a boiling water bath for 10 minutes and cooled in cold water. The contents of the tube were then adjusted to 10 ml, remixed, and centrifuged.

A radioassay was then carried out using the milk extract as outlined in Table II. Samples of the extract (100 µl) were added to each of five tubes containing the standard $N^5CH_3FH_4$ dilutions. In addition, duplicate background samples containing buffer, extract and ^3H PteGlu were analyzed to correct for the milk's non-specific protein binding capacity. Duplicate samples containing buffer, extract, binding protein and ^3H PteGlu were assayed in order to determine the folate content of the powdered milk. When extract background counts were found to be greater than 20% of extract and standard samples, the total count rate for ^3H Pte Glu was also measured.

Table II. Volumes of solutions used in $N^5CH_3FH_4$ recovery study.[a]

Tube No.	Buffer	Milk Extracts	Standard Dilution	Binding Protein	^3H PteGlu
T_1T_2	1,100	---	---	--	50
1,2	500	---	---	--	50
3,4	500	---	---	50	50
5-14	400	---	100	50	50
5-14M	300	100	100	50	50
15,16	400	100	---	--	50
17,18	400	100	---	50	50

[a] All volumes in microliters

Tubes:-

T_1T_2	Total count
1,2	Binding control background
3,4	Binding control
5,6	Standard dilution (5ng/100µl)
5,6M	Standard dilution (5ng/100µl) + milk extract
7,8	Standard dilution (1.667ng/100µl)
7,8M	Standard dilution (1.667ng/100µl) + milk extract
9,10	Standard dilution (0.556ng/100µl)
9,10M	Standard dilution (0.556ng/100µl) + milk extract
11,12	Standard dilution (0.185 ng/100µl)
11,12M	Standard dilution (0.185 ng/100µl) + milk extract
13,14	Standard dilution (0.062 ng/100µl)
13,14M	Standard dilution (0.062 ng/100µl) + milk extract
15,16	Milk extract binding control background
17,18	Milk extract assay

RESULTS

$N^5CH_3FH_4$ Standard Curves

Microbiological Assay

Regression analysis was carried out on the data from the ten standard curves obtained by the microbiological assay method. This is shown in Figure 3. The coefficient of determination (r^2) of 0.61 illustrates that there is a significant ($p \geq 0.01$) relationship between concentration of $N^5CH_3FH_4$ and growth response of L. casei as measured by absorbance at 660 nm. The standard error of estimate was found to be 0.11.

Radioassay

Standard curves were obtained by plotting the percent tracer bound for each amount of standard on semilog paper. The percent of tracer bound for each level of standard was calculated from the normalized (quench corrected) counts per minute, thus:

$$\%\ {}^3H\ \text{PteGlu bound} = \frac{(CPM_n - CPM_{BG})}{(CPM_B - CPM_{BG})} \times 100\%$$

where CPM_n = Average counts per minute for standard samples

CPM_{BG} = Average counts per minute for background controls

CPM_B = Average counts per minute for binding controls

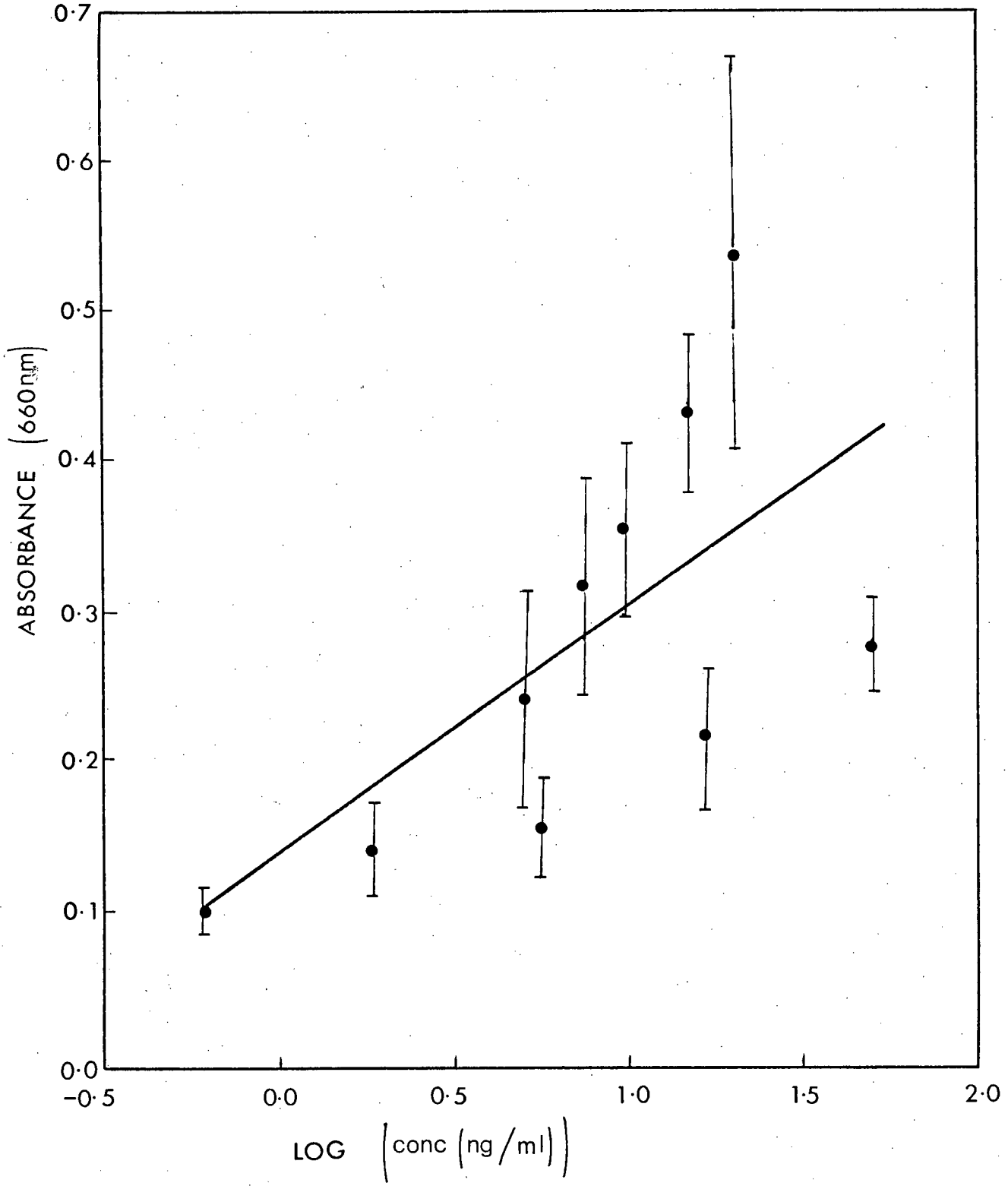


Figure 3. Regression analysis performed on $N^5CH_3FH_4$ standard curves obtained by L. casei microbiological assay method.

Regression analysis performed on the ten sets of data is shown in Figure 4. A cubic equation was fitted to the data to improve the goodness of fit. (The coefficient of determination (r^2) for the cubic equation was 0.98 compared to a coefficient of determination of 0.97 obtained when a quadratic equation was fitted to the data). There is a highly significant ($p \geq 0.01$) relationship between concentration of $N^5CH_3FH_4$ and percent binding of tracer. The standard error of estimate was found to be 4.01.

Comparison of Folate Concentrations Using Both Methods

Folate concentrations for the 25 unknown $N^5CH_3FH_4$ samples were calculated by reference to standard curves obtained by both methods. Results obtained by the radioassay and microbiological methods are shown in Figure 5. Radioassay values ranged from 0.62 to 35 ng/ml while microbiological assay values ranged from 0.75 to 38 ng/ml. The coefficient of determination (r^2) for values obtained by the two methods was 0.93 and the regression line was found to have a slope of 0.76 and an intercept of 3.10 ng/ml. The standard error of estimate was 3.65 ng/ml.

Comparison of Free and Total Folate Levels of Baby Foods Obtained Using Both Methods

Free and total folate concentrations of the food extracts were measured by the radioassay and microbiological assay methods.

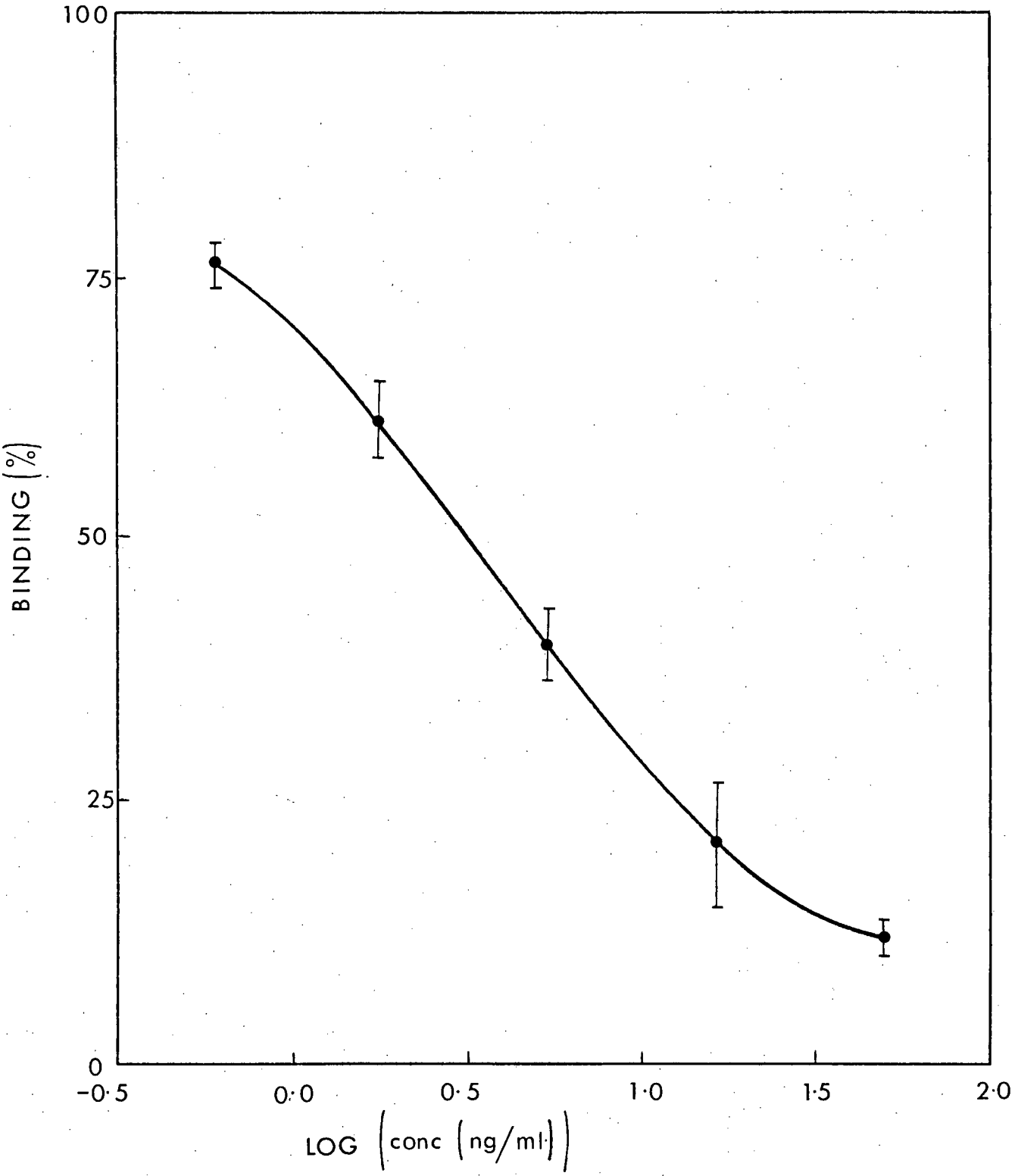


Figure 4. Regression analysis performed on $N^5CH_3FH_4$ standard curves obtained by radioassay method.

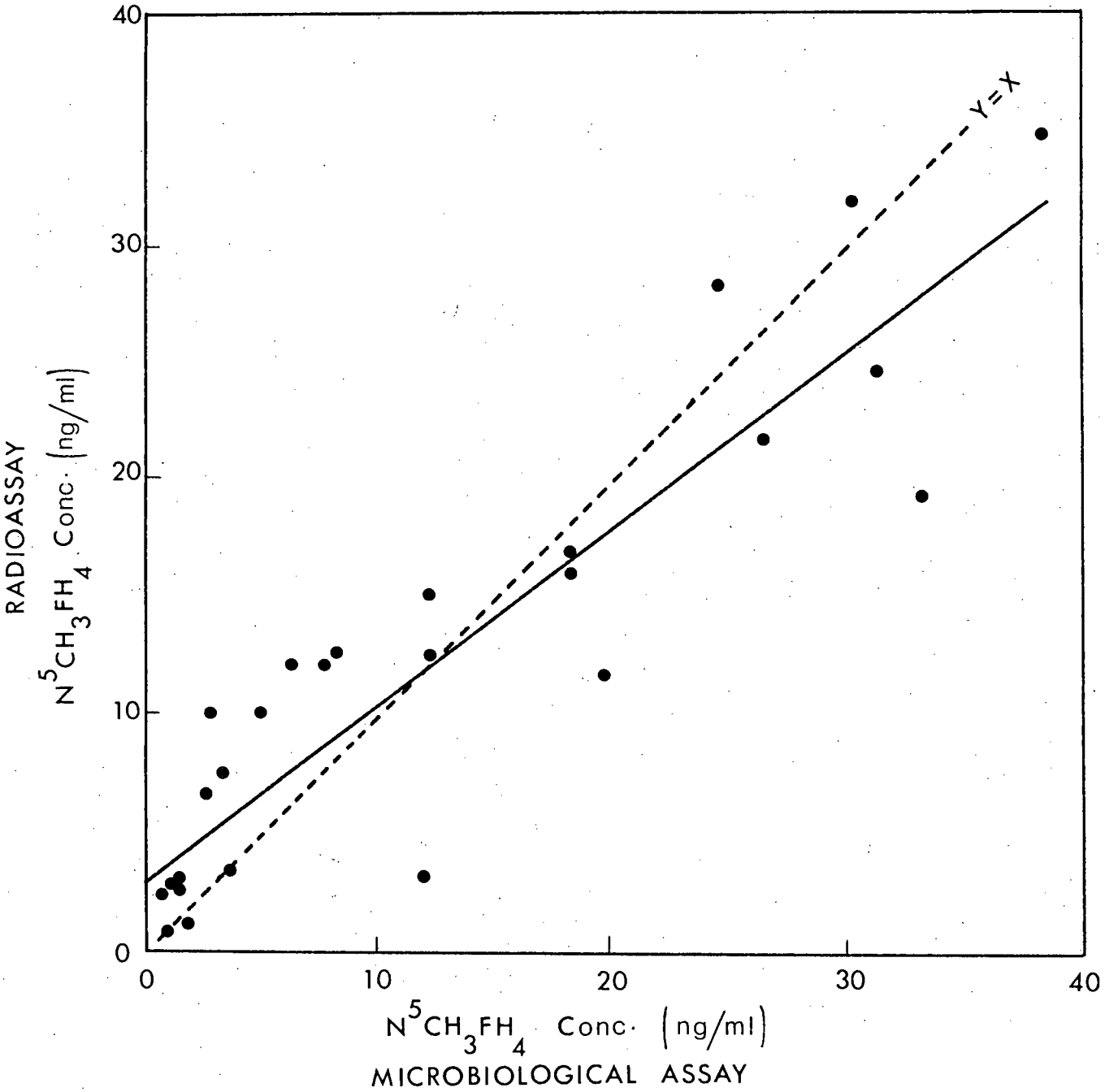


Figure 5. Regression analysis of radioassay on L. casei microbiological assay method of measuring $N^5CH_3FH_4$ concentration.
y = x is the regression line that would be obtained had the microbiological assay values equalled the radioassay values.

For the radioassay method, the binding of each food extract was first corrected for its own non-specific binding thus:

$$\%\ {}^{3}\text{H PteGlu bound} = \frac{(CPM_n - CPM_{BGn})}{(CPM_B - CPM_{BG})} \times 100\%$$

where CPM_n = Average counts per minute for extract sample

CPM_{BG_n} = Average counts per minute for extract backgrounds

CPM_B = Average counts per minute for binding controls

CPM_{BG} = Average counts per minute for background controls.

Where food extract background counts were found to be greater than 20% of the extract sample counts a correction was made to the calculation of values to take into account the lower tracer concentration available for binding during the extract sample incubation. The following equation was used:

$$\%\ {}^{3}\text{H PteGlu bound} = \frac{(CPM_n - CPM_{BGn}) \times 100\%}{(CPM_T - CPM_{BGn}) \times \frac{(CPM_B - CPM_{BG})}{(CPM_T - CPM_{BG})}}$$

where CPM_T = Total counts per minute

This equation reduces to:

$$\%\ {}^{3}\text{H PteGlu bound} = \frac{(CPM_n - CPM_{BGn}) \times 100\%}{(CPM_T - CPM_{BGn}) \times \%\ \text{Total Bound} \div 100}$$

Normalized (quench corrected) counts per minute were used in all calculations.

Values of free and total folatesobtained by the two methods of assay for the three strained baby food samples are shown in Table III.

Comparison of $N^5CH_3FH_4$ and PteGlu Standard Curves

Regression analyses were performed on the five sets of standard curves produced by both methods of assay using the two forms of folate. Results are presented in Figures 6 and 7. Paired comparison Student's t-tests were carried out on the data. These results are presented in Table IV.

A significant difference in the two standard curves is evident. This is particularly the case at higher concentrations of folates when the radioassay method is used. At concentrations lower than 6.0 ng/ml the difference in the two standard curves is less evident in the radioassay method where similar percent binding values are frequently obtained for both standards. The difference in the growth response of L. casei with PteGlu and $N^5CH_3FH_4$ is significant at all concentrations of folate. This is particularly the case at lower concentrations of folate although the difference does not appear to be evident from the regression analysis.

It would appear from the limited data presented that the milk protein shows a greater affinity for binding to PteGlu than to $N^5CH_3FH_4$ at pH 7.3 and that PteGlu causes a greater growth response of L. casei than $N^5CH_3FH_4$.

$N^5CH_3FH_4$ Recovery Study

Results of this study are shown in Table V. The recovery of $N^5CH_3FH_4$ is shown to be between 75-80% for the lowest concentrations of

Table III. Free and Total Folate Activity of Baby Foods.

	Free Folate Activity (µg/100g product)		Total Folate Activity (µg/100g product)	
	Range	Mean	Range	Mean
MIXED VEGETABLES				
Radioassay	2.0 - 4.3	3.2	13.0 - 18.0	17.5
L. casei	4.0 - 4.6	4.2	17.5 - 19.0	18.3
HAM WITH VEGETABLES				
Radioassay	2.6 - 5.7	4.9	18.5 - 22.4	21.0
L. casei	6.5 - 9.5	7.8	10.0 - 13.7	12.6
VEGETABLES AND LIVER WITH BACON				
Radioassay	4.7 - 7.0	6.5	20.0 - 24.5	22.5
L. casei	8.0 -13.5	12.1	26.0 - 29.0	27.0

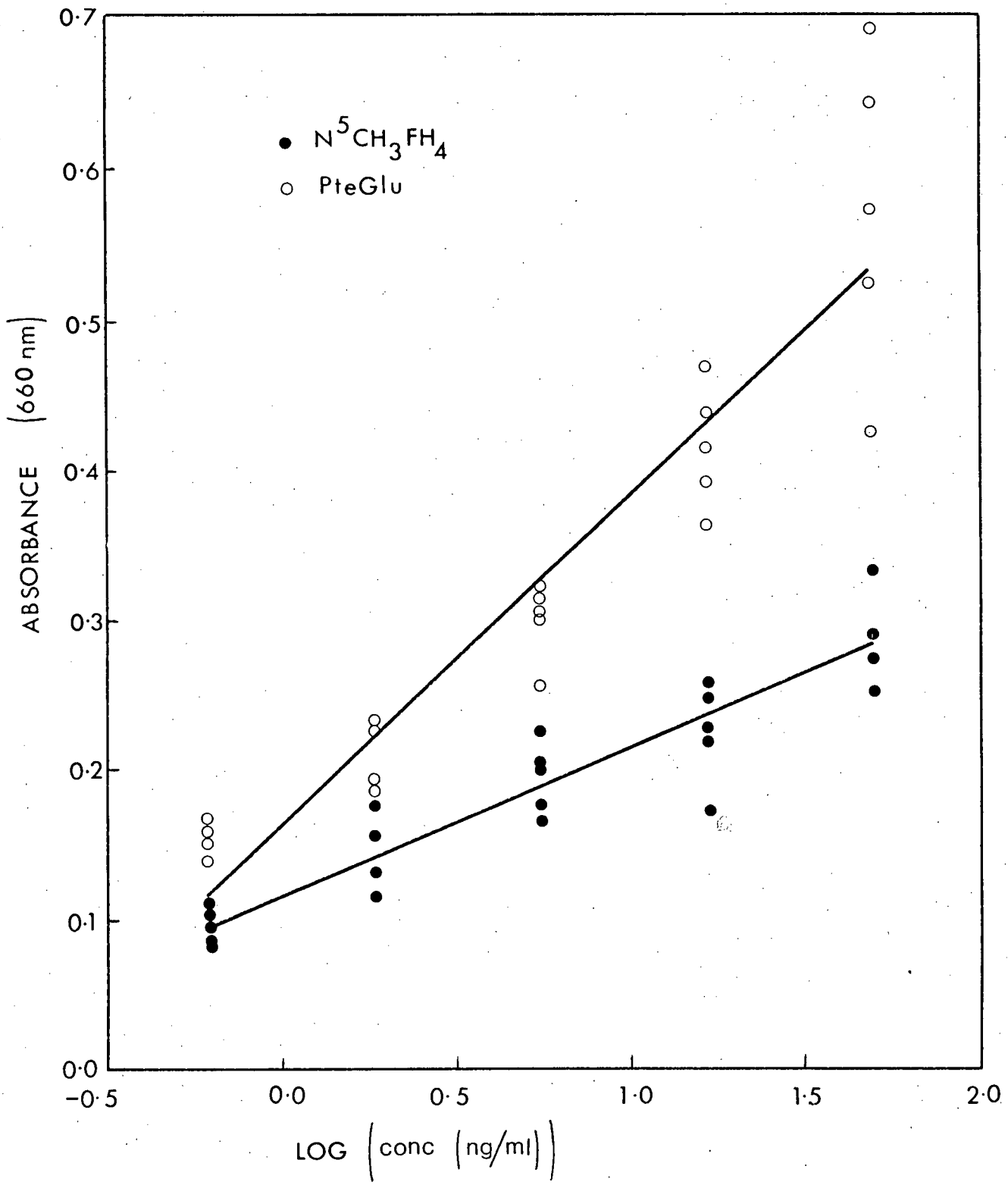


Figure 6. Regression analyses performed on PteGlu and $N^5CH_3FH_4$ standard curves obtained by L. casei microbiological assay method.

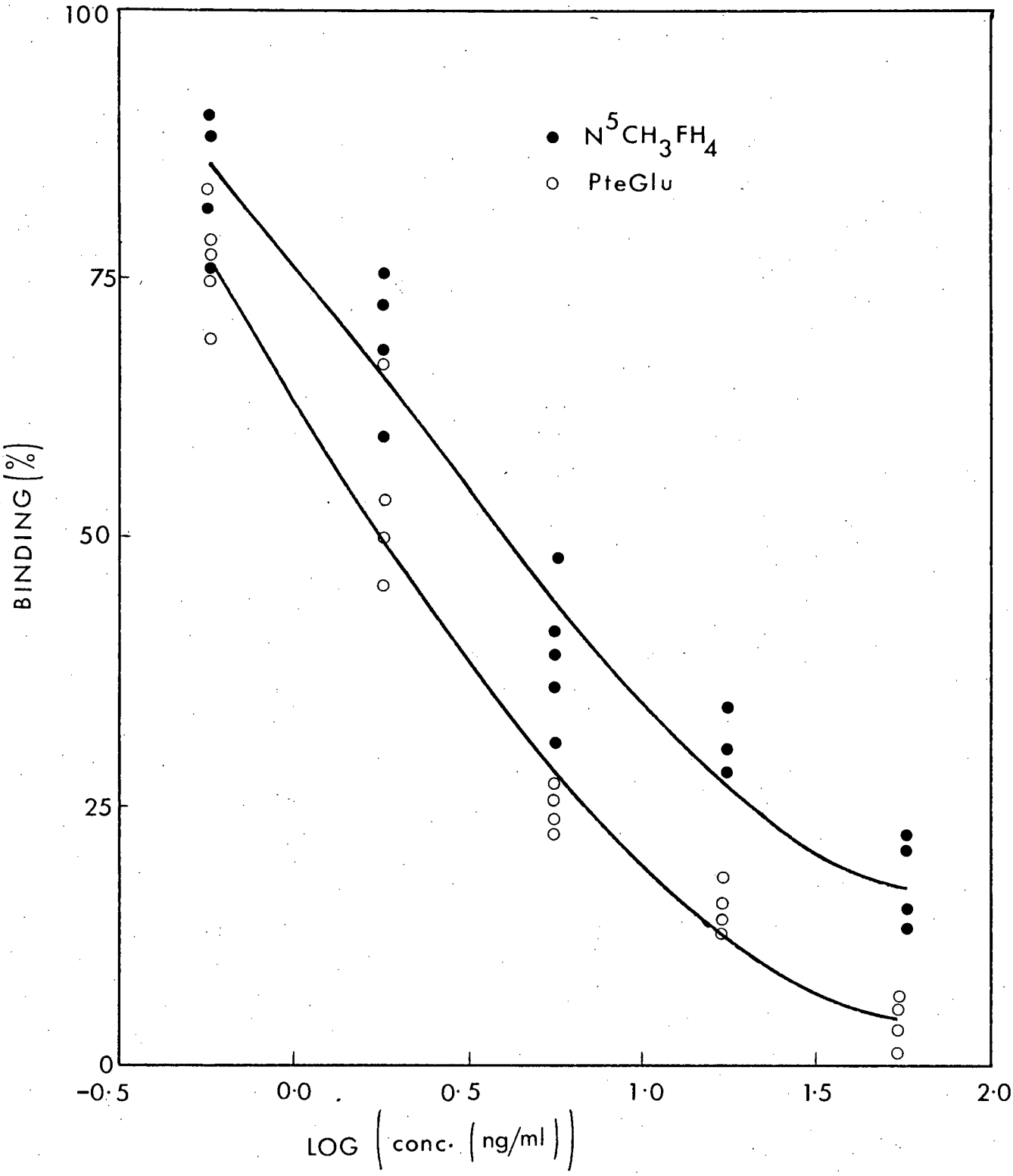


Figure 7. Regression analyses performed on PteGlu and $N^5CH_3FH_4$ standard curves obtained by radioassay method.

Table IV. Paired comparison Student's t-test values of PteGlu and $N^5CH_3FH_4$ standard curves obtained by microbiological and radioassay techniques.

	Concentration of Folate (ng/ml)				
	50.00	16.70	5.57	1.85	0.62
MICROBIOLOGICAL ASSAY					
t value	8.10	6.27	9.07	4.06	12.06
degrees of freedom	4	4	4	4	4
$p \geq$	0.01	0.01	0.001	0.02	0.001
RADIOASSAY					
t value	11.51	7.08	4.74	2.50	3.74
degrees of freedom	4	4	4	4	4
$p \geq$	0.001	0.01	0.02	0.10	0.05

Table V. Recovery of $N^5CH_3FH_4$ from milk extract as measured by the radioassay method.

Concentration of $N^5CH_3FH_4$ added (ng/100µl)	Recovery %
5.000	35
1.667	63
0.556	76
0.185	80
0.062	76

added $N^5CH_3FH_4$. The percent recovery drops rapidly as the concentration of added $N^5CH_3FH_4$ increases however. This is due, in part, to the very high counting rate recorded for the milk extract background sample. In all cases, the milk extract background was found to be greater than 20% of the milk extract plus standard sample. A correction was made to the calculation of values to take into account the lower tracer concentration available for binding during the extract plus standard sample incubation. (See "Comparison of Free and Total Folate Levels of Baby Foods Obtained Using Both Methods".)

DISCUSSION

Microbiological assays have been the most frequently used methods for measuring folate levels. However, such assays are extremely time consuming requiring both maintenance of the stock culture and an overnight incubation period.

L. casei has been the most frequently used microorganism for folate assay but it has been found to be affected by several unidentified growth factors (Stokstad and Hutchings, 1947). It is capable of converting various other growth factors to folate during periods of stress (Baker, et al., 1971), and it can also be subject to metabolic changes such that folate is no longer required. These facts, together with the various effects on growth of different commercial media, temperature and time of autoclaving, temperature and time of incubation, make the use of this method unsuitable for accurate determination of folate levels.

The radioassay method, by comparison, is rapid and can be carried out with simple laboratory equipment over a period of 3 to 4 hours. Although milk folate binding is influenced by such factors as temperature and time of incubation, pH and charcoal preparation (Givas and Gutcho, 1975; Zettner and Duly, 1974, 1975), the effects of these factors are not as great as the aforementioned factors affecting the microbiological method. Thus, as shown by these results, a much more reproducible standard curve can be obtained using the radioassay method than by the microbiological assay.

It should be noted that when using commercially prepared kits, there may be a difference in the folate binding activity of the milk protein from one kit to another causing differences in standard curves to occur.

Previous publications pertaining to the comparison of folate levels obtained using the radioassay and microbiological assay methods, vary considerably in their conclusions. Serum folate levels were compared in every case. Archibald et al. (1972), Waxman et al. (1971) and Tajuddin and Gardyna (1973) showed good agreement between the two methods. Mincey et al. (1973) and Waddell et al. (1976) found that mean serum folate levels as determined by the radioassay method were slightly lower than those obtained by the microbiological method but still offered the same degree of diagnostic information. Rothenberg et al. (1972) found that radioassay folate concentrations were approximately half those obtained by the microbiological assay and Rudzki et al. (1976) concluded that the microbiological assay gave the more accurate indication of folate levels in serum. In all cases, $N^5CH_3FH_4$ was used as standard for the radioassay method but PteGlu or an unidentified standard was used for preparation of the microbiological standard curve.

It can be seen from the results obtained in this experiment that the growth of L. casei and the milk protein binding ability are dependent, in part, on the form of folate present in the system. It is to be expected, therefore, that comparisons of the two methods of assay using PteGlu as standard for the microbiological assay and $N^5CH_3FH_4$ as standard for the radioassay will yield different results.

Since PteGlu and $N^5CH_3FH_4$ react differently in each system of assay, it may also be expected that results read from a standard curve prepared from a singular pure form of folate will not give an accurate estimate of folate contained in a multiple system. It has been well documented that $N^5CH_3FH_4$ is the predominant form of folate found in serum (Herbert et al., 1962). However, forms of folates other than $N^5CH_3FH_4$ may also be present which are able to show a different binding affinity for the milk protein or cause a greater growth response of L. casei. This may partially explain why Shaw et al. (1974) obtained a poor correlation coefficient for serum folate levels measured by both methods even though $N^5CH_3FH_4$ was used as standard for both assays.

This may also be one reason for the great variation in the results obtained when baby food was assayed for folate by both methods. Food folates have been assayed using both L. casei (Hoppner, 1971; Hoppner et al., 1972; 1973; Butterfield and Calloway, 1972; and other) and S. faecalis (Lin et al., 1975; Dong and Oace, 1973 and others). However, the response to the different forms of folate vary among these organisms (Stokstad and Koch, 1967; Dong and Oace, 1973).

Other factors and materials present within the complex food system may also affect assay results particularly when the radioassay method is used.

Many proteins have been shown to exhibit folate binding properties. Binders of folate and/or bound forms of folate have been identified in milk (Ghitis, 1967; Metz et al., 1968) and many other biological materials (Rothenberg et al., 1977). Indeed, the ability to bind folate forms the basis of the radioassay method and possibly also, the microbiological assay method. Thus the presence of other proteins with folate binding ability in the system to be assayed, may cause errors unless corrections are made for their specific folate binding ability. For this reason, Tajuddin and Gardyna (1973) suggested the addition of a serum blank to correct for non-specific binding ability of ^{3}H PteGlu to the assay material. Ford et al., (1969) stated that heating milk for 10 minutes at 100°C released the protein bound folate and destroyed the protein binding ability. Thus, Dunn and Foster (1973) extracted their serum samples by boiling in a water bath for 15 minutes in 0.05 M lysine buffer, pH 10.5. This procedure denatured the serum binder and released the bound folate. (It is interesting to note that no reducing agent was added to their serum extracts during heating).

In the two experiments using food materials described here, the extraction process involved heating the food samples for 10 minutes in a boiling water bath and assaying an extract background sample. Thus, the folate binding proteins not denatured by heating were compensated for in the background sample. In all foods tested with the exception of the mixed vegetables, the extract background counts were found to be greater than 20% of the extract sample counts; indicating that the heating process was not sufficient to destroy all the protein binding activity. Therefore, a

correction was made to the calculation of percent ^{3}H PGA bound to each food sample. High extract background counts however, tend to decrease the validity of the assay.

Other factors such as pH, metal catalysts and the presence of pro- or anti- oxidants within the food system may also affect the validity of the assay.

The recovery of $N^{5}CH_{3}FH_{4}$ from a food system does indicate however, that particularly at lower concentrations of folate the experimental procedure is valid. As shown in this experiment, at high concentrations of folate, the folate binding protein becomes saturated and is thus unable to bind all the folate present in the sample thus decreasing the validity of the method.

CONCLUSIONS

These experiments demonstrate that the radioassay method herein described is a faster, simpler and more accurate method for measuring levels of $N^{5}CH_{3}FH_{4}$ than the L. casei microbiological method. When $N^{5}CH_{3}FH_{4}$ standard curves are used, concentrations of buffered $N^{5}CH_{3}FH_{4}$ as determined by the radioassay method compare well with concentrations obtained by the L. casei microbiological assay. When free and total folate levels in strained baby foods were assayed results obtained by the two methods did not compare well.

When the radioassay method of folate assay is carried out at pH 7.3 there is a marked difference in the ability of the milk protein to bind PteGlu and $N^{5}CH_{3}FH_{4}$. Similarly L. casei also exhibits a difference in its growth response to the two forms of folate. The two assay systems therefore, when carried out by the methods herein described, are suitable only for

measuring singular forms of folate using standard curves constructed from data obtained using the same folate.

$N^5CH_3FH_4$ and PteGlu are just two of the many forms of folate that have been identified.Other forms may show greater or lesser binding affinities and may exhibit further differences in the growth response of microorganisms. In addition, the presence of two or more folate types together in any one material may also cause variations. It becomes obvious therefore, that the folate levels of foodstuffs which are known to contain many forms of folate cannot be estimated accurately by either of the two methods described until more information pertaining to the number, forms and distribution of folate in foodstuffs and the interaction of the various forms with each other and with biological materials is obtained.

At best, presently available data on food folate levels can only be compared to other values obtained by an identical method. It should also be recognized that any folate concentration values available at this time may not necessarily be a reflection of their nutritional significance to man.

CHAPTER III. KINETIC STUDIES

INTRODUCTION

Quality deterioration in the form of nutritional degradation can occur in any food during heat processing. The rates of the reactions responsible for nutritional degradation are dependent upon such factors as temperature and length of time of heating, amount of oxygen present and presence or absence of other chemicals such as pro- or anti-oxidants. The presence or absence of other chemicals makes the prediction of nutritional degradation particularly difficult since within most food systems, there are many substances present which influence the rate of degradation of the various nutrients.

Very little research has been conducted on the effect of processing on folate content of foods. Cheldin et al. (1943), Schweigert et al. (1946), Stokstad et al. (1947), Hanning and Mitts (1949), Ghitis, (1966), Herbert (1967, 1968b), Ford et al. (1968), Schroeder (1971) and Malin (1977) have studied the effects of various processing methods on the nutritional stability of folates within different food systems and have found them to be very susceptible to degradation by oxidative heating and by the action of ultraviolet light.Herbert (1968b) estimated that as much as 95% of the initial folates in food could be lost by oxidative heating processes. Ford et al. (1968), investigating the effects of UHT processing and subsequent storage of milk on the vitamin content, concluded that variation in the stability of folates was related directly to the presence of oxygen in the milk, the deaeration of milk before processing effectively reducing the vitamin loss.

$N^5CH_3FH_4$ is a form of folate reported to be very susceptible to oxidation by various means (Waters and Mollin, 1961). Ghitis (1966) found a heat labile fraction present in milk resembling $N^5CH_3FH_4$. The fraction could be protected from oxidation by the presence of ascorbic acid although the author found that levels of ascorbate naturally present in milk were not sufficient to protect the labile folate fraction during boiling. Ghitis used the Lactobacillus casei microbiological assay method for quantitatively measuring $N^5CH_3FH_4$ levels.

The complexity of foods in which folate levels have been measured, makes it difficult to predict the thermal degradation characteristics of folate within a food system other than the one tested. Several recent studies have involved measurement of other nutrient degradation rates using model systems. Examples of such studies are those of thiamine degradation carried out by Mulley et al. (1975a, b).

In this section, a study of the reaction kinetics of $N^5CH_3FH_4$ degradation by heating in oxygen was carried out using a simple model system of $N^5CH_3FH_4$ in phosphate buffer. The radioassay method as described in Chapter II was used for measuring $N^5CH_3FH_4$ concentrations. Since the degradation of $N^5CH_3FH_4$ by heating in oxygen was thought to be an oxidation reaction, an attempt was made to identify the reaction mechanism by repeating the above experiment in the absence of any reducing agent. $N^5CH_3FH_4$ was assayed, therefore, in the absence of mercaptoethanol. Singh et al. (1976) have shown that the overall reaction of ascorbic acid degradation and oxygen uptake follows second order reaction kinetics. The degradation of $N^5CH_3FH_4$ in the presence of a limited oxygen supply was therefore also studied.

In order to study the effects of a heat treatment, it is desirable to obtain nearly instantaneous and uniform heating to the required temperature and, after a holding time at the required temperature, a nearly instantaneous cooling to room temperature. Erdtsick and Beumer (1976) developed a new method for determining the heat resistance of micro-organisms using a thermal death time pouch. They found that the come-up time of the pouch compared favorably with the conventional thermal death time tube. Thus, the thermal death time pouch was used in the initial kinetic studies reported here.

METHODS

Preparation of $N^5CH_3FH_4$ Solutions

Solutions of $N^5CH_3FH_4$ were made up in 0.1 M phosphate buffer pH 7.3. The approximate starting concentration was 35 µg/ml for all kinetic studies. No mercaptoethanol or ascorbic acid was added to the standard solutions.

Kinetic Studies Using Thermal Death Time Pouches

Pouches were prepared from 40 µm Rilsan (American Lecithin Company, Atlanta, USA) a material normally used for making meat and agar sausages. From this material, rectangular flat pouches (4 cm x 6 cm between seals) were made. Each envelope contained 1.5 ml of $N^5CH_3FH_4$ solution. After filling, the envelopes were sealed and used for the

determination of $N^5CH_3FH_4$ degradation curves. The pouches were stretched flat in a vertical position by weights attached to the lower seal (Fig. 8). By applying this procedure the pouch thickness was found to be less than 1 mm. Heating and cooling lag times in the pouches weredetermined prior to nutrient destruction studies (Appendix C).

Pouches containing $N^5CH_3FH_4$ solution were heated to 100°C in a water bath. Concentrationsof $N^5CH_3FH_4$ after various timesof heating were assayed using the radioassay method as previously described.

Kinetic Studies Using Unlimited Oxygen Supply

A glass flask was placed in an agitated water bath. A condenser was placed on the top of the flask and a tube carrying oxygen to the base of the flask was introduced through an arm (Fig. 9). Samples of $N^5CH_3FH_4$ in 0.1 M phosphate buffer (pH 7.3) were placed in the flask and oxygen was bubbled constantly through the sample while it was heated to various temperatures. All apparatus were kept in diffuse light to minimize $N^5CH_3FH_4$ degradation due to ultraviolet radiation. Duplicate 100 µl samples were removed from the flask, at regular intervals and assayed by the radioassay method.

$N^5CH_3FH_4$ samples were heated to 100°, 90°, 75°, 60°, 55° and 40°C. Preliminary studies similar to those outlined in Appendix C indicated that heating and cooling lag times were negligible in this experiment.

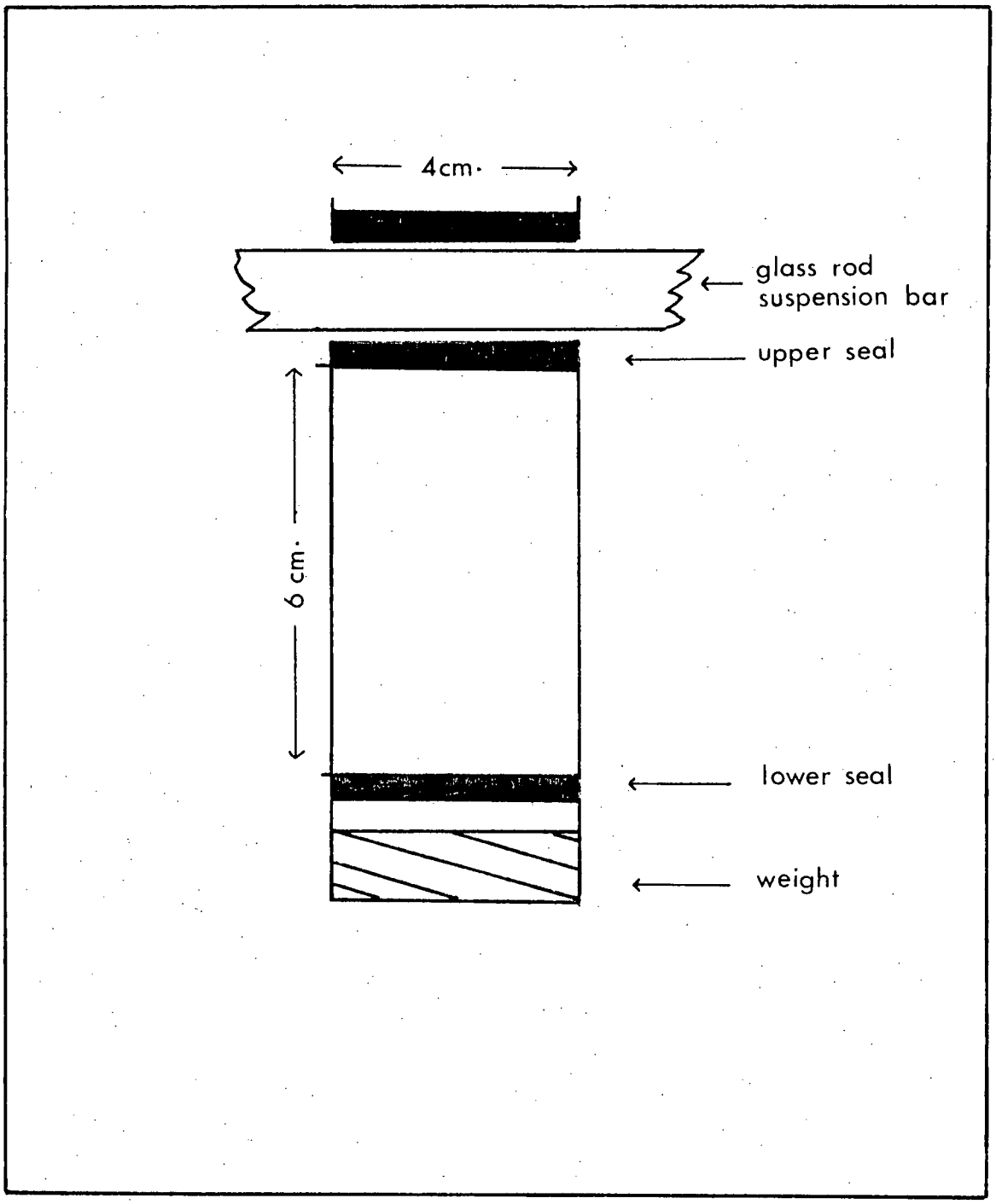


Figure 8. Thermal death time pouch with weight attached.

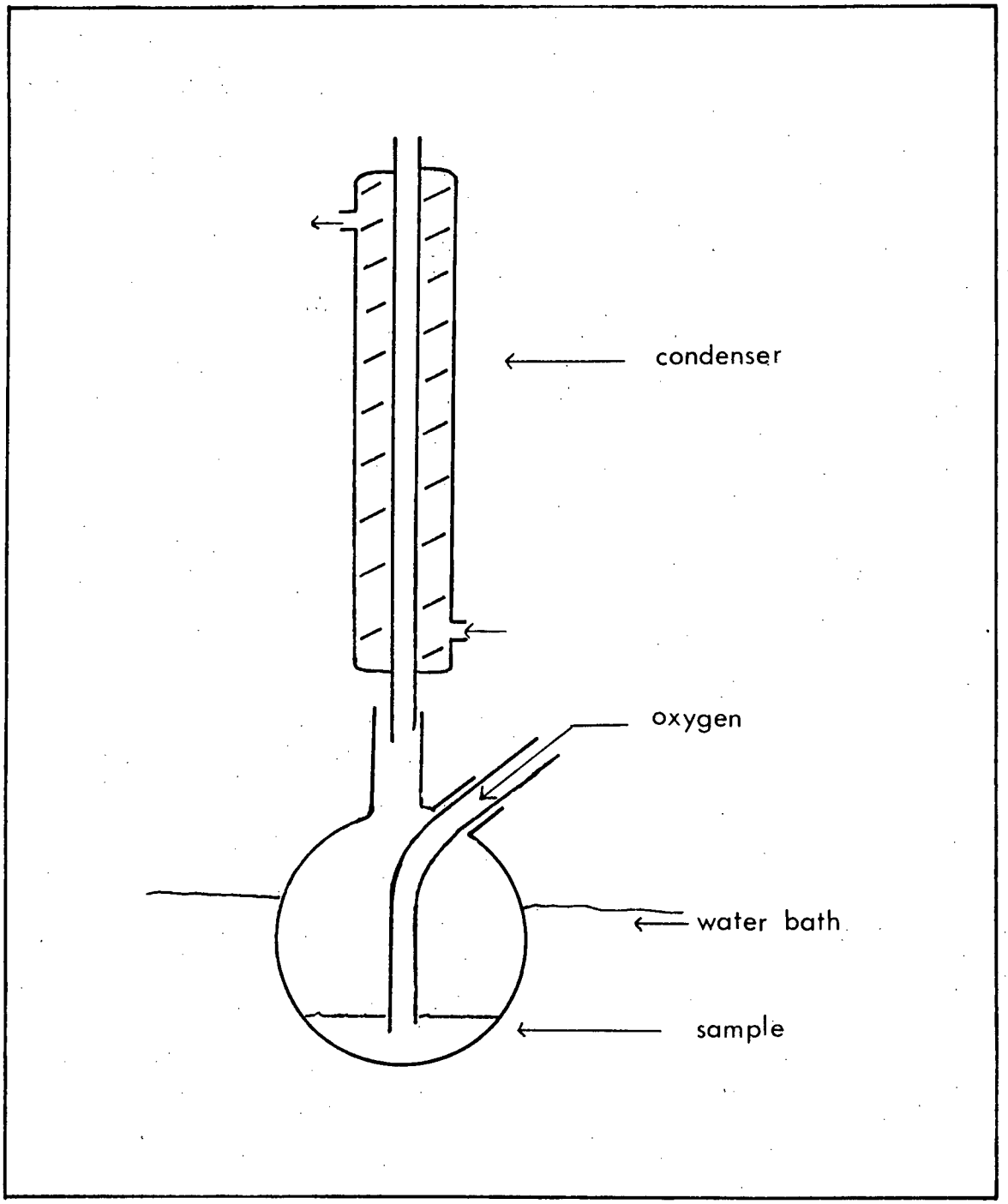


Figure 9. Apparatus used for kinetic studies of the nutritional degradation of $N^5CH_3FH_4$.

Kinetic Studies Using Unlimited Oxygen Supply and a Mercaptoethanol -Free Assay System

Samples of $N^5CH_3FH_4$ were treated in an identical manner to that described in the previous experiment. In the assay system, however, no mercaptoethanol was used in the buffer, the milk binding protein or the tritiated PteGlu. All reagents were kept on ice and in diffuse light throughout the experiment to minimize degradation.

$N^5CH_3FH_4$ samples were heated to 90, 75, and 40°C in this experiment.

Kinetic Studies Using Limited Oxygen Supply

Since a dissolved-oxygen meter (Model 57 Yellow Springs Instrument Co., Ohio) was used for measuring changes in oxygen concentration, $N^5CH_3FH_4$ samples were dissolved in tris/NaCl buffer (pH 7.3) for this experiment. Samples of buffer were bubbled with oxygen or deaerated to give initial oxygen concentrations of 6.3, 9.6 and 14.4 ppm by volume. $N^5CH_3FH_4$ was then added to each buffer and the oxygen levels recorded. The samples were then drawn up into hypodermic syringes which were subsequently sealed and placed in a constantly stirred water bath at 20°C. $N^5CH_3FH_4$ and oxygen concentrations were measured at intervals.

The radioassay system containing mercaptoethanol was used for evaluating $N^5CH_3FH_4$ concentrations. A sample of $N^5CH_3FH_4$ in tris/NaCl buffer at 20°C with an unlimited supply of oxygen was also assayed over the same time period.

THEORETICAL CONSIDERATIONS

First Order Reactions

Experimental results indicated that the degradation of $N^5CH_3FH_4$ (in the presence of an unlimited oxygen supply) follows a first order mechanism. The reaction scheme at a constant temperature and pressure may thus be written:

$$A \xrightarrow{k} \text{Product} \qquad (1)$$

$(N^5CH_3FH_4)$ (degradation product)

where k is the rate constant.

Thus the rate of reaction may be expressed as:

$$\frac{-d[A]}{dt} = k[A] \quad ng \quad ml^{-1} \quad min^{-1} \qquad (2)$$

which may be integrated and rearranged to

$$k = \frac{1}{t} \ln \frac{[A]_o}{[A]_t} \quad min^{-1} \qquad (3)$$

where $[A]_o$ is the initial concentration of $N^5CH_3FH_4$ and $[A]_t$ equals the concentration after t minutes.

However, should oxygen be involved in the degradation of $N^5CH_3FH_4$ then the reaction must be considered to be pseudo first order. This is the case since the concentration of oxygen is very much greater than that of $N^5CH_3FH_4$ and therefore the change in its concentration is negligible compared with the change in concentration of the other reactant present. Thus, the overall order is reduced.

This reaction scheme is written:

$$\underset{(N^5CH_3FH_4)}{A} + \underset{(O_2)}{B} \xrightarrow{k} \underset{\text{(degradation product)}}{\text{Product}} \qquad (4)$$

where k is the rate constant.

The rate of reaction may be expressed as

$$\frac{d\left[\text{Product}\right]}{dt} = k\left[A\right]\left[B\right] \quad \text{ng ml}^{-1} \text{ min}^{-1} \qquad (5)$$

If the oxygen concentration is essentially unchanged throughout the reaction compared to the concentration of $N^5CH_3FH_4$, then it is regarded as a constant and the rate expression is written

$$\frac{d\left[\text{Product}\right]}{dt} = k'\left[A\right] \quad \text{ng ml}^{-1} \text{ min}^{-1} \qquad (6)$$

where

$$k' = k\left[B\right] \quad \text{min}^{-1} \qquad (7)$$

Thus the reaction exhibits first order kinetics although it involves more than one reactant and is referred to accordingly as a pseudo first order reaction.

The Arrhenius Equation

Arrhenius (1889) found that the experimentally observed variation of the rate constant (k) with absolute temperature (T) can be expressed:

$$k = Ae^{-E/RT} \qquad (8)$$

where A (the frequency factor) and E (the activation energy) are constants for the particular reaction and R is the universal gas constant. Both A and E are determined by the properties of the reacting molecules. Once A and E are known for a reaction, its rate may be calculated for any temperature from the above equation.

Second Order Reactions

Experimental results indicated that the degradation of $N^5CH_3FH_4$ (in the presence of a limited oxygen supply) could follow a second order mechanism. In this case, the reaction scheme is as follows:

$$\underset{(N^5CH_3FH_4)}{A} + \underset{(\text{oxygen})}{B} \xrightarrow{k} \underset{(\text{degradation product})}{\text{Product}} \qquad (9)$$

where k is the rate constant.

If the dissolved oxygen, B, undergoes a second order reaction of finite speed with the dissolved $N^5CH_3FH_4$, A, the rate of reaction can be expressed as:

$$\frac{-d\left[A\right]}{dt} = k\left[A\right]\left[B\right] \quad ng \quad ml^{-1} \quad min^{-1} \tag{10}$$

Let $\left[A\right]_o$ and $\left[B\right]_o$ be the initial concentration of reactants and $\left[A\right]_t$ and $\left[B\right]_t$ be the concentration of reactants at time 't'. Let X be the amount of A or B that has reacted at time 't'. Then at any time

$$\left[A\right]_t = \left[A\right]_o - \left[X\right] \tag{11}$$

$$\left[B\right]_t = \left[B\right]_o - \left[X\right] \tag{12}$$

Therefore,

$$\frac{-d\left[A\right]_t}{dt} = \frac{d\left[X\right]}{dt} \tag{13}$$

Substituting into the rate equation gives

$$\frac{-d\left[A\right]_t}{dt} = \frac{d\left[X\right]}{dt} = k\left(\left[A\right]_o - \left[X\right]\right)\left(\left[B\right]_o - \left[X\right]\right) \tag{14}$$

Integration from t = 0 gives the concentration of A or B or X at any time

$$\ln\left(\frac{[A]_o - [X]}{[B]_o - [X]}\right) = \ln\left(\frac{[A]_t}{[B]_t}\right) = \left([A]_o - [B]_o\right)kt + \ln\left(\frac{[A]_o}{[B]_o}\right) \quad (15)$$

or

$$kt = \frac{1}{[A]_o - [B]_o} \ln \frac{[A]_t \; [B]_o}{[B]_t \; [A]_o} \quad (16)$$

RESULTS

It should be noted that throughout these experiments, an increase in the binding of ^3H PteGlu to the milk protein in the radioassay system is referred to as a decrease in the concentration of $N^5CH_3FH_4$ as determined from the radioassay standard curve. Thus, the assumption is made that the degradation product of $N^5CH_3FH_4$ does not bind to the milk protein.

Kinetic Studies Using Thermal Death Time Pouches

The degradation curve for $N^5CH_3FH_4$ at 100°C in phosphate buffer (pH 7.3) is shown in Fig. 10 as a semilog plot of $N^5CH_3FH_4$ retained versus heating time at constant temperature. Because it was difficult to eliminate all air from the pouches during sealing, and because oxygen is able to diffuse slowly through nylon it was found to be impossible to accurately monitor the amount of oxygen present in the system throughout

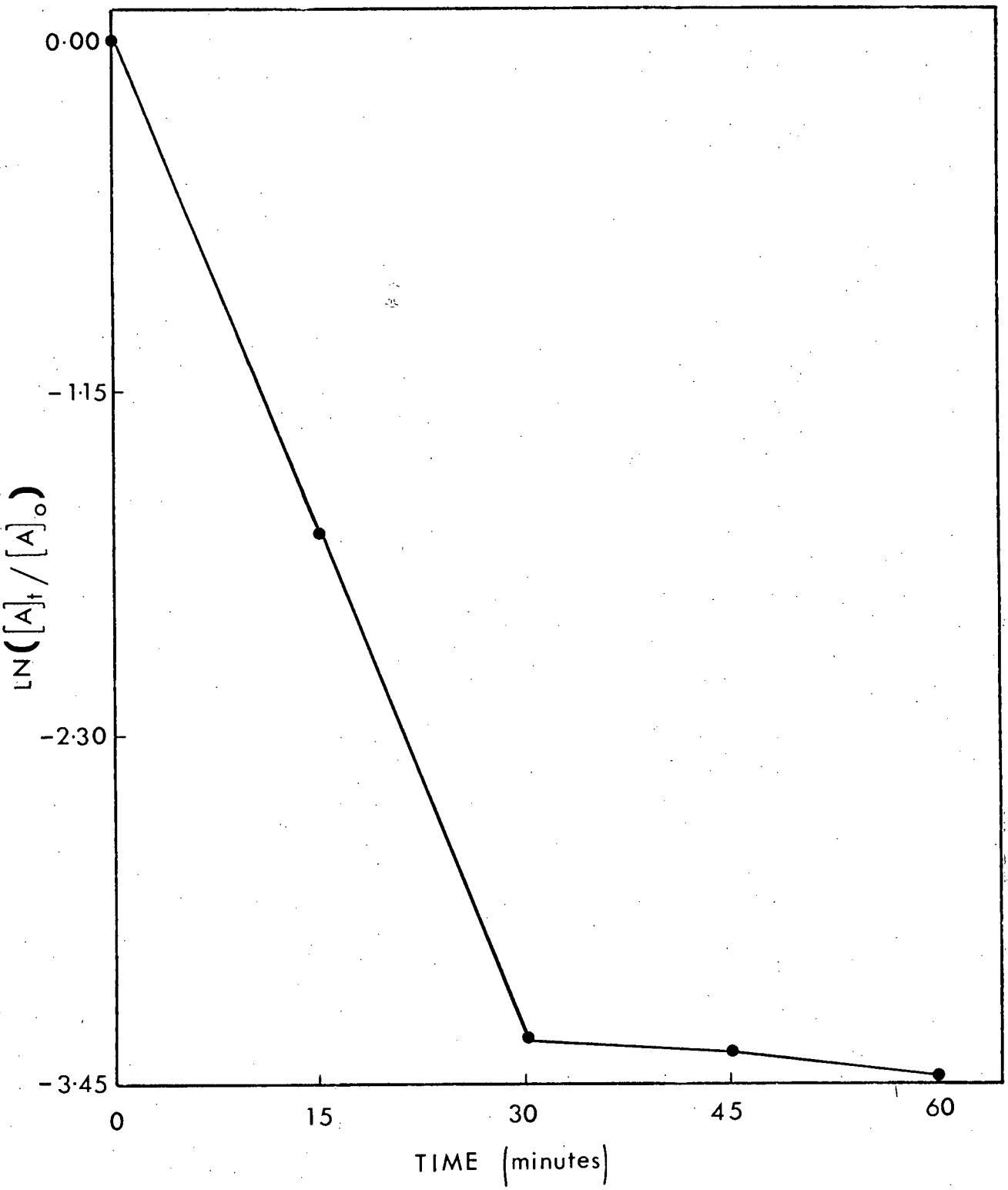


Figure 10. Degradation of $N^5CH_3FH_4$ heated to 100°C in thermal death time pouches.

the heating period. Since oxygen is thought to be important in the nutritional degradation of $N^5CH_3FH_4$, the thermal death time pouches were only used in this preliminary study to indicate the fact that $N^5CH_3FH_4$ indeed degrades when heated to 100°C.

Kinetic Studies Using Unlimited Oxygen Supply

The rate of degradation curves for $N^5CH_3FH_4$ at 100, 90, 75, 60, 55 and 40°C in phosphate buffer (pH 7.3) (as a semilog plot of $N^5CH_3FH_4$ retained versus heating time at constant temperature) may be seen in Figure 11. Two stages to the reaction are apparent. The first part of the reaction occurs along the horizontal portion of the curve (shown as X-Y for 40°C) where no appreciable difference in protein binding ($N^5CH_3FH_4$ concentration) is occurring. The second part of the reaction occurs along the slope portion of the curve (shown as Y-Z for 40°C) where an increase in protein binding takes place as shown by a decrease in folate concentration.

The Horizontal X-Y

The length of time from X to Y was found to increase as the temperature of reaction decreased. A plot of temperature of reaction versus log time of reaction is shown in Figure 12 and indicates that these intervals are temperature dependent.

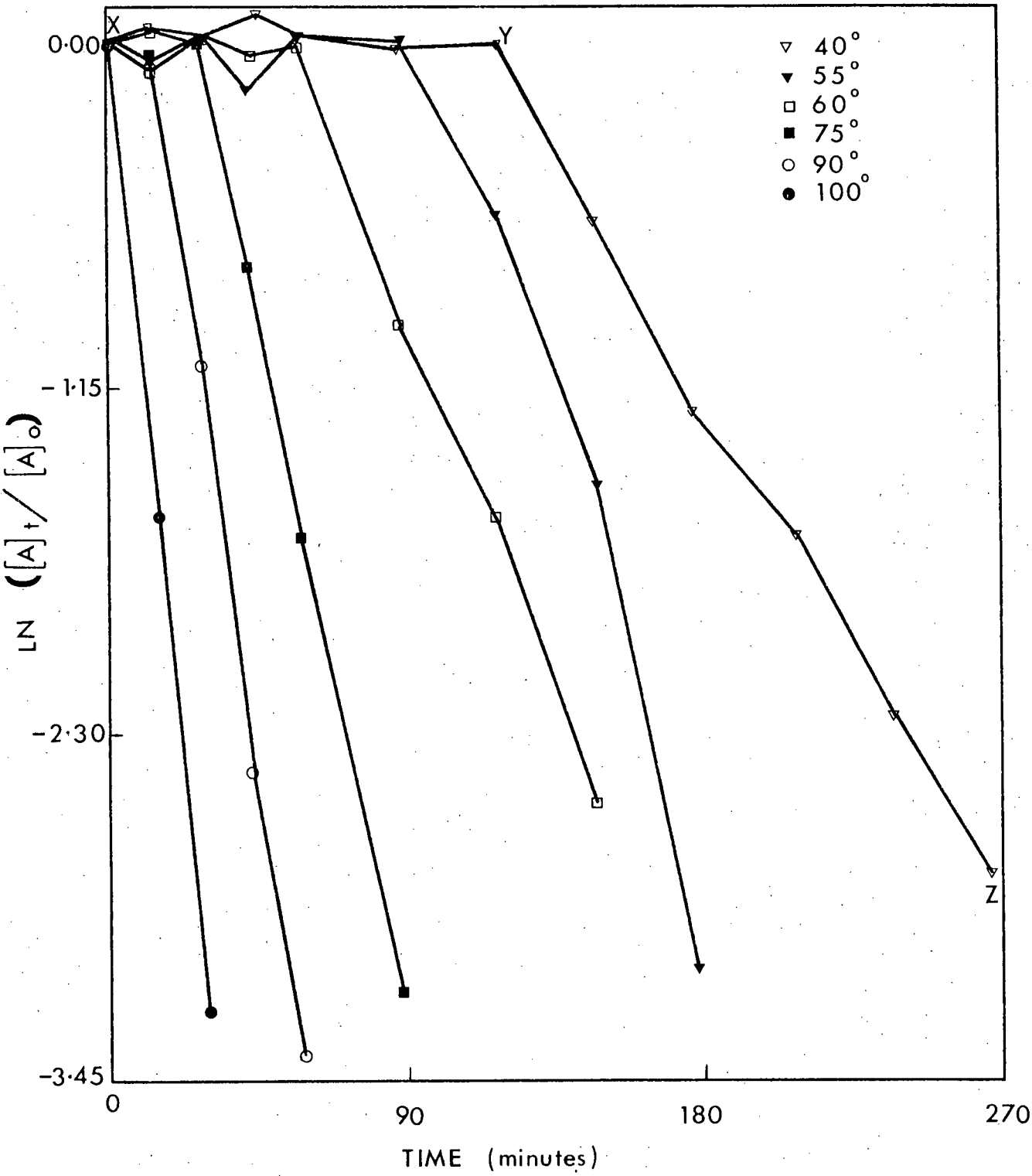


Figure 11. Degradation of $N^5CH_3FH_4$ heated to various temperatures in an unlimited oxygen supply.

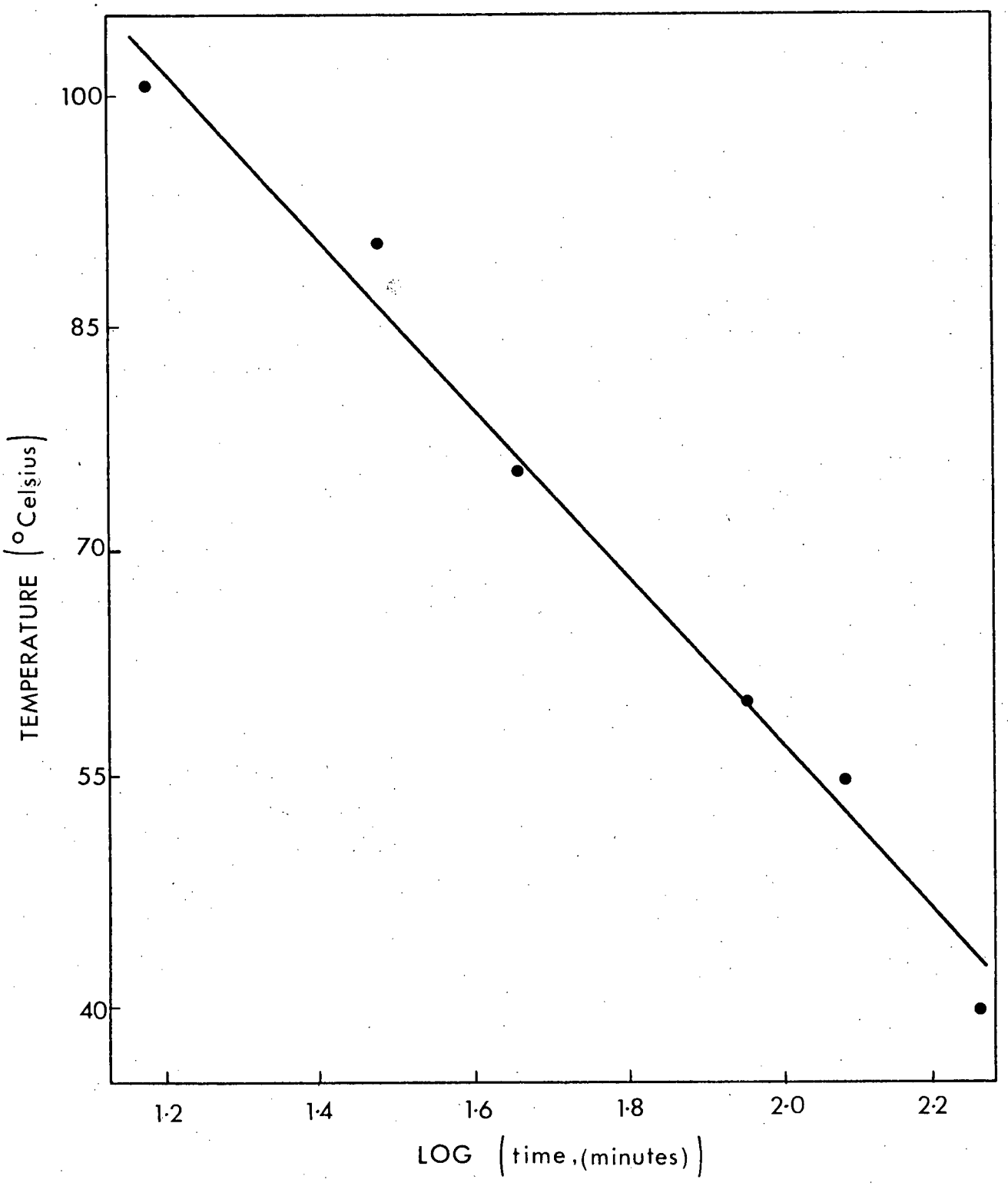


Figure 12. Influence of temperature on length of time to beginning of $N^5CH_3FH_4$ degradation reaction in the presence of an unlimited oxygen supply.

The Slopes Y-Z

Since oxygen has been cited as necessary for the degradation of $N^5CH_3FH_4$, the data used to construct the sloping portion of the curves were plotted according to a pseudo first order kinetic equation for each temperature. The regression analyses for the data are illustrated in Fig.13.

The high coefficients of determination obtained (Table VI) indicate that the reaction follows pseudo first order reaction kinetics. As discussed earlier, the reaction is referred to as 'pseudo first order' since oxygen was present in an abundant supply throughout the reaction and thus its change in concentration was negligible compared to the change in concentration of $N^5CH_3FH_4$ molecules present. The calculated pseudo first order rate constants for this experiment are reported in Table VI.

A plot of the rate constants (k') against temperature of reaction is shown in Figure 14. Since the rate constant was found to increase as the temperature of the reaction was raised, an Arrhenius-type plot was constructed (Figure 15). The straight line obtained demonstrates the validity of the Arrhenius equation in this reaction. From these data, A (the frequency factor) was found to be 1440 min^{-1} while E (the activation energy) was found to be 7.10 kcal $mole^{-1}$. Thus the complete rate expression may be written.

$$k' = 1440\, e^{\frac{-7100}{RT}} \quad min^{-1}$$

This rate expression is for a pseudo first order reaction.

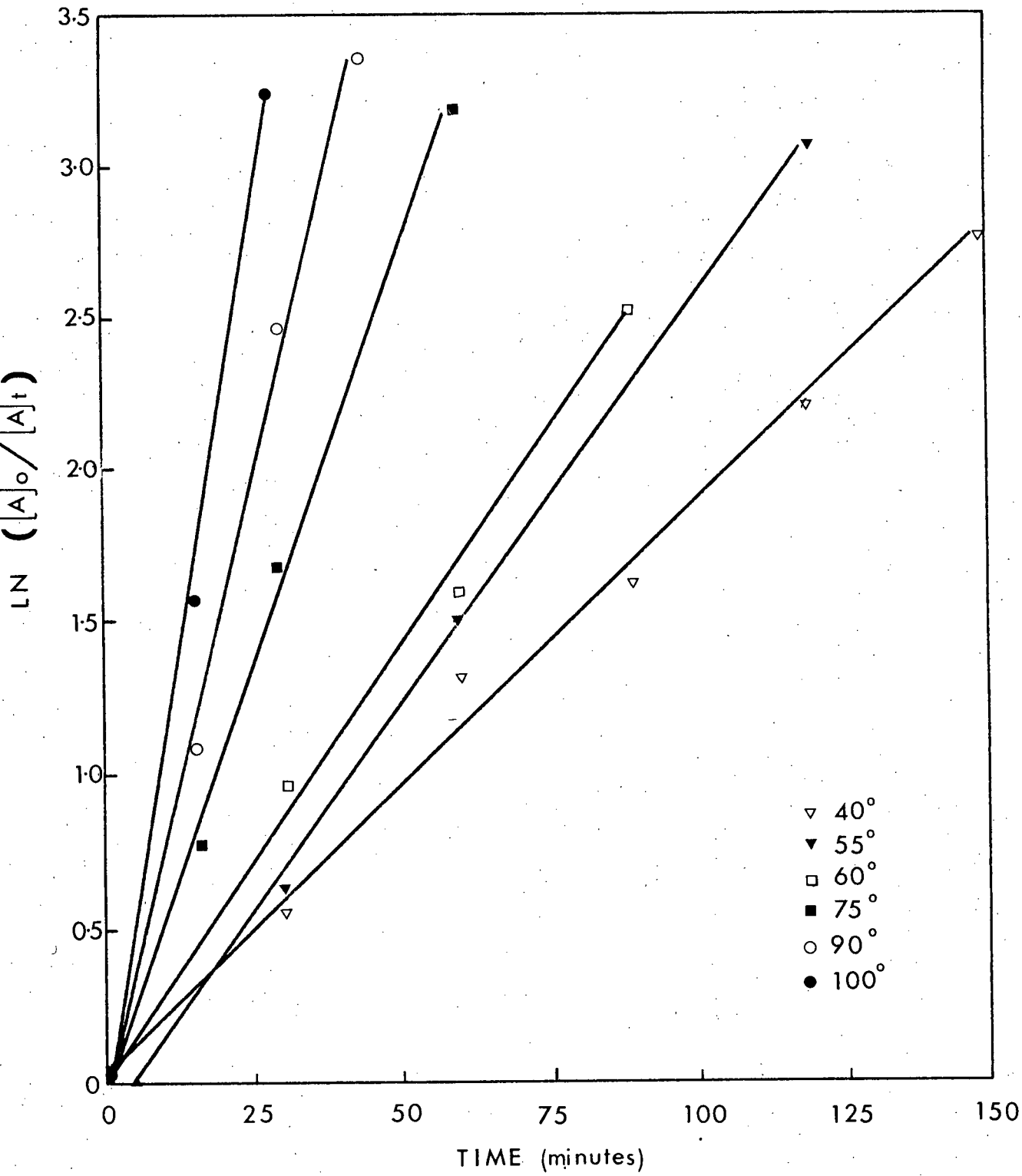


Figure 13. Regression analyses performed on data presented in Figure 11 according to pseudo first order reaction equation (6).

Table VI. Pseudo first order rate constants (k') of $N^5CH_3FH_4$ degradation in an abundant supply of oxygen.

Temperature of Reaction (^oC)	Intercept	Rate Constant (min^{-1})	Coefficient of determination (r^2)
100	0.012	0.108	0.96
90	0.009	0.076	0.96
75	0.011	0.053	0.96
60	0.032	0.028	0.94
55	0.087	0.026	0.96
40	0.056	0.018	0.96

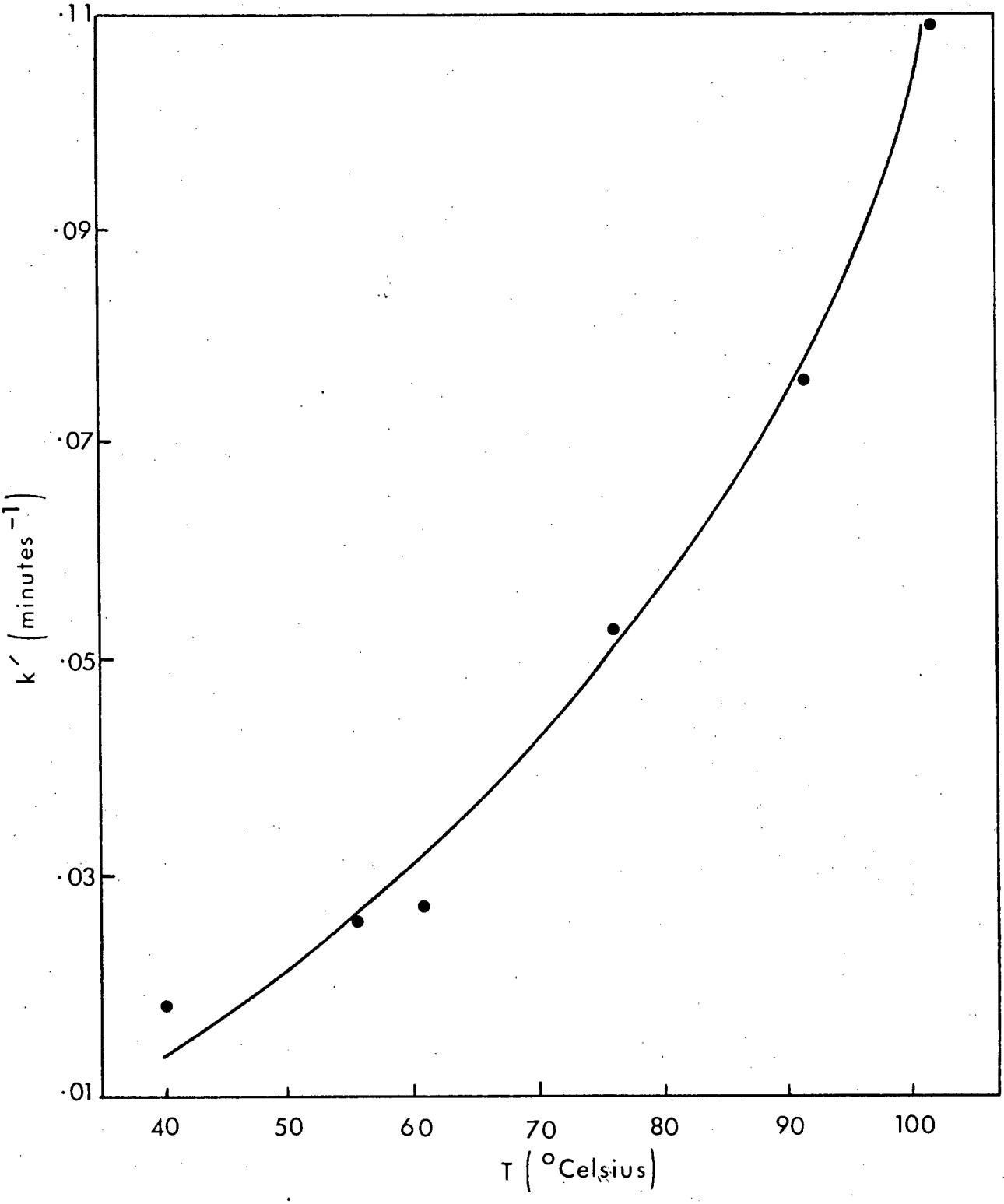


Figure 14. Variation of rate constant (k') with temperature of reaction.

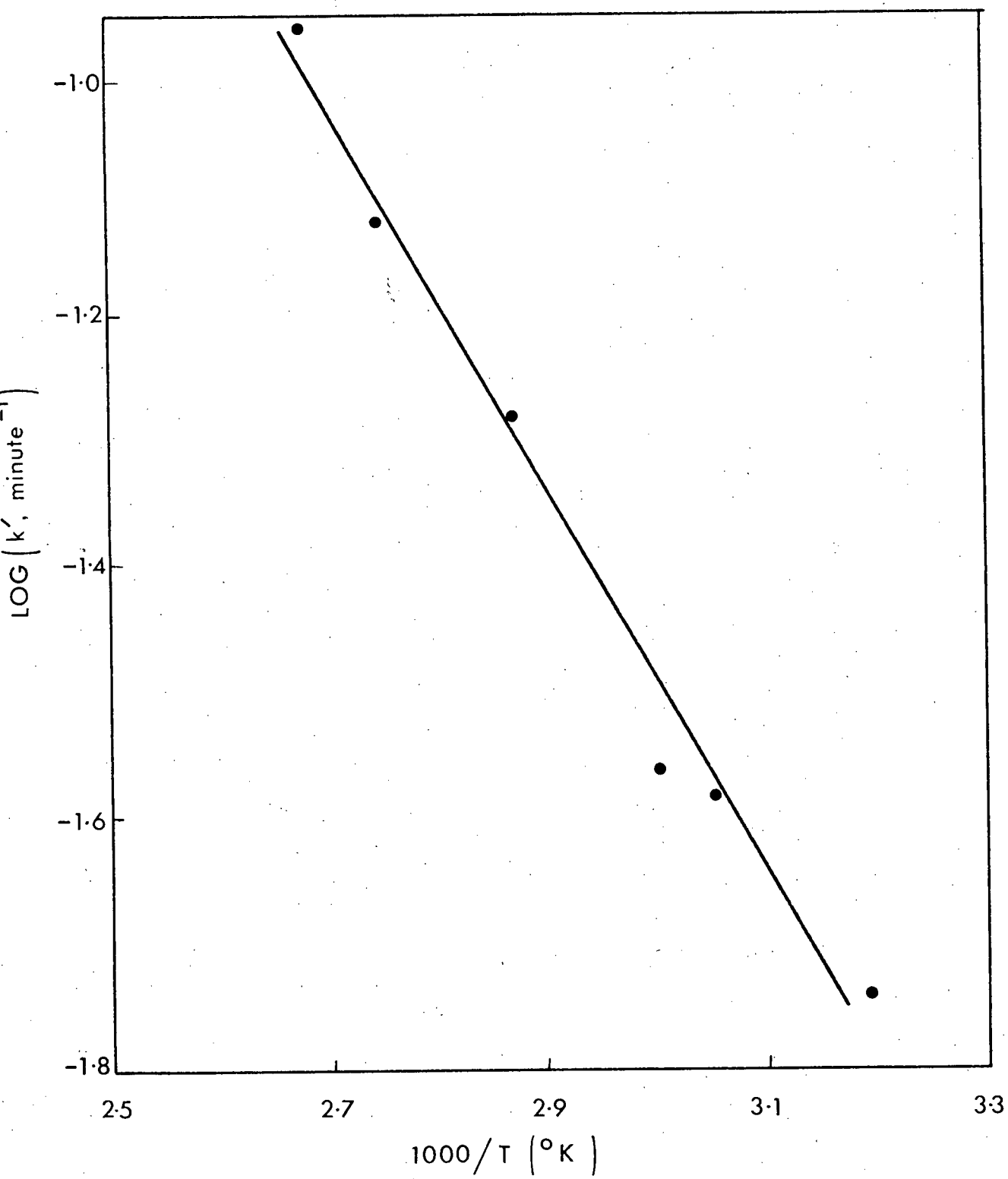


Figure 15. Plot of data in Figure 14 according to Arrhenius equation (8).

Kinetic Studies Using Unlimited Oxygen Supply and a Mercaptoethanol - Free Assay System

The rate of degradation curves for $N^5CH_3FH_4$ at 40, 75 and 90°C in phosphate buffer (pH 7.3) measured in a mercaptoethanol - free assay are shown as a semilog plot of $N^5CH_3FH_4$ retained versus heating time at constant temperature in Figure 16.

The degradation of $N^5CH_3FH_4$ is observed to occur immediately when samples are heated to 90° and 75°C in the presence of an unlimited oxygen supply. Degradation occurs after 90 minutes in the sample heated to 40°C.

A pseudo first order kinetic equation was applied to all the data of the samples heated to 75° and 90°C and to data recorded after 90 minutes in the sample heated to 40°C. Linear regression analyses of the data are shown in Figure 17. The high coefficients of determination obtained (Table VII) indicate that the reaction follows pseudo first order reaction kinetics. The calculated pseudo first order rate constants (k') are reported in Table VII and are observed to be very similar to the values obtained when the $N^5CH_3FH_4$ was assayed in a system containing mercaptoethanol.

Kinetic Studies Using a Limited Oxygen Supply

The rate of degradation curves, for $N^5CH_3FH_4$ at 20°C in phosphate buffer (pH 7.3) and in the presence of a limited oxygen supply are shown in Figure 18. The figure also includes results for samples in which dissolved oxygen was maintained at levels above saturation.

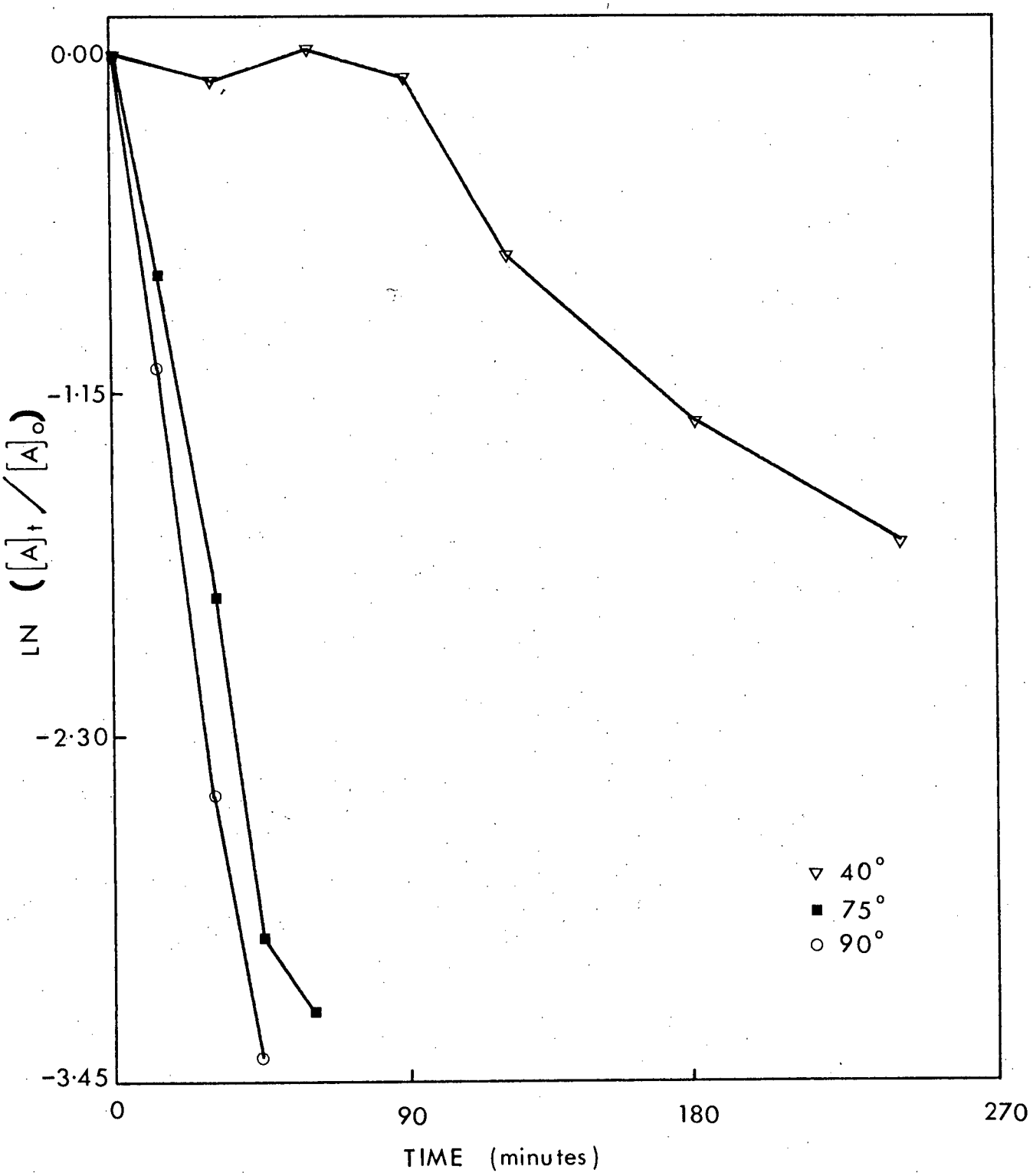


Figure 16. Degradation of $N^5CH_3FH_4$ heated to various temperatures in an unlimited oxygen supply and assayed using a mercaptoethanol-free system.

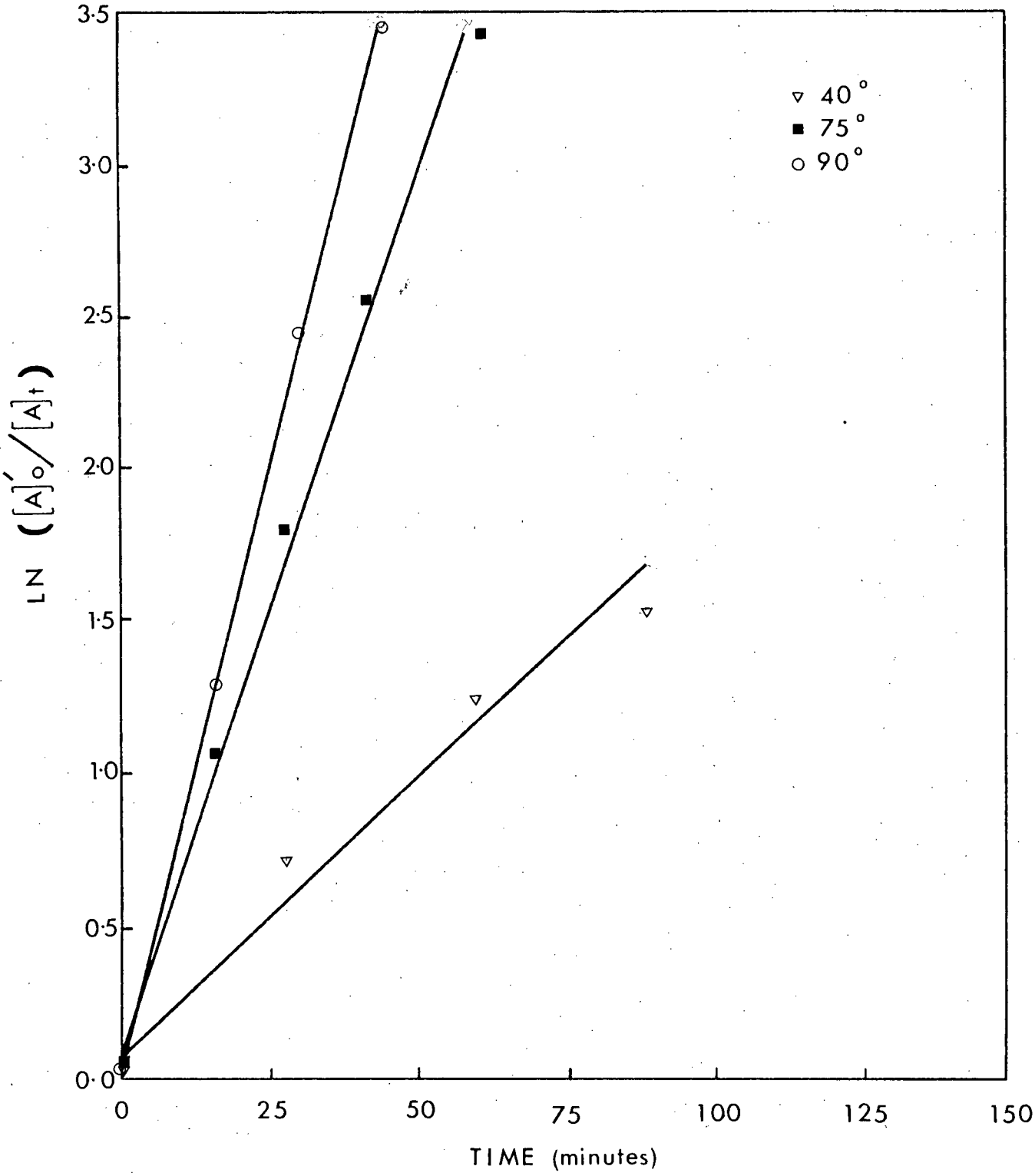


Figure 17. Regression analyses performed on data presented in Figure 16 according to pseudo first order reaction equation (6).

Table VII. Pseudo first order rate constants (k') of $N^5CH_3FH_4$ degradation in an abundant supply of oxygen measured by a mercaptoethanol-free assay system.

Temperature of Reaction (^oC)	Intercept	Rate Constant (min^{-1})	Coefficient of Determination (r^2)
40	0.060	0.018	0.96
75	0.062	0.057	0.94
90	-0.012	0.077	0.96

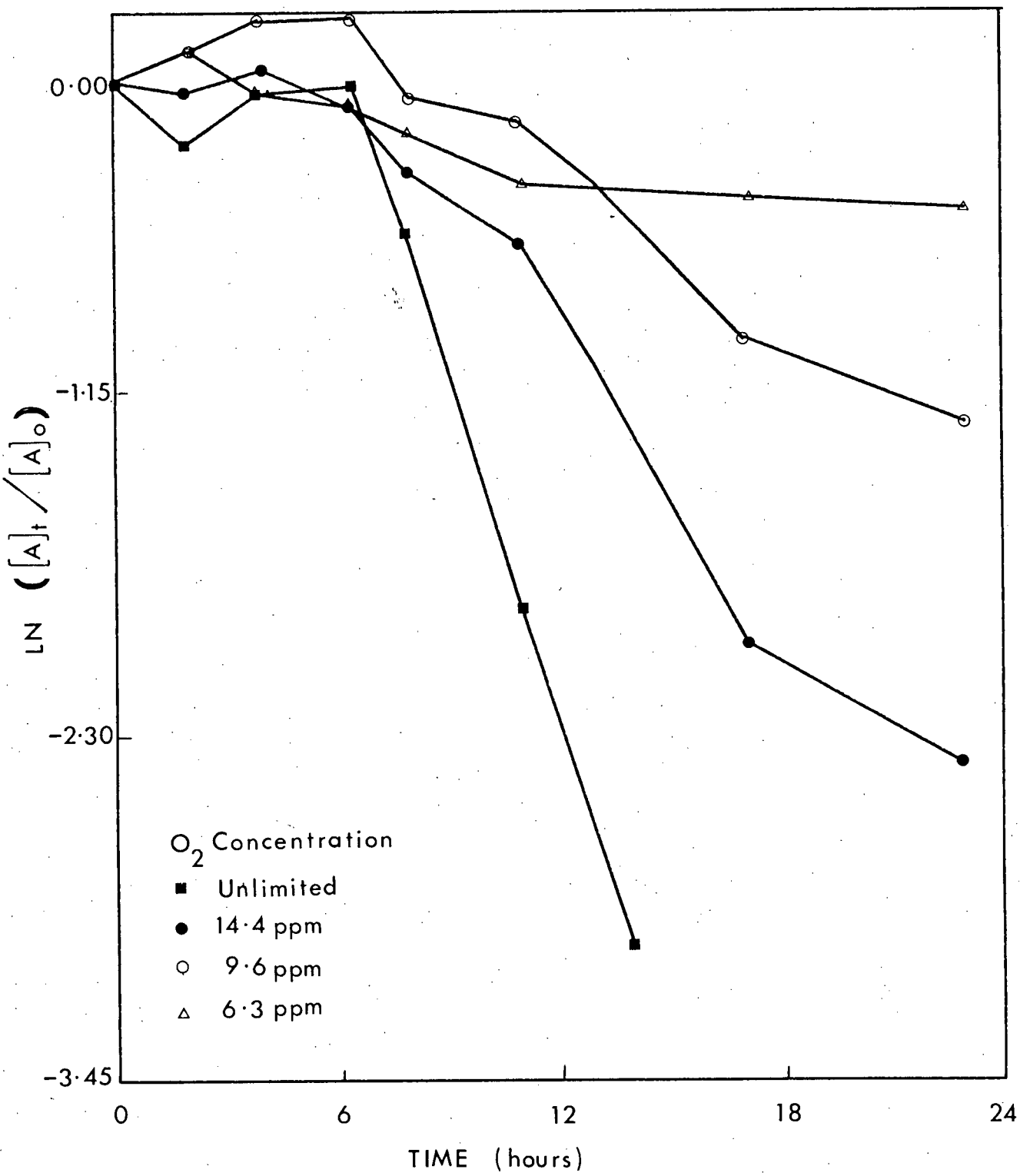


Figure 18. Influence of dissolved oxygen content on $N^5CH_3FH_4$ degradation at 20°C.

As previously observed, the concentration of $N^5CH_3FH_4$ is essentially constant and does not change appreciably during the first six to eight hours of the experiment (mercaptoethanol was present in the radioassay system).

A plot of change in concentration of oxygen throughout the duration of the experiment, however, indicates that oxygen concentration was decreasing more rapidly during the first 6 to 8 hours than during the remainder of the experiment (Figure 19). This is indicative of an oxidation reaction taking place throughout the 23 hours of the experiment but the rate of oxidation decreases after the first 6 to 8 hours.

The degradation of $N^5CH_3FH_4$ appears to depend on the concentration of dissolved oxygen. This is observed from Figure 18 where the rate of degradation of $N^5CH_3FH_4$ is faster for higher initial dissolved oxygen concentration. It is also apparent that in the presence of a limited oxygen supply the data do not fit a straight line plot on semilog coordinates. To test the assumption that the reaction follows second order kinetics, the data from the sloping portion of each curve were plotted according to a second order kinetic equation (Figure 20). The units of vitamin concentration (A) and dissolved oxygen concentration (B) were mg/liter and parts per million, respectively. Since oxygen was present in a greater concentration than the vitamin, the integrated second order rate equation was reversed to avoid the use of negative values. Plots of the integrated second order rate equation for samples containing initial dissolved oxygen concentrations of 14.4 and 9.6 parts per million are shown in Figure 20. The calculated second order rate constants for the two initial dissolved oxygen concentrations were found to be very similar as illustrated in Table VIII.

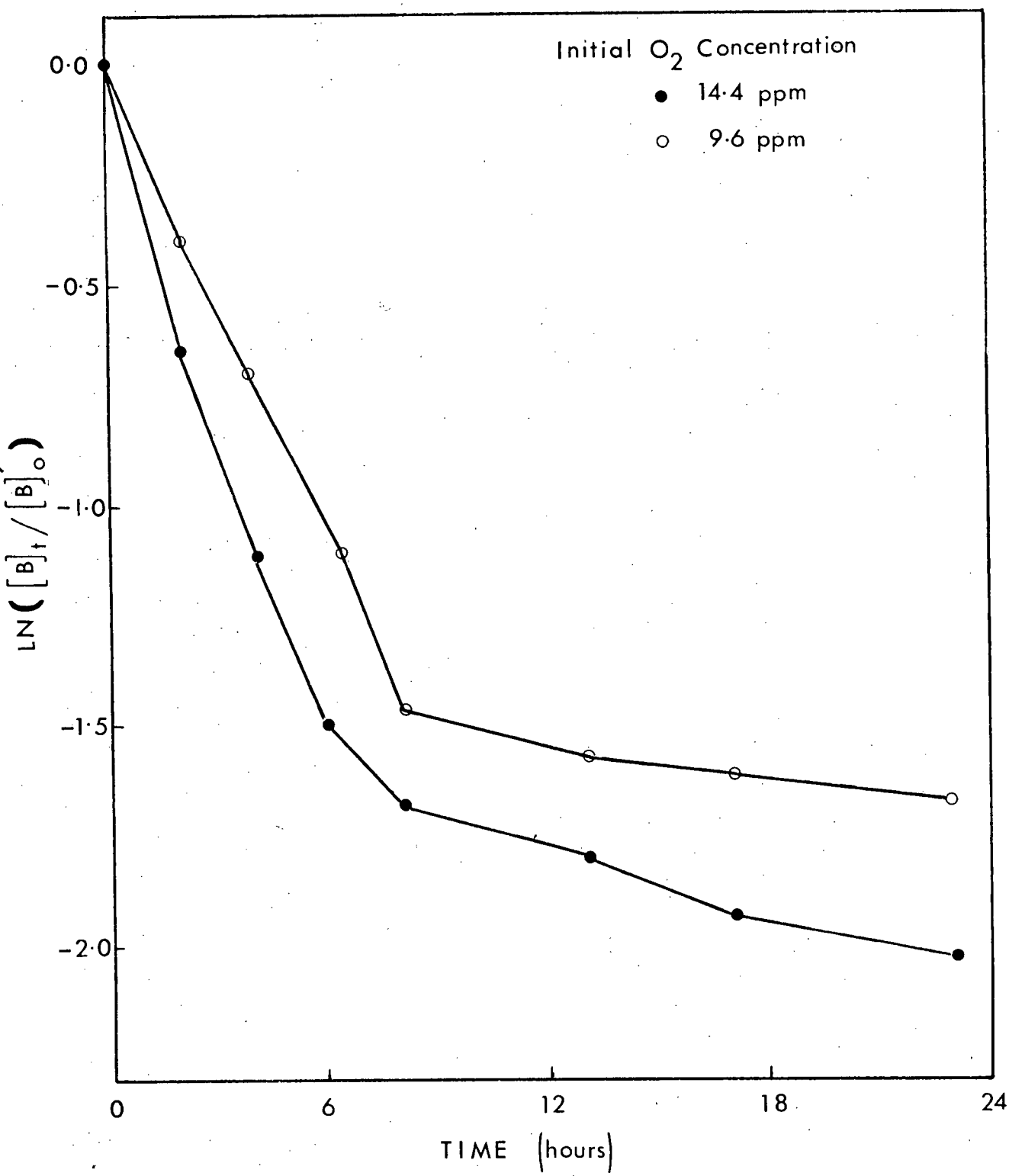


Figure 19. Change in oxygen concentration at 20°C in the presence of $N^5CH_3FH_4$.

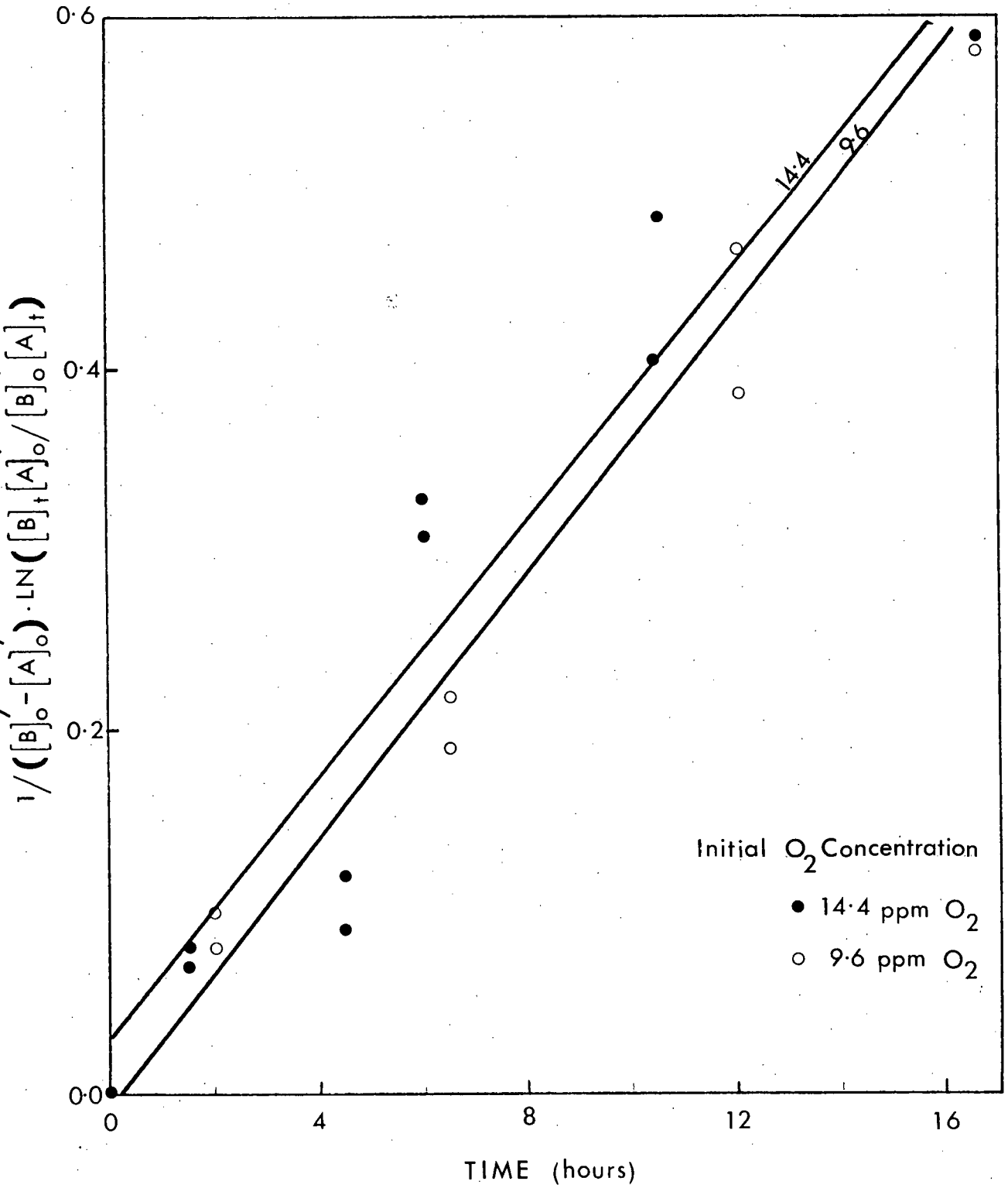


Figure 20. Regression analyses performed on part of the data presented in Figure 18 according to second order reaction equation (16).

Table VIII. Second order rate constants (k) for $N^5CH_3FH_4$ degradation in a limited supply of oxygen at 20°C.

Initial dissolved O_2 conc. (ppm)	Intercept	Rate Constant ($mg\ l^{-1}\ hr^{-1}$)	Coefficient of determination (r^2)
9.6	0.028	0.036	0.92
14.4	-0.011	0.038	0.94

The rate constants are based on duplicate trials.

DISCUSSION

Folic acid has been shown by many workers to be very susceptible to degradation by oxidative heating. (Cheldin et al., 1943; Schweigert et al., 1946; Stokstad et al., 1947; Hanning and Mitts, 1949; Ghitis, 1966; Herbert, 1967, 1968b; Ford et al.,1968; Schroeder, 1971; Malin, 1977). Methods of measurement of folic acid degradation in all these experiments have been microbiological in nature and have been based on the assumption that the degradation products do not support the growth of the test microorganisms.

Throughout the experiments reported here the assumption was made that the product of degradation of $N^5CH_3FH_4$ did not bind to the folate binding protein of the radioassay method.

Initial experiments utilising the thermal death time pouches showed that, when $N^5CH_3FH_4$ in a phosphate buffer was heated in a boiling water bath, a change in the structure of $N^5CH_3FH_4$ took place such that it no longer had the ability to bind to the folate binding protein.

Since oxygen was thought to be involved in the degradation reaction, $N^5CH_3FH_4$ was then heated to various temperatures while oxygen was constantly bubbled through the sample. Degradation curves indicated an initial period of time, (which increased as the temperature of heating decreased), when the $N^5CH_3FH_4$ concentration, as measured by the radioassay method, did not appear to change. This was followed by a rapid decrease in $N^5CH_3FH_4$ concentration. This reaction appeared to follow pseudo first order kinetics and the pseudo first order rate constants were shown to decrease as the temperature of reaction decreased.

Conformity with the Arrhenius equation made it possible to calculate the activation energy for the thermal breakdown of $N^5CH_3FH_4$ in a phosphate buffer system and in the presence of an unlimited oxygen supply.

Gupta and Huennekens (1967) have shown that oxidation of $N^5CH_3FH_4$ yields a dihydro compound, presumably 5 methyl-5, 6 dihydrofolate. This compound was shown by the authors to be reduced readily back to $N^5CH_3FH_4$ upon the addition of mercaptoethanol.

Since mercaptoethanol was present in the buffer, the milk protein and the ^3H PteGlu of the radioassay method, it was speculated that this may be influencing the first part of the degradation reaction where no appreciable change in $N^5CH_3FH_4$ concentration was taking place. Mercaptoethanol was therefore eliminated from the assay system and the degradation of $N^5CH_3FH_4$ was examined as in the previous experiment.

The effect of the elimination of mercaptoethanol was to decrease the period of time in the reaction when little change in $N^5CH_3FH_4$ concentration was observed. However, samples heated to 40^oC still exhibited an initial time period before $N^5CH_3FH_4$ concentration changes were detected. The absence of mercaptoethanol did not affect the rate constants for the degradation reaction.

When the degradation of $N^5CH_3FH_4$ was observed in a limited oxygen supply, the reaction appeared to follow second order reaction kinetics. The rate constants calculated must be regarded with a certain element of caution, however. Oxygen concentrations, although limited, were still in relatively large amounts compared to the $N^5CH_3FH_4$ concentrations. Thus, the reaction could still be regarded as pseudo first order although coefficients of determination for the regression lines

obtained when a pseudo first order kinetic equation was applied to the data were not as high as when a second order kinetic equation was applied. Greater initial concentrations of $N^5CH_3FH_4$ are necessary in order to accurately ascertain the rate constants for this reaction. It does appear, however, that the presence of oxygen is necessary for the degradation of $N^5CH_3FH_4$.

The decrease in oxygen concentration in the initial stages of the reaction indicates that oxidation is taking place although this is not detected by a change in $N^5CH_3FH_4$ concentrations. Thus, it appears that the mercaptoethanol present in the assay system is reducing the degradation product back to $N^5CH_3FH_4$ during the initial stages of the reaction.

CONCLUSIONS

$N^5CH_3FH_4$ degradation in the presence of an unlimited oxygen supply can be described by a pseudo first order reaction. Pseudo first order rate constants increase as the temperature of reaction increases and this relationship can be described by the Arrhenius equation.

In the presence of a limited oxygen supply the overall reaction appears to be second order in nature. Oxygen concentrations decrease throughout the reaction but, when mercaptoethanol is present in the assay system, a decrease in $N^5CH_3FH_4$ concentration is not observed immediately. When mercaptoethanol is eliminated from the assay, $N^5CH_3FH_4$ concentrations were observed to decrease earlier. No change in the degradation rate constant for each temperature was detected.

The degradation of $N^5CH_3FH_4$ is therefore thought to be an oxidation reaction probably to the N^5 methyl dihydrofolate. This compound does not bind to the folate binding protein in the radioassay method. The oxidation product is readily reduced back to $N^5CH_3FH_4$ in the presence of mercaptoethanol.

Care must be taken, therefore, to exclude the presence of mercaptoethanol in the assay system when attempting to measure the rate of breakdown of $N^5CH_3FH_4$ since, in the initial stages of the reaction, no degradation will be observed if it is used. The presence of other reducing agents in a food system may have a similar effect on the rate of degradation of $N^5CH_3FH_4$.

CHAPTER IV. IDENTIFICATION OF THE DEGRADATION PRODUCT AND THE EFFECT OF ASCORBIC ACID ON RATE OF DEGRADATION OF N^5 METHYLTETRAHYDROFOLIC ACID

INTRODUCTION

$N^5CH_3FH_4$ has been shown to be susceptible to degradation by heat in the presence of oxygen. There are several publications pertaining to the identification of the $N^5CH_3FH_4$ degradation product. O'Broin et al. (1975) reported that $N^5CH_3FH_4$ was "nutritionally very unstable" particularly at acid pH values. The authors stated however, that the relatively rapid nutritional inactivation of this compound was unlikely to be due to cleavage of the $C_9 - N_{10}$ bond, since this form of the vitamin being substituted in the 5 position would be expected to be resistant to such reactions.

Donaldson and Keresztesy (1962) have shown that $N^5CH_3FH_4$ is oxidized to the dihydrofolate form by a number of oxidizing agents. The oxidized derivative was characterized as 5 methyl 5, 6, dihydrofolic acid. Gupta and Hennekens (1967) confirmed these results.

In this part of the research an attempt was made to identify the degradation product of $N^5CH_3FH_4$ using ultraviolet spectroscopy.

Donaldson and Keresztesy (1962) showed that various reducing agents are required for the conversion of $N^5CH_3FH_4$ to 5, 10 methylene-tetrahydrofolic acid by the enzyme 5, 10-methylenetetrahydro folic acid reductase. The authors speculated that the role of the reducing agent was to prevent the degradation of $N^5CH_3FH_4$ to the dehydro-compound. Homocysteine, mercaptoethanol and ascorbic acid were all found to be

effective reducing agents. O'Broin et al. (1975) stated that ascorbic acid in phosphate buffer afforded better protection against oxidation than mercaptoethanol at lower concentrations of folate.

The previous chapter indicated that, mercaptoethanol was able to reduce the degradation product of heating $N^5CH_3FH_4$ in oxygen back to the $N^5CH_3FH_4$ compound. It was suggested that the presence of other reducing agents within a food system may have an effect on the rate of oxidation of $N^5CH_3FH_4$. Ascorbic acid is a reducing agent commonly found in foods. To observe its effect on the rate of degradation of $N^5CH_3FH_4$ a known amount of ascorbate was added to a food, the food was heated and the degradation of $N^5CH_3FH_4$ was measured using the radioassay method.

METHODS

Ultraviolet Spectroscopy

A sample of $N^5CH_3FH_4$ in phosphate buffer (pH 7.3 0.1M) was heated to 70°C in a water bath. Oxygen was bubbled through the sample while it was heated.

Samples were removed for analyses by both ultraviolet spectroscopy and the radioassay method every 15 minutes. No mercaptoethanol was used in the radioassay method.

Samples of $N^5CH_3FH_4$ at greater concentrations than previously used were then heated to 100°C for 2 hours. Oxygen was bubbled through the sample throughout the heating period. The resulting sample was then analyzed by ultraviolet spectroscopy.

The Effect of Ascorbic Acid on The Degradation of $N^5CH_3FH_4$ in a Food

A baby food was chosen for this experiment since this negated a blending process prior to the assay. Care was also taken to ensure that the food chosen contained little or no ascorbic acid (according to manufacturer's nutrient content tables).

Two 100g samples of "bacon and egg breakfast" baby food were placed in flasks. 15 mg of ascorbic acid were added to one sample. The flasks were then placed in a boiling water bath and oxygen was bubbled through the samples. Both samples were constantly stirred throughout a two-hour heating period. Care was taken to ensure that very little evaporation of the sample took place.

Samples were removed every 15 minutes for the first hour of heating and every 30 minutes thereafter. After cooling in an ice water bath samples were assayed for free and total folate by the radioassay method described in Chapter 2.

RESULTS

Ultraviolet Spectroscopy

A single absorption peak at 265 nm was observed when a sample of unheated $N^5CH_3FH_4$ in phosphate buffer was analyzed. No other absorption peaks were detected at the concentrations tested. The decrease in absorption peak height with length of time of heating the $N^5CH_3FH_4$ sample in phosphate buffer is shown in Figure 21. After

30 minutes of heating at 70°C, the absorption peak was no longer detectable (care was taken to ensure that samples were not exposed to ultraviolet light for too long a period prior to obtaining an ultraviolet scan as it was found that the absorption peak disappeared very rapidly upon exposure to light at this wavelength).

Corresponding radioassay results shown in Table IX indicate that a decrease in $N^5CH_3FH_4$ concentration is not detected immediately. After heating the sample for 45 minutes, a 54 percent decrease in concentration was observed. It is assumed therefore that the residual ascorbate present in the sample protected the $N^5CH_3FH_4$ from oxidation during the initial stages of the reaction.

Samples of $N^5CH_3FH_4$ at greater concentrations than those used in the previous experiment yielded two ultraviolet absorption peaks at 290 and 249 nm after heating to 100°C for 2 hours in the presence of an unlimited oxygen supply. This is shown in Figure 22.

The Effect of Ascorbic Acid on The Degradation of $N^5CH_3FH_4$ in a Food

A standard curve constructed using $N^5CH_3FH_4$ samples was used for calculating food folate concentrations.

The rate of degradation curves for folate (as measured by the radioassay method) in a food system, heated in a boiling water bath with an unlimited supply of oxygen are shown in Figure 23. Free and total folate concentrations of the two food samples are shown.

The free folate concentration of the food sample containing no ascorbate is shown to decrease during the first 15 minutes of heating. A similar decrease in the free folate concentration of the sample containing 15 mg ascorbic acid per 100 ml of food does not occur until after 30 minutes of heating. It is of interest to note that very little

Figure 21. Decrease in ultraviolet absorption with length of time (in minutes) of heating $N^5CH_3FH_4$ samples in phosphate buffer to 70°C.

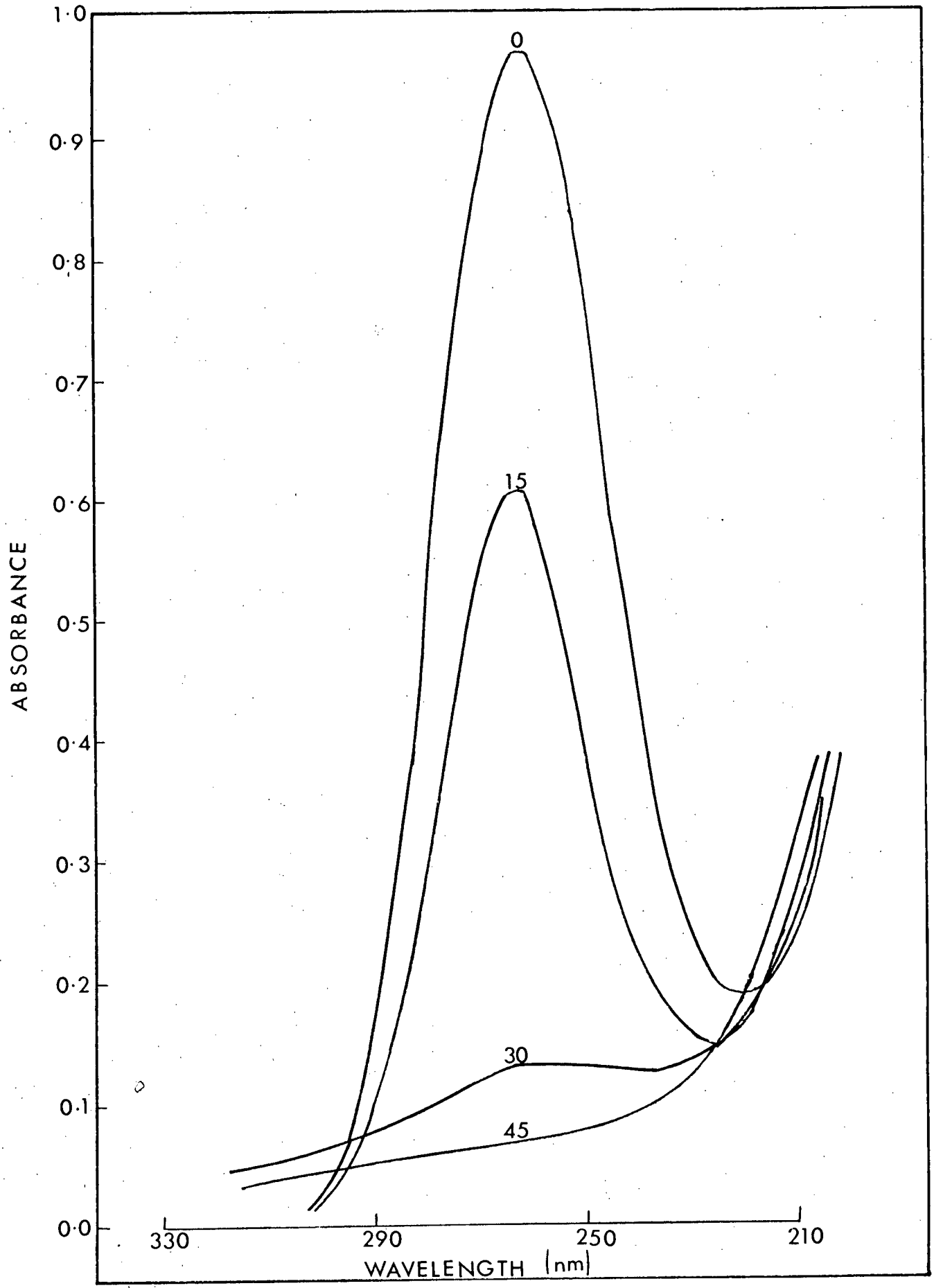
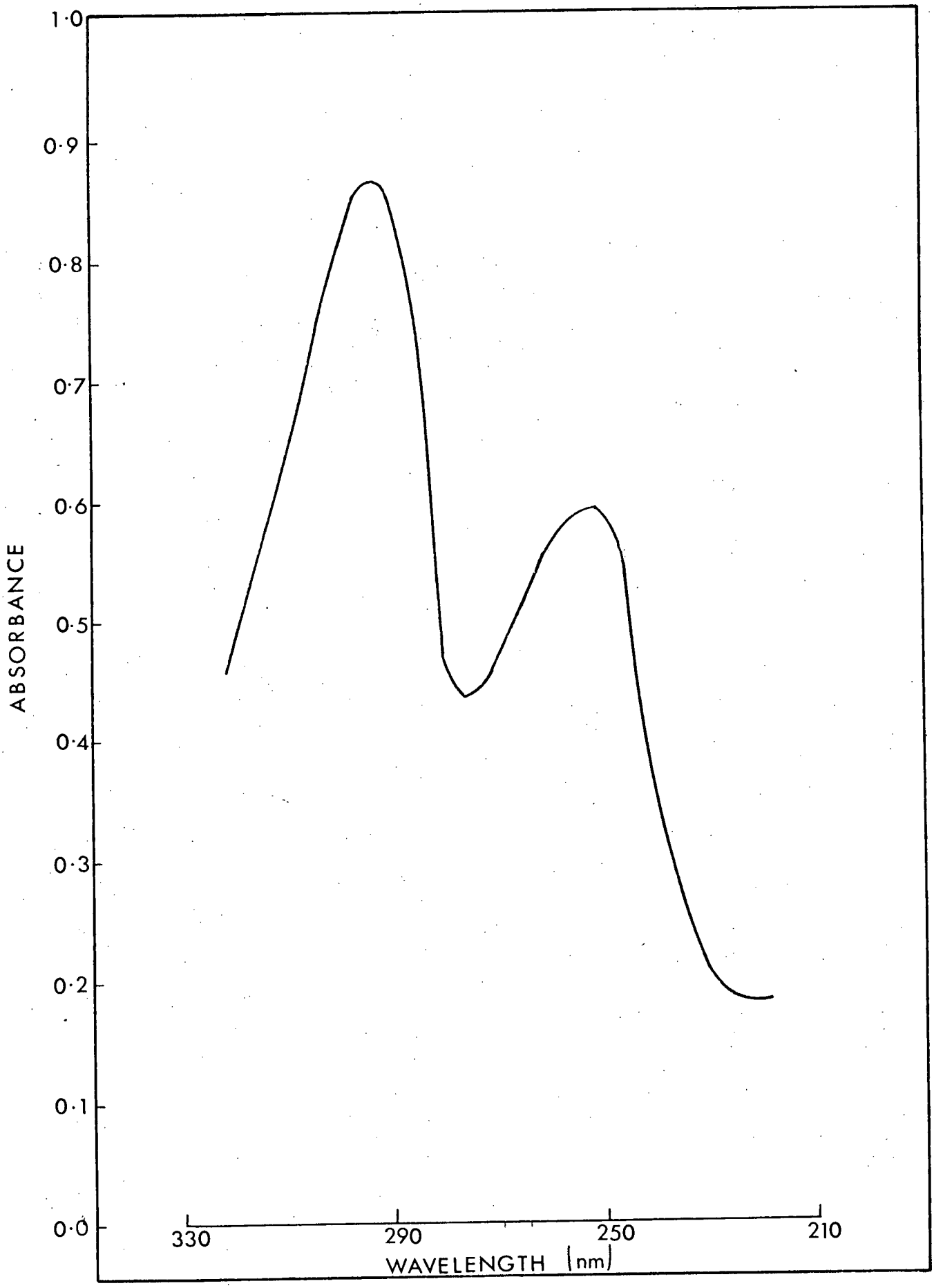

1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·0
ABSORBANCE
0
15
30
45
330
290
250
210
WAVELENGTH (nm)

Table IX. Effect of heating on $N^5CH_3FH_4$ stability.

Time of Heating (mins)	Concentration of $N^5CH_3FH_4$ (ng/ml)	Change in Concentration (%)
0	27.31	0
15	26.70	2
30	27.54	0
45	12.64	54
60	5.23	81

Figure 22. Ultraviolet absorption spectrum of N^5 methyldihydrofolic acid.

$\lambda_{max} = 290$ nm ($\epsilon = 31.0 \times 10^3$ M^{-1} cm^{-1})


1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·0
ABSORBANCE
330
290
250
210
WAVELENGTH (nm)

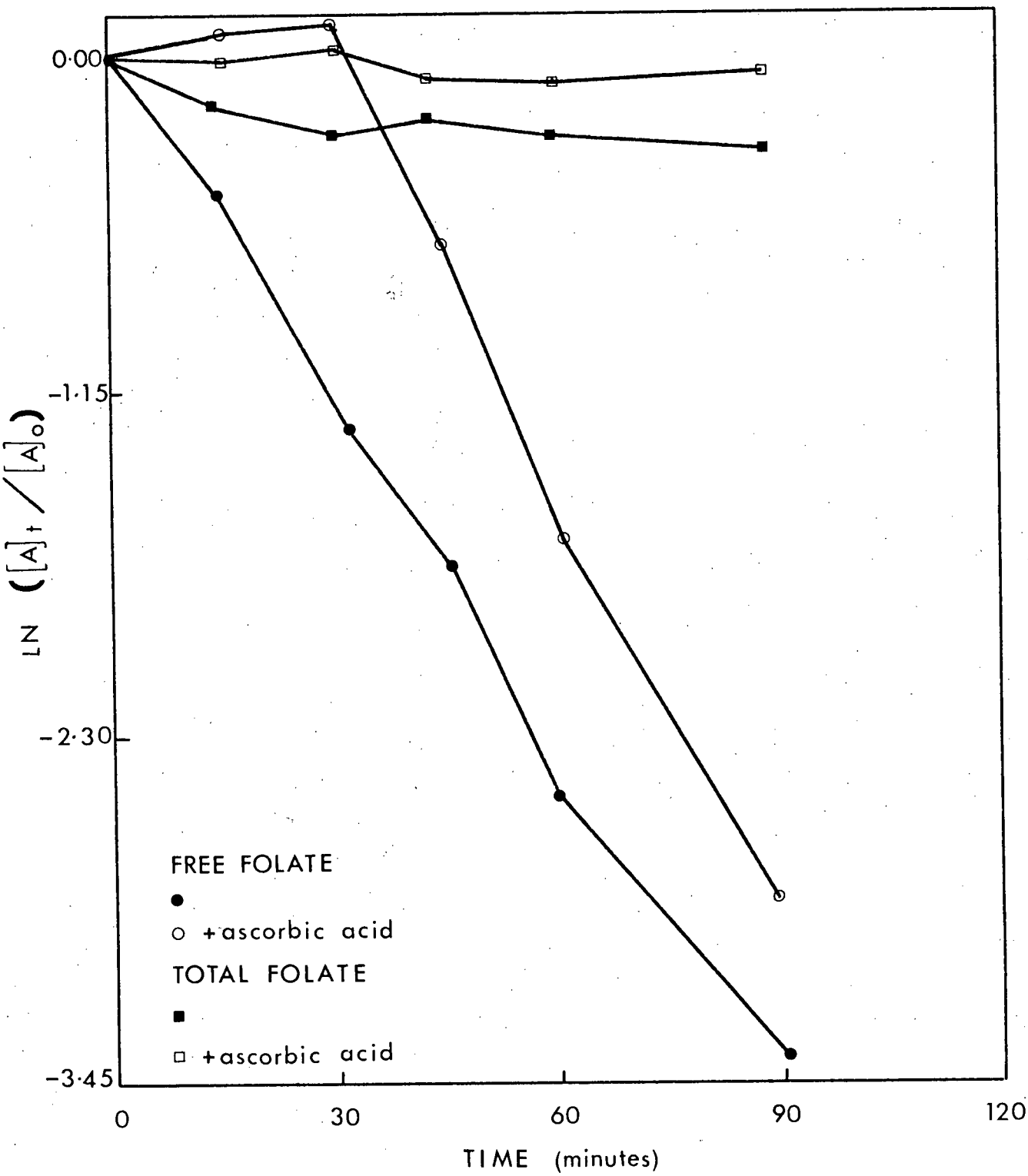


Figure 23. The influence of ascorbic acid on the degradation of free and total folate in a food heated to 100°C in the presence of an unlimited oxygen supply.

difference is observed in the total folate content of the two samples. Within the two-hour heating no appreciable decrease in total folate concentration of either sample is observed.

DISCUSSION

Consultation with Clinical Assays (Massachusetts) confirmed that the peak at 265 nm observed when $N^5CH_3FH_4$ samples were subjected to ultraviolet spectroscopy was due to the presence of residual ascorbic acid added to the $N^5CH_3FH_4$ prior to lyophilization. The presence of this peak made it impossible to observe the characteristic ultraviolet absorption spectra of the pure $N^5CH_3FH_4$ sample. Donaldson and Keresztesy (1962) and Gupta and Huennekens (1967) have indicated that the maximum absorption for pure $N^5CH_3FH_4$ occurs at 290 nm ($E = 30.8 \times 10^3 \ M^{-1} \ cm^{-1}$).

The presence of ascorbic acid in the sample is shown to affect the rate of degradation of $N^5CH_3FH_4$. It would appear that ascorbic acid reduces the degradation product back to $N^5CH_3FH_4$, itself being oxidized to the dehydroascorbic acid which does not absorb ultraviolet light. Hence, the absorption peak at 265 nm is observed to decrease when the sample containing $N^5CH_3FH_4$ is heated.

The presence of ascorbic acid in the $N^5CH_3FH_4$ samples explains why the sample heated to 40°C and assayed in a mercaptoethanol - free system (described in Chapter III) exhibited a decrease in $N^5CH_3FH_4$ concentration only after 1½ hours of heating. Thin layer and column chromatography of $N^5CH_3FH_4$ samples heated for short periods of time yielded only the original starting product for the same reason thus

making the identification of the degradation product impossible.

The ultraviolet absorption peaks at 290 and 249 nm observed when higher concentrations of sample were used could only be detected when the absorption peak for ascorbic acid had disappeared. These absorption peaks confirm that the product of $N^5CH_3FH_4$ oxidation is the dihydro-compound.

Since ascorbic acid is an effective reducing agent in this reaction, its effect on the folate degradation rates in foods is of interest. Ghitis (1966) and Ford et al. (1968) have observed that the presence of ascorbate in milk is necessary to stabilize folate retention during heating processes.Ghitis (1966) stated, however, that the ascorbic acid content normally found in milk was not sufficient to protect $N^5CH_3FH_4$ from degradation during boiling. The author suggested that if ascorbic acid is used to enrich processed milks then it should be added in sufficient amounts to protect the folates from oxidation during heating.

Many foods rich in folate are also rich in ascorbic acid. Liver and green vegetables are examples of such foods. However, other foods such as eggs, which are relatively rich in folates, do not contain ascorbic acid. From the experiment described herein, it is observed that ascorbic acid can protect free folate from degradation during heating. The total folate content of the food examined did not change during two hours of heating in a boiling water bath.

CONCLUSIONS

When $N^5CH_3FH_4$ is heated in the presence of oxygen, the degradation product is the dihydrofolate compound. This confirms the work of Donaldson and Keresztesy (1962) and Gupta and Hennekens (1966). The degradation of $N^5CH_3FH_4$ by heating in oxygen is delayed by the presence of ascorbic acid. The ascorbic acid acts as a reducing agent, converting the dihydro - compound back to the tetrahydro - compound.

The presence of ascorbic acid in food rich in folate is therefore of great importance if the food is to be subjected to heating. Foods which are rich in ascorbic acid may be heated for longer periods than foods which contain little or no ascorbic acid before exhibiting a decrease in folate content. If foods are to be enriched with ascorbate, then levels should be sufficient to protect the folate from oxidation.

Future studies should be directed towards the determination of the resistance of other folate compounds to food processing operations. The effects of other food constituents on the rate of folate degradation and the determination of reducing agent levels necessary to protect folates during heat processing should also be studied.

Only as a result of a better understanding of this complex vitamin can attempts be made to ensure maximum retention of the nutrient in food during processing and cooking operations.

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APPENDIX A

Replacement of Tris-NaCl Buffer with Phosphate Buffer in the Radioassay Method

Tris-NaCl buffer as supplied by Clinical Assays Inc. was replaced by 0.1M phosphate buffer (Na_2HPO_4:$NaH_2PO_4H_2O$) pH 7.3 in the radioassay method. Standard curves obtained using this method did not differ significantly as shown by the values of percent ^3H PteGlu bound for each standard used in Table A-1.

Table A-1. ^3H PteGlu bound in radioassay method using two buffer systems.

Concentration of Standard (ng/100 ul)	^3H PteGlu bound (%) (Tris/NaCl)	^3H PteGlu bound (%) (Phosphate)*
5.0	11.3	11.5
1.67	24.1	23.7
0.555	32.0	31.6
0.185	62.1	63.0
0.062	72.6	74.1

* Results for duplicate samples.

APPENDIX B

Method of Quench Correction

Liquid scintillation counting is a method of detecting radiation and is used mainly for low energy beta emitters. The method is based on the energy transfer from the ionizing particle to the solvent molecules and hence to the primary solute (fluor) which emits some of the energy as light. The light is detected by the photomultiplier tube which responds by producing a charge pulse which when amplified, is counted. It is desirable that the maximum number of emitted particles be detected. However, in liquid scintillation counting several processes may reduce the efficiency of energy transfer and thus the number of emitted particles that are detected. This is known as 'quenching'. A number of methods have been used to correct for the loss of detectable activity by counting. In this research, the channels ratio method of quench correction was utilized.

A standard curve relating counting efficiency to the ratio of the net count rates of a sample in two different channels is constructed. This is done by counting variously quenched samples containing known amounts of radioactivity in two appropriate channels. Unknown samples of the same radionucleide can then be counted in these same two channels and, by reference to the standard curve, the efficiency corresponding to the observed channels ratio is obtained. Such information is necessary in cross-comparing the counting results of samples. A series of quenched samples were prepared with compositions shown in Table B-1.

Table B-1. Composition of quenched samples.

Sample No.	Buffer (µl)	Scintiverse (ml)	^{3}H PteGlu(µl)	Chloroform (µl)
1	500	12	50	0
2	"	"	"	100
3	"	"	"	200
4	"	"	"	300
5	"	"	"	400
6	"	"	"	500

All samples were counted in the Nuclear Chicago Isocap 300 Liquid scintillation counter using the tritium program. The A/B channels ratio for each sample was automatically calculated by the counter. The counting efficiency for each of the samples was then calculated. Counting efficiency versus channels ratio was then plotted for all the samples. The plot so constructed is shown in Figure B1. From a linear regression performed on this plot it was then possible to calculate the counting efficiency for any sample given its channels ratio. The quench corrected counts per minute could then be calculated for each sample. The majority of the samples counted throughout this research had channels ratio values of .50 - .75.

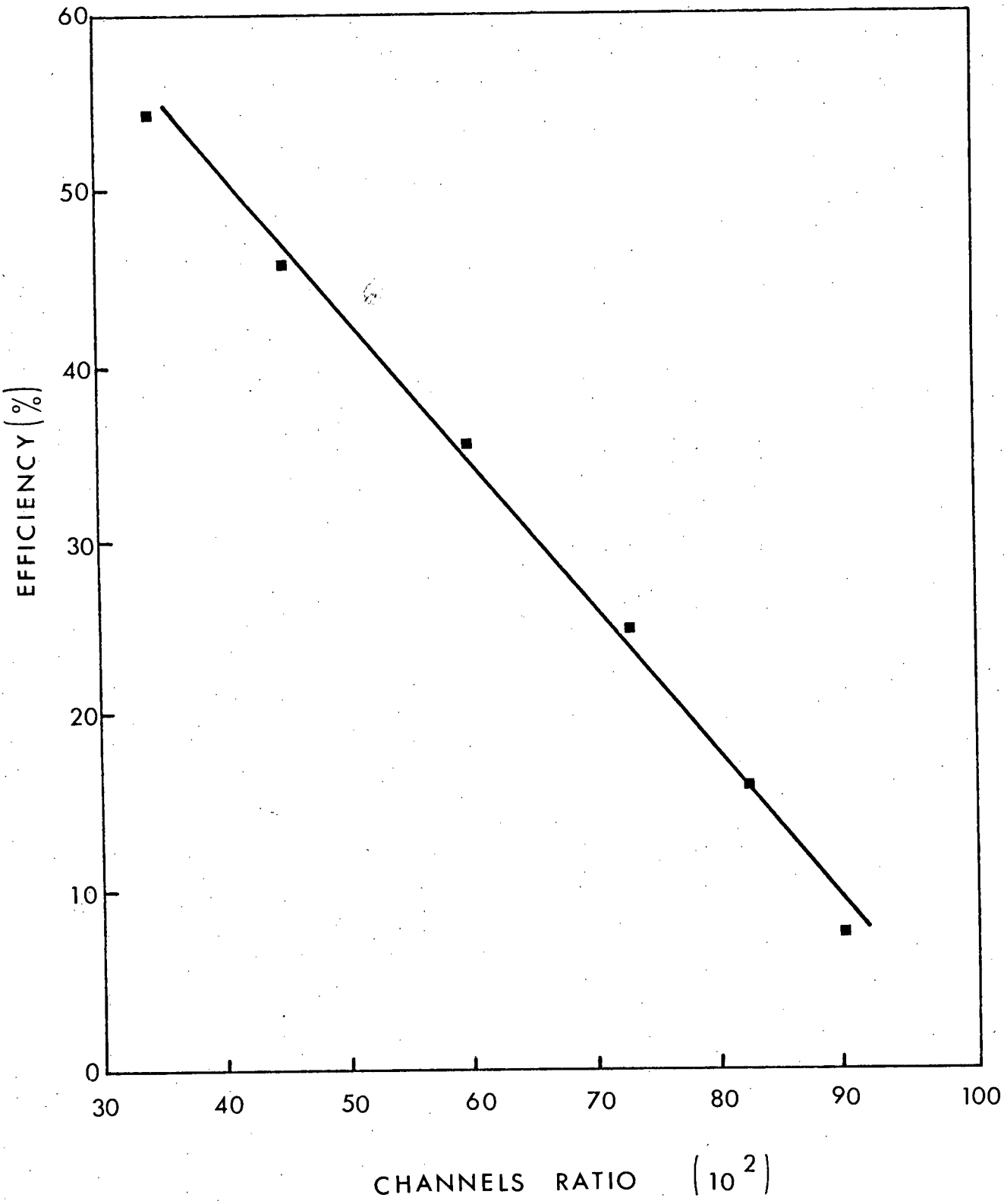

Figure B1. Quench correction curve.

APPENDIX C

Determination of Heating and Cooling Lag Times for Thermal Death Time Pouches

Thermocouples (copper/constantan, wire diameter .005mm) were used for measuring the heating and cooling lag times in the thermal death time pouches. The thermocouples were incorporated into the end seal. A Digitec 128 data logger was used for temperature recording.

Pouches (with thermocouples incorporated into the end seal) were filled with 1.5 ml 0.1M phosphate buffer and, while eliminating as much air as possible, the ends were heat sealed. Each pouch was then placed in a boiling water bath and internal pouch temperature was recorded at one second intervals using the millivolt channel of the recorder. When the internal pouch temperature reached the temperature of the boiling water bath, the pouch was removed and immediately placed in a cold waterbath. Once again, the internal pouch temperature was recorded until it reached the temperature of the water bath. Ten series of values were obtained for heating and cooling.

After conversion of millvolt readings to ^{o}C it was found that the heating and cooling lag times for pouches containing 1.5 ml 0.1M phosphate buffer were of the order of 11 seconds $\pm$ 2 seconds. Lag times of this order were regarded as negligible in the reaction under investigation.