A STUDY OF SOME CHEMICAL CHANGES OF PROTEINS OF FOODS DURING STORAGE WITH PARTICULAR REFERENCE TO LYSINE

A Thesis

by

John Harold Hare, M.Sc.

Submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

McGill University

September, 1949

ACKNOWLEDGEMENTS

The author gratefully acknowledges his indebtedness to Professor R. H. Common for his continued interest and guidance, and to Dr. R. A. Chapman, presently of the Department of National Health and Welfare, Ottawa, for his invaluable counsel during the course of this investigation. A Swift Research Fellowship was held throughout this study and was sincerely appreciated.

TABLE OF CONTENTS

GENERAL INTRODUCTION

Food dehydration has been an occupation of man from the earliest times. The ancients dried fish and meat in the sun as a means of preservation, and sun dried fruits have been familiar foods for centuries. More recently the commercial dehydration of a wide range of food products has become one of the world's major food preservation industries. Commercial food dehydration appears still to be in its infancy in spite of these recent developments.

Several advantages of drying foods to a low moisture content are obvious, such as facilitation of transportation, convenience in use, prolongation of the storage period and decrease of storage space requirement. On the other hand, a few disadvantages remain, chief of which is the decrease of palatability and nutritional quality of many dehydrated products during storage. Research during recent years has vastly improved the keeping qualities of dried foods, but the fundamental causes of many types of spoilage are by no means completely understood.

The last five years have witnessed a steadily increasing flow of researches directed to the study of protein fractions of foodstuffs. A large body of data relating to the changes undergone by the proteins in dried food has been published during this period. It is well recognized that proteins and amino acids are closely involved in food deterioration,

since many changes have been reported to occur in these food components during spoilage. However, few definite conclusions concerning the role of proteins in food deterioration have been made. The chief reason for this fact is that dried foods are complex mixtures of fats, carbohydrates, proteins, water, vitamins and other minor groups. The fat in one dried food may be the major cause of deterioration in that particular food; the protein in another; the carbohydrates in another. The safest working assumption is to assume that every component substance of a dehydrated food is contributing, by some definite chemical change or changes, to the overall deterioration of that food product. It is therefore extremely unwise to attribute food spoilage to a specific component of a food product.

It may be realized from the above discussion that the solution of the problems of dehydrated food deterioration is a most complex task and that a single investigator is unlikely to elucidate more than a few facets of the problem within a short time.

The present investigation has been concerned with the changes in the proteins of dried foods during storage. The specific aim of the work has been to examine the possible relation of these changes to deterioration as measured by taste, odor, colour, etc. The role of lysine in the deterioration process has received special attention. Several types of foods and feeds have been studied with the idea that a comparison of results secured with foods of widely different composition might throw some light on the general problem.

Part I

Studies on the Determination of Lysine

REVIEW OF LITERATURE

Isolation Procedures

The first isolation of lysine is usually ascribed to Dreschel (23). Dreschel separated this amino acid in 1889 from a casein hydrolysate. Kossel (44) and Kossel and Kutscher (45) subsequently developed a quantitative method for the separation of the basic amino acids, including lysine, from protein hydrolysates. The method included the precipitation of histidine with mercuric chloride, removal of arginine as the silver salt after saturation of the solution with barium hydroxide, the separation of lysine from the remaining amino acids by precipitation with phosphotungstic acid and its final crystallization as the picrate. This scheme has been variously modified by several investigators (99, 100, 17, 98) and microdeterminations have been suggested but the principles of the method have remained substantially the same.

Foster and Schmidt (30) proposed an electrical transport method for separation of amino acids from protein hydrolysates. This procedure is carried out in a three-compartment cell. At pH 5.5 - 5.8 the acidic amino acids migrate to the anode, the basic amino acids migrate to the cathode while the electrically neutral amino acids remain in the central compartment (53, 56, 1, 21). At pH 7.5 only arginine and lysine were transported to the cathode chamber

(29). Sperber (80) added an amberlite to the centre compartment and thereby prevented an increase of pH during electrodialysis. Arginine and histidine may then be removed from the solution in the cathode chamber as the monoflavianate and mercury salt respectively and lysine calculated from the nitrogen in the residual solution (1).

The use of ion exchange as a means of separating basic amino acids from protein hydrolysates has been proposed by Block (13). He pretreated the hydrolysate to remove excess acid and added an acid-binding resin. The treated hydrolysate was then passed through a cation exchange column which retained the basic amino acids. These were quantitatively eluted with hydrochloric acid. Freudenberg et al (31) proposed a modification of this method which included preferential elution of the amino acids after adsorption on either an acid-binding resin or a cation exchanger. Englis and Feiss (26) have investigated some of the factors governing exchange of amino acids on organic and other ion exchange substances. These authors present evidence suggesting that adsorption proceeds essentially by salt formation. A similar study was carried out by Cleaver et al (20) who investigated the effect of type of resin, particle size, rate of flow, concentration of amino acid in solution, pH of the solution and length of the adsorbent column.

A quantitative method has been developed in which Lloyd's reagent was used for the separation of lysine from

protein hydrolysates (11). The non-basic amino acids were washed from the column with 0.5 N hydrochloric acid and the basic amino acids were then removed from the column by preferential elution with 1N hydrochloric acid, 0.125M sodium bicarbonate and pyridine. The authors reported quantitative recovery of lysine from the 1N hydrochloric acid eluate using a modified ninhydrin procedure for the determination of lysine. Nelson (57) proposed a method using a Decalso column on which only arginine and lysine remained after elution with a large volume of ten per cent pyridine. These amino acids were subsequently washed out with ten per cent hydrochloric acid.

Dakin (22) proposed a method for separating protein hydrolysates into five groups characterized by their solubilities in butyl alcohol, ethyl alcohol and water. The five groups are as follows:

1. Monoaminomonocarboxylic acids, both aliphatic and aromatic, insoluble in ethyl alcohol but extracted with butyl alcohol.

2. Proline, soluble in absolute ethyl alcohol and extracted with butyl alcohol.

3. Peptide anhydrides, extracted by butyl alcohol, but sparingly soluble in alcohol or water.

4. Dicarboxylic acids, not extracted by butyl alcohol.

5. Diamino acids, not extracted by butyl alcohol but separated from group four by any of the basic amino acid

precipitants.

The advantages of the above procedure lie in the fact that an almost quantitative separation of the groups occurs, while the amino acids are obtained in an un-racemized condition.

Chemical Methods (Lysine)

During the first decade of the twentieth century most of the methods for determining amino acids were gravimetric, the amino acid being precipitated as a salt and the weight calculated in terms of the amino acid (44, 45). Modifications of the gravimetric procedures have since been proposed (98, 99, 17, 12, 90). Lysine was generally determined as the picrate.

In 1911 Van Slyke drew attention to the fact that the methods available for the determination of amino acids in protein hydrolysates were very lengthy and required considerable amounts of protein. He consequently devised his well known nitrogen distribution method (92) for the basic amino acids. The method is based on the fact that phosphotungstic acid precipitates, prepared from ammonia- and humin-free hydrolysates contain all the cystine, arginine, histidine and lysine. Cystine nitrogen may be calculated from the total sulfur in this precipitate. Arginine nitrogen may be calculated from the amount of ammonia liberated by concentrated alkali less eighteen per cent of the cystine nitrogen, since cystine liberates this proportion of its nitrogen

under the same conditions. Histidine nitrogen was calculated from the total non-amino nitrogen of the precipitate minus the arginine non-amino nitrogen. Lysine nitrogen was then calculated from the total nitrogen minus the cystine, arginine and histidine nitrogen. The method has been shown to be of value only where nitrogenous impurities are absent (3).

Albanese (1) determined the arginine and histidine of a phosphotungstic precipitate by colorimetric methods and then calculated lysine by difference using the microkjeldahl technique.

A definite relationship between the free amino groups and the lysine content of proteins has been demonstrated (75, 93). Lieben and Loo (51) have made use of this fact in the estimation of the lysine content of the unhydrolysed protein. These authors determined the quantity of amino nitrogen released from proteins by nitrous acid in the Van Slyke apparatus after thirty, sixty and ninety minutes. The amino nitrogen released in thirty minutes is produced from the ϵ-amino group of lysine plus other amino groups with which nitrous acid reacts. These other amino groups represent amino groups liberated by the action of the acetic acid on the intact protein, the rate at which these groups are liberated falling off uniformly over the first hour and a half. By plotting the difference in amino nitrogen between thirty and sixty minutes and between sixty and ninety minutes, and extrapolating to zero time, one finds the

correction which must be subtracted from the thirty minute value in order to derive the lysine ϵ-amino nitrogen. Lieben and Loo secured results in very close agreement to those already reported in the literature for the lysine contents of casein, gelatin, gliadin and zein.

The ninhydrin reaction was first reported in 1911 (68). In this reaction, free amino acids are decomposed to yield ammonia, carbon dioxide and an aldehyde. Van Slyke et al (94, 95, 96) have devised a quantitative method for the determination of lysine utilizing the ninhydrin reaction. Lysine is precipitated from the protein hydrolysate by phospho-24-tungstic acid. After removal of the reagents, the quantity of lysine is determined by the formula:

Lysine nitrogen = 2(Amino nitrogen - carboxyl nitrogen)

The amino nitrogen is determined by the nitrous acid method and carboxyl nitrogen determined by the ninhydrin procedure. In this latter method, the carbon dioxide evolved by ninhydrin is measured either manometrically or by titration procedures. Other workers have modified the method somewhat (19, 54). The colorimetric application of the ninhydrin reaction has not been altogether successful due to differences in colour produced by slight impurities (58, 62).

A new colorimetric procedure has recently been developed (57, 14) in which lysine is converted by a halogen into a reducing compound. This reducing compound can be quantitatively estimated by means of Folin's phenol reagent (28).

Chlorine was shown to be a better reagent than bromine for the reaction with lysine (14). The reaction may be similar to that suggested by Langheld (48, 49) and studied by several investigators since that time (16, 60, 105). More recently a method (2) has been proposed for the determination of alanine, valine and leucine using hypochlorites. The aldehyde formed by the reaction of the hypochlorite and amino acid is trapped in bisulfite and determined by titration. It would seem, however, that the methods of Nelson (57) and Boulet (14) are the only available procedures for the determination of lysine which are based on a colorimetric determination of the products formed by chlorination of the lysine after its isolation from other amino acids.

Microbiological Assay

Microbiologists have studied the dietary requirements of microorganisms for several decades, but it was not until 1939 that the use of microorganisms for biological assay of vitamins became a practical possibility. The development of such methods became a reality only when it became possible to devise media deficient in the single nutrient substance under assay and yet adequate for growth in all other respects. Such media could not be developed until the requirements for many specific nutrients were known and until suitable sources of these nutrients became readily available. The first application of the above procedure was a microbiological method for the determination of riboflavin devised by Snell and

Strong (77). The response of the organism to additions of riboflavin was measured by the amount of acid which it produced or by its growth as measured by turbidimetry. Very shortly thereafter microbiological methods for other vitamins were published (61, 79) and methods for the determination of amino acids in protein hydrolysates by this new and accurate technique were rapidly developed (46, 47, 24). A medium for the determination of the ten essential amino acids has recently been devised (84).

Lysine has been determined microbiologically by the use of five organisms:

1. Lactobacillus arabinosus (64, 47).
2. Streptococcus faecalis (64, 84, 8).
3. Leuconostoc mesenteroides P-60 (4, 5, 38, 64, 24, 40, 6). A micro method using this organism has also been described recently (55).
4. Streptococcus lactis R (7).
5. Neurospora crassa (63, 69).

Neurospora crassa was suggested for use with unhydrolysed proteins because this organism is itself capable of hydrolysing the proteins; however its use without preliminary hydrolysis has not been perfected. Recently (43) it has been shown that lysine is required for the protozoan Tetrahymena gelii and that bound as well as free lysine may be utilized. Other microorganisms have been shown to utilize the unhydrolysed proteins (63, 69). Realizing that hydrolysis

of proteins is inconvenient and possibly may destroy some of the amino acids, Rockland and Dunn (67) investigated the lysine and other amino acid requirements of T. gelii. While pH measurements and titratable acidity curves showed little promise as a means of checking growth response, the turbidimetric method was found to be more favourable. As yet, however, the microbiological determination of lysine on unhydrolysed proteins has not been placed on a satisfactory quantitative basis.

Media containing small amounts of one amino acid in the presence of a great excess of a related acid are often unsatisfactory for assay purposes. Typical "imbalances" have been observed between serine and threonine (41), aspartic and glutamic acids (7), norleucine and methionine (39), valine and leucine (73), phenylalanine and tyrosine (73), and glycine and alanine (76). Steele et al (83) studied the effect of the ratios of the amino acids as they occur in hydrolysed casein on the growth curves of Leuconostoc mesenteroides P-60 and Leuconostoc citrovorum 8081. From their results they developed a medium which could be applied to the determination of eighteen amino acids using the above microorganisms.

The preferential destruction or removal of certain amino acids from protein hydrolysates, or from partially hydrolysed protein material such as peptone, offers the possibility of developing media for amino acid assays which are less expensive and simpler to prepare than media which contain from

fifteen to twenty different pure amino acids. Hydrolysed casein (79, 5, 4, 70) and peptone (78) have long been a source of amino acids for the media used in the microbiological determination of vitamins and some of the amino acids. Lyman et al (52) proposed using peptone treated with hydrogen peroxide as part of the basal media, since the treated peptone is lacking in methionine, cystine, tryptophane and tyrosine.

A part of the work described in the present thesis is concerned with the use of hydrolyzed zein as a source of amino acids in media used for the determination of lysine by the growth response of Leuconostoc mesenteroides P-60. So far as the author is aware, the use of a protein naturally deficient in a particular amino acid has not previously been applied to microbiological assay of amino acids. Zein has been suggested, however, as a protein source in nutrition studies where lysine is not required in the diet (59). The use of acid hydrolysates of casein for the microbiological assay of tryptophane is, of course, well known, but in this case the deficiency of tryptophane is a result of the acid treatment and not a consequence of an original deficiency of the amino acid.

Enzymatic Methods

The decarboxylation of lysine by Bacillus coli and estimation of the cadaverine produced (101) was the first enzymatic procedure to be proposed for the determination of

this amino acid. More recently Gale and Epps (35) described a coliform organism, Bacterium cadaveris 6578, which specifically decarboxylated l-lysine and gave a quantitative yield of carbon dioxide. They suggested its use for lysine determination. Zittle and Eldred (106) studied the above principle and have furnished a quantitative method for the determination of this amino acid by measuring the carbon dioxide produced by the specific enzyme. The accuracy of the method has been substantiated by Neuberger (59) and Gale and Epps (36, 37) and others (25). The method has been improved and applied to the estimation of a variety of amino acids by the manometric procedure (27, 85, 37).

Isotope Dilution Methods

A modern method of increasing importance for estimation of amino acids in protein hydrolysates makes use of the isotope technique and has been shown to allow an error within one to two per cent (72, 65, 91). This isotope dilution method is based on the fact that a compound which has an abnormal isotope content is inseparable from its normal analogue by the usual laboratory procedures. The chief operations after hydrolysis of the proteins are: i. Addition to the hydrolysate of a known amount of isotopic dl-amino acid whose N^{15} content has been determined. ii. Isolation from the hydrolysate a portion of the amino acid under investigation in a high state of purity. iii. Determination

of the excess of N^{15} in the isolated sample, from which the quantity of amino acid of the original material may be calculated.

The advantages of the method are: i. that the error of a determination is independent of any errors in the method of isolation or yield and ii. that the error is independent of the concentration of the amino acid under investigation. The method has been applied to the estimation of lysine in several proteins (29, 74).

EXPERIMENTAL

Chemical Determination of Lysine

The colorimetric procedure of Boulet, Nelson and McFarlane (15) has been followed in this investigation for the determination of lysine in stored foods and feeds. This method was developed by Nelson (57) and modified by Boulet (14). It is a process involving the isolation of arginine and lysine on an ion exchange column, after hydrolysis of the protein, followed by the elution of these two amino acids. A chlorine solution is added to the eluate and the resultant reducing compound formed by the lysine is measured by means of Folin's phenol reagent.

Description of Method.

Reagents:- 1. Decalso - Permutit Co. N. Y., + 40 - 60 mesh - Sodium form of decalso - water softener.

2. Sodium chloride solution - 3 gms. NaCl (Reagent) per 100 ml. water.

3. Pyridine Solution - 100 ml. of redistilled pyridine B. P. 114 - 115°C. Diluted to 1 litre with water. Allow to stand a few hours before using to permit escape of air bubbles.

4. Sodiumcarbonate solution - (0.2M) 2.12 grams Na_2CO_3 (Reagent) per 100 ml. water.

5. 2.0 N HCl.

6. Chlorine solution - Electrolytic

Chlorine gas from a cylinder or chlorine gas prepared by adding three grams manganese dioxide to 12 ml. concentrated hydrochloric acid at a temperature below 20°C, is bubbled through a twenty per cent solution of barium chloride into 50 ml. of 0.2 N HCl for about twenty minutes. This solution should be used within one hour of its preparation.

7. Sodium arsenite - 5 grams of sodium arsenite in 100 ml. water - prepare a fresh sample each week.

8. Mixed alkali - 2 grams sodium citrate (reagent) + 4 grams sodium phosphate dodecahydrate (reagent) per 100 ml. water.

9. Phenol reagent - Prepared according to Folin and Ciocalteu (28) but with 100 grams of lithium sulfate monohydrate instead of 150 grams per litre. Dilute 3:10 before using.

Adsorption column:- 700 mg. Decalso is transferred to a beaker and washed by decantation three times with three per cent sodium chloride and then washed with water until free of chloride. The adsorption apparatus consists of a three-inch longstem pyrex funnel which has been constricted at the outlet. A small pad of cotton is inserted in the bottom, 25 ml. of water is poured into the funnel and the adsorbent, suspended in water is immediately added. By tapping the funnel gently and air-free column about 10 cm. long is obtained. A thin pad of cotton is placed on top of the column and the excess water is poured out of the funnel by tilting. The column is washed with 5 ml. of ten per cent

pyridine solution and is ready for use. If the amino acid solution is not added without delay the column must be kept under liquid.

Hydrolysis:- A sample of protein containing 2 - 15 mg. lysine is refluxed with 10 ml. 6 N hydrochloric acid for twenty-four hours. The hydrolysate is evaporated *in vacuo* to dryness. Five ml. of water is added and this is taken to dryness again. The residue is taken up in exactly 10 ml. of ten per cent pyridine and the suspension centrifuged.

Adsorption:- One ml. of the amino acid solution containing 0.3 - 1.5 mg. of lysine is passed through the column until a depth of about 1 mm. of solution remains above the surface of the adsorbent and three 1 ml. aliquots of ten per cent pyridine are added successively to wash all the lysine into the Decalso. The column is then washed with an additional 100 ml. of ten per cent pyridine and the pyridine washed out of the column with 4 ml. of distilled water. The filtrates are discarded and the lysine and arginine eluted with 20 ml. 0.2 M sodium carbonate solution, the eluate being collected in a 50 ml. volumetric flask. A small piece of indicator paper is added to the contents of the flask, the hydrogen ion concentration adjusted to pH 3 - 4 with 2 N hydrochloric acid and the solution diluted to volume with water.

Chlorination and colorimetry:- A 1 ml. aliquot of the lysine solution containing five to thirty micrograms of

lysine is carefully measured into a 10 ml. graduated glass-stoppered Pyrex cylinder and 1 ml. of the chlorine solution is added. After allowing to stand for five minutes, 1 ml. of the sodium arsenite solution is added. The mixed alkali solution is then added from a microburette in amount sufficient to adjust the solution so that the pH after addition of the phenol reagent will be 6.2 - 6.5--usually 1.5 ml. is required. The solution is diluted to 9 ml. with distilled water, mixed, and the cylinder placed in a water bath at a temperature of at least 90°C but not boiling. After a few minutes 1 ml. of the phenol reagent diluted 3:10 is added from a slow delivery pipette (about 1 ml. in fifteen seconds), the liquid being allowed to flow down the wall of the cylinder. The heating is continued for forty minutes and the solution cooled to room temperature. It is important that the phenol reagent be added in the manner indicated without removing the cylinder from the bath and without mechanical mixing.

The colour intensity is measured with an Evelyn photoelectric colorimeter using a 660 mμ filter. A reagent blank prepared by carrying out the chlorination and colorimetry with the reagents alone is used to obtain the "centre setting" of the colorimeter prior to reading the test solution. The lysine content of the solution may be obtained by reference to a calibration curve prepared from readings obtained with standard solutions of l(+)-lysine monochloride, containing

0.5 - 3 mgm. lysine per 100 ml., which had been carried through the procedure, including the adsorption on Decalso. The most reliable procedure is to carry out simultaneously a control determination with a standard solution containing approximately the same amount of lysine as that contained in the protein hydrolysate. From the reading with the standard solution Beer's law constant K is calculated from the relation $K = \frac{2 - \log G}{C}$ where C is the concentration of lysine in micrograms per ml. of standard solution and G is the galvanometer reading. The intensity of the blue colour obeys Beer's law over a range of concentrations of one to three micrograms of lysine per ml. but deviates slightly at lower concentrations.

A standard curve using Boulet's procedure is shown in Figure 1. The method has been used on several proteins and good results were obtained. To the author's knowledge, the method has been applied to a food product in only one instance (14).

In the present investigation this procedure has given fairly good results for the determination of lysine in food products. However it was noted in initial experiments, that even when the purified chlorine solution was used, slightly different values were obtained for the same standard lysine solutions carried out at different times. It was thought that slight differences in pH of the final solution were the cause of the variation. This was substantiated by adding small

quantities of acid or alkali to the chlorinated lysine solution prior to the addition of the phenol reagent. Consequently, precise measurement of the pH must be carried out in the colorimetric procedure.

Modification of the chemical procedure.

Use of hypochlorites:- The preparation of pure chlorine as required in Boulet's method was not only inconvenient but time-consuming and often caused unpleasant odours in the laboratory. Since a relatively large proportion of free chlorine is available in solutions of hypochlorites, it was thought that calcium or sodium hypochlorite might effectively replace the chlorine solution of the original procedure. Calcium hypochlorite was used initially since the solid material has greater stability and is more readily available than sodium hypochlorite. The method was similar to the original procedure with the exception that 1 gm. of calcium hypochlorite, dissolved in 50 ml. 0.2 N hydrochloric acid replaced the chlorine solution. A calibration curve, using standard lysine solutions, was prepared and yielded a straight line (Figure 1) but due to the presence of calcium ions in solution, a precipitate formed on the addition of sodium arsenite. It was necessary to allow this precipitate to settle in the colorimetry tubes before taking a reading.

It is of interest to note that the original colorimetric procedure devised by Nelson (57) gave a precipitate which was allowed to settle before the colorimetry was

carried out.

Sodium hypochlorite would appear to overcome the difficulty of the formation of a precipitate since the resultant sodium arsenite should be soluble. Therefore the same procedure was repeated using sodium hypochlorite in acid solution to replace the chlorine. The method was similar to the original procedure with the exception of the chlorination and colorimetry.

One ml. of the lysine solution (from the adsorption column) is transferred to a 10 ml. graduated cylinder. A similar amount is added to a separate test tube for pH calculation. 0.5 ml. of the sodium hypochlorite solution, prepared by adding 10 ml. sodium hypochlorite (five per cent available chlorine) to 1.5 ml. 6 N hydrochloric acid, was added to both the cylinder and test tube and allowed to react for five minutes. One ml. of the sodium arsenite is then added to both tubes. To the sample in the test tube, 1 ml. of the phenol reagent (diluted 3:10 with water) is added. The quantity of mixed alkali (prepared as directed in the original method) required to adjust the pH to 6.3 is determined by the use of the Beckmann pH meter. This amount of mixed alkali (generally about 2 ml.) is then added to the twin sample in the 10 ml. graduate cylinder and the volume made up to 9 ml. with distilled water. The contents are well mixed by shaking and the cylinder placed in a water bath above 90°C but below 100°C for a few minutes until the

liquid has reached the temperature of the bath. Then 1 ml. of the phenol reagent (diluted 3:10 with water) is added from a slow-delivery pipette. The solution is kept at the same temperature for forty minutes and cooled to room temperature. The colour intensity is measured as previously, on the Evelyn photoelectric colorimeter, using a 660 mμ filter. A calibration curve for lysine obtained by the use of the sodium hypochlorite method is shown in Figure 1.

Effect of hypochlorite concentration:- In order to determine the effect of concentration of available chlorine on the reaction with lysine, the sodium hypochlorite solution was diluted to give four solutions as follows: 5, 2.5, 1.25, and 0.675 per cent available chlorine. 0.5 ml. of each solution was added to 1 ml. samples of lysine solution containing ten and thirty micrograms per ml. The colorimetric procedure was carried through and the quantity of lysine calculated in each sample. Results of the experiment are shown in Table I.

The modified procedure was used to determine the lysine content of the foodstuffs after six, twelve and eighteen months storage. Similar determinations were conducted by means of microbiological assay from which a comparison of the two methods was made. The results are shown in Tables VI and VII.

Microbiological Determination of Lysine

The microbiological assay of lysine has been carried out by the use of several microorganisms, most promising of which were Leuconostoc mesenteroides P-60 (4, 64), Streptococcus faecalis R (8, 64) and Lactobacillus orabinosus (47, 64). In order to determine the most effective organism for lysine assay, standard curves using each of the above bacteria were prepared. The basal medium for L. mesenteroides is shown in Table II. Similar media were used for the other organisms with the exception that sodium acetate and ammonium chloride were omitted in the case of Strep. faecalis. The standard curves using each microorganism are shown in Figure 2 from which it may be seen that the method utilizing Leuconostoc mesenteroides produced the steepest and straightest curve. This microorganism was therefore used throughout the investigation.

The method followed was similar to that of Barton-Wright (5) but was modified slightly to overcome minor difficulties observed in preliminary investigations.

Description of method.

Organism:- Stock cultures of Leuconostoc mesenteroides P-60 were carried on a yeast-water-glucose agar, to which one per cent of sodium acetate (hydrated) had been added. The cultures were preserved in the refrigerator at 5° and renewed every two weeks.

Composition of the Basal Medium:- The basal medium was slightly modified from that of Barton-Wright (5) and was prepared as shown in Table II. Glass-distilled water was used throughout the procedure.

The vitamins, except biotin and folic acid, were made up in a solution ten times their required concentration, to facilitate weighing, and a tenth of the solution was used for the assay. Biotin and folic acid solutions were made up separately and stored in the refrigerator; biotin at a concentration of one microgram per ml. of water and folic acid at two micrograms per ml. of fifty per cent ethyl alcohol.

When dissolved, all of the ingredients were added together and the volume made up to about 350 ml. The pH was adjusted to 6.8 with alkali and the solution finally diluted to 500 ml. This gave sufficient medium for 100 assay tubes at 5 ml. each.

Preparation of inoculum:- A transfer was made from a stock culture of Leuconostoc mesenteroides P-60 to a similar medium from which the agar had been omitted. This was incubated for eight to twelve hours at 35°C or until noticeable growth was observed. The cells were centrifuged aseptically for a few minutes and taken up in 10 ml. of sterile 0.9 per cent saline solution. One drop of this inoculum was added to each tube in the subsequent assay procedure.

Preparation of hydrolysates:- The material to be analysed, usually a 1 gm. sample, was added to a 20 x 150 mm.

test tube along with 25 ml. of 6 N hydrochloric acid. The tube was sealed in an oxygen flame and the substance hydrolysed by autoclaving for ten hours at fifteen pounds pressure. The hydrolysate was then cooled, broken out of the test tube and 2 ml. of 2.5 M sodium acetate was added. The pH was adjusted to 4 - 4.5 with sodium hydroxide and the solution made up to 100 ml. An aliquot was filtered, the filtrate adjusted to pH 6.8 with more sodium hydroxide and diluted to a suitable volume. The concentration of lysine in this final dilution should be in the neighbourhood of forty micrograms per ml.

Standard lysine solution:- A stock solution was prepared by dissolving 100 mg. of dl-lysine monohydrochloride (the d-enantiomorph is inactive toward L.mesenteroides) in 100 ml. of glass-distilled water. For a working solution, an aliquot of the stock solution was diluted ten times, and thus contained forty micrograms of l-lysine per ml.

Assay procedure:- Five ml. of the basal medium is transferred to each tube (20 x 150 mm.). Both the standard lysine solution and the hydrolysate were assayed in triplicate at six levels of concentration. This was obtained by adding from zero to 5 ml. of the standard or the hydrolysate solution to the tubes of basal medium. Thus eighteen tubes were used to assay the hydrolysate and eighteen for computing the standard curve. A reciprocal volume of water was added to bring the total volume of liquid in each tube to 10 ml. It

was found convenient to use aluminum test-tube racks as shown in Figure 3. These racks were advantageous in that a lid (shown in the picture), whose under surface was covered with a quarter-inch sheet of absorbent cotton, could be placed conveniently over all the tubes, thus dispensing with cotton plugs. The racks also facilitated handling, sterilization and inoculation of each set of tubes.

When the unknown and standard lysine solutions had been added and the volumes in each tube diluted to 10 ml., the cover was placed on the tubes. The complete rack was placed in a large size domestic pressure cooker and autoclaved at fifteen pounds pressure for ten minutes. The tubes were cooled to about 37°C in a cold water bath and the racks placed in a constant temperature room previously sterilized by ultra-violet radiation. The lid of the rack was removed, one drop of the inoculum-saline solution was added to each tube from a sterile micro-pipette and the lid quickly replaced. To ensure that the inoculum penetrated the sample, the racks were shaken in a horizontal direction for a few seconds. Incubation was at 35°C for three days. At the end of this time the acid produced by the organism was determined by titration with 0.1 N sodium hydroxide to pH 7.0 using a Beckman pH meter with glass electrode.

The volume of alkali required in titration of the standard tubes is plotted against the lysine concentration. A straight curve was observed for lysine in the range of forty to 200 micrograms per ml. From this standard curve, the

lysine content of each concentration level of the hydrolysate was determined. For reliable results less than five per cent variation between the concentration levels should be obtained.

It was obvious that to carry out the assays of lysine on the stored foods by the standard microbiological method, considerable quantities of the pure amino acids would be required. Since many of these pure substances are extremely costly, the expense of the method was found to be prohibitive. Therefore, it was thought wise to investigate the possibility of a less expensive substitute. The use of hydrolysed zein, having been proved satisfactory (see page 42) was consequently adopted for the determination of lysine in the food products. An occasional check, using the pure amino acids in the medium was carried out to determine the accuracy of the zein method. It was estimated that the cost of the amino acid portion of the medium was decreased from one dollar and eighty-four cents per 100 tubes to less than six cents* by the use of the hydrolysed zein.

Microbiological Assay of Lysine Using Hydrolysed Zein as a Source of Amino Acids

Introduction.

The fact that zein, due to its lack of lysine, might be utilized in nutritional studies where lysine is not desired in the diet (59), has suggested a means whereby the

---

* Calculated from present prices - Nutritional Biochemicals Corporation.

microbiological assay of this amino acid may be simplified and reduced in cost. Acid-hydrolysed zein, supplied with added tryptophane was found to be a good source of amino acids, yet without the addition of lysine would not support the growth of Leuconostoc mesenteroides. Table III has been constructed from values taken from the literature and shows the amino acid composition of zein. No values have been observed for either norvaline or norleucine, although these amino acids have been shown to be unnecessary for Leuconostoc mesenteroides (24). Zein is almost entirely lacking in tryptophane and with the exception of one case (106), completely deficient in lysine. Therefore it was necessary to add tryptophane to the hydrolysed zein since this amino acid is a definite requirement for the micro-organism (83). Glycine is similarily absent but some doubt still remains as to its necessity for growth of L. mesenteroides.

According to the requirements of the Barton-Wright (5) basal medium, zein is deficient in threonine, cystine, arginine and aspartic acid. On the other hand, it may be observed that glutamic acid and leucine comprise a relatively large proportion of the amino acid composition of zein. It was considered possible that these discrepancies might affect the normal utilization of other amino acids in the medium, thereby introducing errors. Therefore it was necessary to establish the optimum conditions for growth of

Leuconostoc mesenteroides when hydrolysed zein was used as the source of amino acids in the basal medium.

Effect of quantity of zein used.

An inspection of the medium of Barton-Wright (Table II) shows that the amino acid portion of the medium weighs approximately five grams. Almost one half of this is in the form of the non-usable d-enantiomorph so that the weight of the l-forms would be in the neighbourhood of three grams. The effect of using excessive amounts of zein could perceivably affect the amino acid "imbalances" (83), whereas a deficiency of the hydrolysed protein would not yield sufficient of the essential amino acids to support growth of the microorganism. Consequently the optimum quantity of zein to use per 100 tubes was determined as follows:

One, three and five grams of zein were hydrolysed for ten hours at fifteen pounds pressure with fifteen, thirty and fifty ml. respectively of 6 N hydrochloric acid. Each hydrolysate was filtered, neutralized and substituted for the amino acids of the normal basal medium. One hundred mg. of tryptophane was added in each case. The media were adjusted to pH 6.8 as in the usual method and diluted to 500 ml. Standard curves, using solutions of l-lysine, were determined for the three different media. These curves are shown in Figure 4.

Effect of strength of acid and length of hydrolysis of zein.

Preliminary work had revealed that if the zein was not completely hydrolysed, unusual results (Figure 5) were obtained for the lysine standard curve, a decided acceleration of growth of the organism being observed at the lower levels. Similar observations have been reported by Sprince and Woolley (81, 82) and others (89, 102) who have suggested that a peptide-like substance, termed strepogenin, is responsible for this phenomenon. The literature does not reveal evidence for the presence or absence of strepogenin in zein, but its occurrence is a possibility. Consequently, the method of hydrolysis of zein becomes extremely important in this microbiological procedure.

Five gram samples of zein were hydrolysed for ten hours at fifteen pounds pressure with 50 ml. portions of hydrochloric acid of the following normalities: (a) 0.1 (b) 0.5 (c) 2.0 (d) 6.0. The zein was then incorporated into basal media and the lysine standard curves were determined on each medium. Similarily the effect of the length of hydrolysis using 6 N hydrochloric acid was determined by autoclaving the zein for one, four, seven and ten hours at fifteen pounds pressure. The results of these two experiments are depicted graphically in Figures 6 and 7.

During this investigation it was observed that the samples containing the partially hydrolysed zein showed a much earlier growth of the organism (increase in turbidity)

during the three day incubation period than those containing the completely hydrolysed protein. To obtain a quantitative measurement of this growth, the turbidity of the samples containing the highest level of lysine per tube was measured at twelve hour intervals during the incubation period. These measurements were accomplished by means of an Evelyn photo-electric colorimeter using a 620 mμ filter. The results are shown in Figures 8 and 9.

Effect of salt concentration.

It is known that a relatively high salt concentration markedly affects the growth of microorganisms (24). Due to the fact that 6 N hydrochloric acid was to be used for the hydrolysis of zein, a high concentration of salt would result when the hydrolysate was neutralized with sodium hydroxide. Consequently, it was necessary to determine the effect of this and of added salt on the growth of L. mesenteroides.

Basal media containing hydrolysed zein as the amino acid source, were made up from which the sodium chloride, as required by Barton-Wright's media (Table II) was omitted. The medium was then divided into five equal parts and sodium chloride was added at the proportion of 0, 2.5, 5.0, 10.0 and 25 grams per 100 tubes. Again, lysine standard curves were determined and the results are shown in Figure 10.

Effect of the addition of other amino acids.

Those amino acids in which zein is deficient:-

It may be observed from Table III that some of the amino acids required for the growth of Leuconostoc mesenteroides P-60 are not supplied in sufficient quantity, by 5 gm. of zein, to meet the requirements of Barton-Wright's medium. These include arginine, aspartic acid, glycine and cystine. It is also possible that some of the cystine may be lost on acid hydrolysis. Since several of these amino acids may be limiting factors for the growth of L. mesenteroides it was considered wise to investigate the effect of the addition of these compounds on the lysine standard curve. The method used was similar to previous procedures. The amino acid in question was added to the zein medium at the same proportion as listed in Table II. Tryptophane was added in each case. Standard curves obtained by the above procedure are shown in Figure 11.

Those amino acids of which zein contains an excess:- Zein contains relatively large proportions of both glutamic acid (25 - 35 per cent) and leucine (20 - 25 per cent). Due to this fact it is possible that the availability or activity of some of the other amino acids might be affected (83). Consequently the effect of large excesses of these two amino acids on the lysine standard curve was investigated.

The complete medium of Barton-Wright (Table II) was made up with the exception of glutamic acid, leucine and lysine. For the effect of excesses of glutamic, leucine was added to the medium at the normal rate and vice versa. To three equal portions of the glutamic acid-free medium, this

amino acid was added at three levels: (a) 500 mg., (b) 1,000 mg., and (c) 1,500 mg. per 100 tubes. To three equal portions of the leucine-free medium this amino acid was also added at three levels: (a) 500, (b) 1,000, (c) 1,500 mg. per 100 tubes. The lysine standard curves were determined in a manner similar to the previous methods. The results are depicted in Figures 12 and 13.

Histidine:- From preliminary experiments it had been shown that the amounts of several other amino acids affected the position of the standard curve. Of these, the most noticeable differences were due to the presence of histidine which gave added impetus to the growth of L. mesenteroides. A quantitative experiment, similar in nature to those carried out with leucine and glutamic acid was undertaken. Levels of histidine studied were 100 (normal amount), 250, 500, 750 and 1,000 mg. per 100 tubes. The curves using standard lysine solutions were determined as in previous tests and are shown in Figure 14.

Determination and recovery of lysine from casein and casein hydrolysate.

The standard lysine curve prepared by the use of the original Barton-Wright procedure was compared with the standard curve prepared by using hydrolysed zein as the amino acid source. Figure 15 shows the similarity of the curves.

The lysine content of casein* was determined using the

---

*Casein - Fat and Vitamin-Free. British Drug Houses, London.

zein method and the results are reported in Table IV.

Lysine recovery was also calculated both on whole casein and casein hydrolysate by adding a known amount of lysine to these products and estimating the total lysine. The reason for the two procedures was to determine the effect of hydrolysis of the protein on the recovery of lysine. Table V shows the results.

Lysine determinations of the stored foods and feedstuffs were carried out by the modified microbiological procedure. The results of the assay of soybeans, meat meal, oats and dehydrated potatoes are shown in Table VI. Only those samples stored at 37°C and under the high humidity conditions were investigated in the case of the above four products. However lysine was determined on both the high- and low-humidity samples of the milk powders at 27° and 37° storage. The results are shown in Table VI.

Included in the above tables are the lysine values on the stored foods as determined by the chemical procedure outlined previously so that a comparison of the two methods may be made.

DISCUSSION OF RESULTS

Chemical Determination of Lysine

The curves of Figure 1 indicate that the method of chlorinating lysine by means of sodium hypochlorite is as satisfactory as that where free chlorine is used. Evidently a aimilar reaction occurs between the hypochlorite solution and lysine as between chlorine solution and lysine, otherwise a much greater variation of the curves would be expected.

Boulet (15) did not attempt to determine the type of reaction which occurred between lysine and chlorine solutions. Langheld (48, 49) suggested that, in alkaline solution, an aldehyde with one less carbon atom was formed by the interaction of an amino acid and chlorine. Wright (105) considered the reaction of hypochlorites on amino acids to depend on the relative proportions of active chlorine and amino acid present; with excess chlorine it was regarded as an oxidation process; with excess amino acid it was regarded as a chlorination. Norman (60) has studied the reaction and suggested that chlorination takes place in acid solution, while oxidation occurs in alkaline solution when hypochlorites are reacted with amino acids. As yet however, the reaction is not satisfactorily explained.

It was found in this investigation that when lysine was mixed with sodium hypochlorite a positive reaction was obtained with Schiff's reagent. This would appear to indicate that even at pH 6.3 some aldehyde may be formed from

lysine.

The calibration curve using calcium hypochlorite (Figure 1) was always somewhat lower than those when chlorine solution or sodium hypochlorite were used. This would indicate that the precipitate in the colorimeter tube, affected the optical density to some degree. As the densities were higher in value it is probable that some of the precipitate did not settle out. Filtration or centrifugation did not affect the colorimeter readings whatsoever.

The concentration of hypochlorite had little effect on the lysine-chlorine interaction as may be seen in Table I. This would suggest that excesses of free chlorine are of no objection in the process. This conclusion was similar to that of Boulet (14) who found that the amount of chlorine solution added to lysine had little or no effect on the reaction. Thus the only precaution necessary in the procedure is to ensure that the excess chlorine is removed before applying colorimetry.

Temperatures within the range of 10 - 30°C had little effect on the reaction between lysine and the chlorine agent. Similarily, the length of time the compounds were allowed to react did not appreciably affect the amount of reducing material produced.

Microbiological Assay of Lysine Using Hydrolysed Zein as a Source of Amino Acids

Quantity of zein used.

Figure 4 reveals that three grams of zein per 100 tubes could be utilized satisfactorily for the microbiological determination of lysine but the curve so-formed is not as straight, as that when five grams of zein were hydrolysed. The blank figure of the five gram sample is appreciably higher than that of the three grams, in all probability due to the higher concentration of other amino acids (see page 41). Despite this latter disadvantage, it was considered wisest to use five grams of zein in preference to the three since a surplus of the required amino acids, unless imbalances of some of the acids were observed, would be preferable to a deficiency and also because of the straight line standard curve.

Strength of acid and length of hydrolysis of zein.

In preliminary experiments, a sample of zein prepared by Pfanstiehl Chemical Co., Waukegan, Illinois, had been used and satisfactory curves were obtained when the protein was hydrolysed with 2 N acid. This zein was approximately ten years old and was in short supply. When a fresh sample arrived from Nutritional Biochemicals Corporation, Cleveland, Ohio, it was found that this material when hydrolysed with 2 N acid and added to the basal medium, gave a double sigmoidal curve with standard amounts of lysine as shown in Figure 5. This indicated that some factor, responsible for additional growth of L. mesenteroides, existed in the new zein. When the zein was more fully hydrolysed, by

increasing either the strength of the acid used, or the time of hydrolysis, this additional growth was not observed. It was concluded that partial hydrolysis of zein liberated peptide-like materials which were stimulatory in their action toward the microorganism. These materials appear to be similar in nature to strepogenin, a peptide first encountered by Sprince and Woolley (81) and shown to be responsible for additional growth of Streptococcus faecalis and Lactobacillus casei by Totter and King (89).

Woolley (102) more recently has suggested that glutamic acid is intimately related to strepogenin activity as derivatives of this amino acid were found to have stimulatory action on Lactobacillus casei. Glutamine and glutathione were most reactive. Zein is exceptionally high in glutamic acid (Table III), as also is casein, a protein of high strepogenin activity. Consequently it appears reasonable to suggest that the growth factor found in casein is of the same nature as that found in zein in this investigation. It may also be reasonable to suggest that the old zein (Pfansteihl) due to its long period of standing, may somehow have lost its strepogenin activity. If this were true it would be concluded that the strepogenin portion of the protein molecule is relatively near the ends of the peptide chains, otherwise it could not be lost so easily from the protein. This is similar to conclusions reached by Woolley (103) who suggested that the strepogenin of insulin is located at the

ends of amino acid chains of the protein.

The results of the standard curves (Figure 6) using different strength acids markedly shows the effect of incomplete hydrolysis of zein. The 6 N hydrochloric acid curve is straight whereas that of the 2 N acid shows the typical additional growth at the lower lysine levels and the same amount of increased growth at the higher lysine levels. This is better illustrated in Figure 8 where the growth response of the microorganism was measured at twelve hour intervals by means of turbidity development. The 2 N acid-hydrolysed zein caused the organism to commence growth almost at the time of inoculation, whereas that hydrolysed by 6 N hydrochloric acid required almost a day before appreciable growth was observed. The sample hydrolysed with 0.5 N acid appeared to release some of the growth factor but not sufficient of the essential amino acids for satisfactory growth.

The time of hydrolysis of zein affected the standard lysine curve in much a similar manner to that of the strength of the acid used in the hydrolysis. A double S-shaped curve was formed when the time of zein hydrolysis was limited to one or four hours while a flat curve was obtained after seven or ten hours (Figure 7). Here also, it was observed that profuse growth appeared much earlier in the short-time hydrolysed samples. Quantitative turbidimetric results are depicted in Figure 9.

It is apparent from these results that autoclaving the

zein for relatively short periods, does not sufficiently hydrolyse the protein, with the consequence that additional growth factors are formed. Whether the growth factors observed here are similar to the strepogenin found after incomplete hydrolysis of casein (81) is difficult to say with any degree of assurance. The method of discovery and the growth characteristics of strepogenin are however, strikingly similar to those of the substances described in this investigation.

Salt requirements.

It is evident (Figure 10) that the neutralization of the zein hydrolysate with sodium hydroxide, adds sufficient salt to the medium for satisfactory growth of the microorganism. The addition of small amounts of sodium chloride (2.5 g. per 100 tubes) does not appreciably lower the curve, while larger quantities materially affect the steepness of the curve and hence the reliability of the method. Large excesses of salt completely inhibited growth of Leuconostoc mesenteroides. As a consequence of these experiments no salt was used in the basal media when hydrolysed zein was substituted for the amino acids.

Amino acid requirements.

The addition of tryptophane to the hydrolysed zein basal medium was imperative for it was found that no growth of L. mesenteroides occurred when this amino acid was omitted from

the medium. It is therefore suggested that, as well as lysine, the method may be applicable to the determination of tryptophane in proteins.

Large excesses of either glutamic acid or leucine, as found normally in zein, have slight effect on the lysine standard curve, as shown in Figures 12 and 13. Excess leucine appears only to increase the steepness of the line, a factor which should add to the sensitivity of the procedure. Glutamic acid does not alter the slope of the curve but increasing amounts of this amino acid show increasingly large blanks, with a resultant higher titration value over the complete lysine range. It is evident from these results that the excess quantity of glutamic acid and leucine do not interfere in the determination of lysine except that they may increase the blank to some extent.

From Figure 11 it may be seen that the addition of those acids in which zein is deficient (according to the Barton-Wright basal medium) does not affect the standard curve to a great extent. The use of only tryptophane along with hydrolysed zein appears to be equally if not more satisfactory than if arginine, aspartic acid, cystine or glycine are added, none of which improve the standard curve.

Histidine has considerable effect on the position of the lysine standard curve as is illustrated in Figure 14. From a comparison of the standard curves using both types of basal media (Figure 15) it may be noted that the blank value of the

normal curve is considerably lower than that of the zein hydrolysate curve. This may in part be due to the effect of the histidine, glutamic acid and perhaps several of the other amino acids of zein. A portion of this blank may be due to the small amount of lysine which was found to occur in zein (0.06 per cent). However, the curve, despite its high blank will still measure quantities of lysine between 40 and 200 micrograms.

Lysine recovery.

Table IV shows the complete determination of casein. The volumes of 0.1 N sodium hydroxide lie within 0.1 ml. in the duplicates. The multiple amounts of casein used showed a maximum deviation of 3.8 per cent which is within the range of the experimental error. Five per cent is the figure usually given as the maximum deviation which should be allowed for microbiological assay. The lysine value (7.85 per cent) obtained for casein by the modified microbiological method is in good agreement with values recorded in the literature.

The recovery experiment as reported in Table V shows that ninety-seven per cent of the lysine added to casein hydrolysate is recovered. However only ninety-two per cent is recovered from casein itself. This would lead us to expect that a small portion of lysine has been lost during hydrolysis. Schein and Berg (71) have shown that autoclaving of casein with 8 N sulphuric acid racemized a considerable

amount of lysine. Since L. mesenteroides utilizes only the l-form of lysine it is possible that some racemization, and hence some loss of lysine, may have occurred in this experiment. However since the figures of Table V are averages of only three assays, their statistical significance is uncertain.

The use of a protein naturally deficient in one or more amino acids being used as a source of amino acids for the estimation of the deficient compound has not been observed in the literature. This study on zein and its use for the determination of lysine has shown the possibility of the procedure. Even though zein was found to contain a small amount of lysine, the amount was not so great that a satisfactory standard curve could not be produced. As long as the amount of the amino acid under question is not so large that a high blank occurs, then the protein may be used as a source of amino acids, provided of course, that other factors such as amino acid imbalances, do not interfere. If the blank value is of such a height that a reasonable slope to the standard curve is not obtained, then the hydrolysed protein is a poor substitute for the pure amino acids. No work was done in this project to follow the matter further, however the possibilities are numerous. Gelatin, for instance, is deficient in serine and cystine, and completely lacking in valine, tyrosine, tryptophane and methionine; silk fibroin is low in arginine, histidine and lysine; zein is deficient in lysine,

tryptophane, glycine and hydroxyproline. Many others will be revealed by a search of the literature.

Toennies (88) has eliminated tyrosine and tryptophane from casein by oxidation. Lyman et al (52) demonstrated that treatment with hydrogen peroxide removed methionine, cystine, tryptophane and tyrosine from casein. The use of oxidized or otherwise treated proteins thus offers another wide field for study in the search for amino acid sources to be used in microbiological assay procedures.

Description of procedure utilizing zein.

The microbiological assay of lysine is carried out as described on page 23 with the exception that the amino acids are not added to the basal medium.

Five grams of zein is hydrolysed for ten hours at fifteen pounds pressure with 6 N hydrochloric acid. The pH is adjusted to approximately 4 with sodium hydroxide and the slight amount of humin which was formed during hydrolysis is filtered off. Then the clear hydrolysate is neutralized with more of the alkali and added to the rest of the basal medium. The pH of this solution is adjusted to 6.8 and the volume is made up to 500 ml. This gives sufficient basal medium for 100 tubes.

Utilizing the above basal medium, the rest of the microbiological procedure is similar to the original method (5).

A Comparison of the Colorimetric and Microbiological Methods for the Determination of Lysine in Stored Foodstuffs

Table VI and Table VII show the results of the two different methods used in this study for the determination of lysine in the food products of Part II. It is clearly evident that the chemical method in nearly all cases yields much lower values than the microbiological assay, although the results were generally parallel in nature. It is only when the lysine content is relatively low that the values obtained by the chemical method approach those of the microbiological procedure. This latter fact is observed after twelve and eighteen months storage at 37° (high humidity) of the milk powders and in several cases of the ground oats and dehydrated potatoes.

It was concluded from the results that although the chemical method yields correct relative values the microbiological assay was the more exact of the two.

SUMMARY

1. A modification of the Boulet procedure for the chemical determination of lysine has been proposed. This procedure has been utilized to determine the lysine content of stored foods.

2. The microbiological determination of lysine by the use of Leuconostoc mesenteroides P-60, has been investigated. A simplified and less expensive procedure whereby hydrolysed zein is used as a source of the amino acids for the basal media, has been outlined.

3. Partially hydrolysed zein has been found to contain an unidentified growth factor for L. mesenteroides.

4. The microbiological procedure has been utilized for the determination of lysine in stored foods. A comparison is made between the chemical and the microbiological methods for the determination of lysine in dried milk powders after different periods and conditions of storage.

REFERENCES

1. Albanese, A. A.
An Electrolytic Method for the Determination of the Basic Amino Acids in Proteins.
J. Biol. Chem., 134, 467; 1940.

2. Aubel, E. and Asselineau, J.
The Use of Sodium Hypochlorite in the Determination of Alanine and Valine.
Analyst, 73, 693; 1948.

3. Ayre, C. A.
The Lysine Content of Feeding Stuffs.
Biochem. J., 32, 1152; 1938.

4. Barton-Wright, E. C.
The Microbiological Assay of Nicotinic Acid in Cereals and Other Products.
Biochem. J., 38, 314; 1944.

5. _____.
The Microbiological Assay of Amino Acids. I. The Assay of Tryptophane, Leucine, Isoleucine, Valine, Cystine, Methionine, Lysine, Phenylalanine, Histidine, Arginine and Threonine.
Analyst, 71, 267; 1946.

6. _____, Emery, W. B. and Robinson, F. A.
Microbiological Assay of Amino Acids with Leuconostoc mesenteroides P-60.
Nature, 157, 628; 1946.

7. Baumgarten, W., Mather, A. N. and Stone, L.
Glutamic Acid Content of Feed Materials.
Cereal Chem., 22, 514; 1945.

8. _____.
Essential Amino Acid Composition of Feed Materials.
Cereal Chem., 23, 135; 1946.

9. _____, Stone, L. and Boruff, C. S.
Amino Acid Composition of Grain Alcohol Fermentation By-Products.
Cereal Chem., 22, 311; 1945.

10. Bennett, M. A. and Toennies, G.
A Nutritional Assay of Casein Modified by the Action of Hydrogen Peroxide and Formic Acid.
J. Biol. Chem., 145, 671; 1942.

11. Bergdoll, M. S., and Doty, D. M.
Chromotography in the Separation and Determination of the Basic Amino Acids.
Ind. Eng. Chem., Anal. Ed., 18, 600; 1946.

12. Block, R. J.
The Determination of the Basic Amino Acids in Small Quantities of Proteins by the Silver Precipitation method.
J. Biol. Chem., 106, 457; 1934.

13. _____.
A New Method for Separation of the Basic Amino Acids from Protein Hydrolysates.
Proc. Soc. Expt. Biol. and Med., 51, 252; 1942.

14. Boulet, M. A.
A Modified Method for the Determination of Lysine and Its Application to Reactions between Reducing Sugars and Amino Acids.
McGill University Thesis, 1948.

15. _____, Nelson, J. A., and McFarlane, W. D.
A Rapid Colorimetric Method for the Determination of Lysine in Protein Hydrolysates.
Can. J. Res., B25, 540; 1947.

16. Brigl, P., Held, R., and Hartung, K.
Protein Chemistry. IV. The Hypobromite Reaction of Amino Acid Derivatives.
Z. Physiol. Chem., 173, 129; 1928.
Chem. Abstracts, 22, 1328; 1928.

17. Calvery, H. O.
Basic Amino Acids. The Estimation of the Basic Amino Acids in Small Amounts of Casein and Edestin by the Modified Method of Vickery and Leavenworth and Other Methods.
J. Biol. Chem., 83, 631; 1929.

18. Chapin, R. M.
The Influence of pH upon the Formation of the Chloro Derivatives of Ammonia.
J. Am. Chem. Soc., 53, 912; 1931.

19. Christenson, B. E., West, E. S., and Dimick, K. P.
A Simple Apparatus and Procedure for Determination of Amino Acids by the Ninhydrin Reaction.
J. Biol. Chem., 137, 735; 1941.

20. Cleaver, C. S., Hardy, R. A., and Cassidy, H. G.
Chromatographic Adsorption of Amino Acids on Organic Exchange-Resins.
J. Am. Chem. Soc., 67, 1343; 1945.

21. Csonka, F. A.
Amino Acids in Oats and Oat Milling Products, Including Rolled Oats.
Cereal Chem., 18, 523; 1941.

22. Dakin, H. D.
Amino Acids of Gelatin.
J. Biol. Chem., 44, 499; 1920.

23. Dreschel, E.
Quoted from Schmidt, C. L. A.
The Chemistry of Amino Acids and Proteins with Addendum.
Thomas, Springfield, Ill., 2nd Ed., (1945), p. 8.

24. Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W. and Rockland, L. B.
The Amino Acid Requirements of Leuconostoc mesenteroides P-60.
J. Biol. Chem., 56, 703; 1944.

25. Eldred, N. R. and Rodney, G.
The Effect of Proteolytic Enzymes on Raw and Heated Casein.
J. Biol. Chem., 162, 261; 1946.

26. Englis, D. T. and Feiss, H. A.
Conduct of Amino Acids in Synthetic Ion Exchangers.
Ind. Eng. Chem., 36, 604; 1944.

27. Epps, H. M. R.
Studies on Bacterial Amino Acid Decarboxylases.
4. l(-)Histidine Decarboxylose from Cl. Welchii Type H.
Biochem. J., 39, 42; 1945.

28. Folin, O. and Ciocalteu, V.
On Tyrosine and Tryptophane Determination in Proteins.
J. Biol. Chem., 73, 627; 1927.

29. Foster, G. L.
Some Amino Acid Analyses of Hemoglobin and B-Lactoglobulin.
J. Biol. Chem., 159, 431; 1945.

30. _____., and Schmidt, C. L. A.
The Separation of the Hexone Bases from Certain Protein Hydrolysates by Electrolysis.
J. Biol. Chem., 56, 545; 1923.

31. Freudenberg, K., Walch, H. and Molter, H.
The Separation of Sugars, Amino Sugars, and Amino Acids.
Naturwissenschaften, 30, 87; 1942.
Chem. Abstracts, 37, 658; 1942.

32. Furth, O. and Minnibeck, H.
Studies on the Proline and Hydroxyproline Contents of Some Proteins.
Biochem. Z., 250, 18; 1932.
Chem. Abstracts, 26, 5108; 1932.

33. _____., Scholl, R. and Herrmann, H.
A Micro Method for the Determination of Alanine in Proteins.
Biochem. Z., 251, 404; 1932.
Chem. Abstracts, 26, 5596; 1932.

34. Gale, E. F.
Studies on Bacterial Amino Acid Decarboxylases.
5. The Use of Specific Decarboxylase Preparations in the Estimation of Amino Acids and in Protein Analysis.
Biochem. J., 39, 46; 1945.

35. _____. and Epps, H. M. R.
l-Lysine Decarboxylase--Preparation of Specific Enzyme and Coenzyme.
Nature, 152, 327; 1943.

36. _____.
Studies on Bacterial Amino-Acid Decarboxylases.
1. l(+)-Lysine Decarboxylase.
Biochem. J., 38, 242; 1944.

37. _____.
Studies on Bacterial Amino-Acid Decarboxylases.
3. Distribution and Preparation of Codecarboxylase.
Biochem. J., 38, 250; 1944.

38. Guirard, B. M., Snell, E. E. and Williams, R. J.
Microbiological Determination of Amino Acids.
IV. Lysine, Histidine, Arginine and Valine.
Proc. Soc. Expt. Biol. and Med., 61, 158; 1946.

39. Harding, W. M. and Shive, W.
Biochemical Transformations as Determined by Competitive Analogue-Metabolite Growth Inhibitions.
VIII. An Interrelationship of Methionine and Leucine.
J. Biol. Chem., 174, 743; 1948.

40. Horn, M. J., Jones, D. B. and Blum, A. E.
Microbiological Determination of Lysine in Proteins and Foods.
J. Biol. Chem., 169, 71; 1947.

41. _____.
Microbiological Determination of Threonine in Proteins and Foods.
J. Biol. Chem., 169, 739; 1947.

42. Kapeller-Adler, R.
The Detection and Determination of Phenylalanine.
Biochem. Z., 252, 185; 1932.
Chem. Abstracts, 26, 5596; 1932.

43. Kidder, G. W. and Dewey, V. C.
Biochemistry of Tetrahymena gelii. I. Amino Acid Requirements.
Arch. Biochem., 6, 425; 1945.

44. Kossel, A.
Uber die Constitution der einfachsten Eiweisstoffe.
Z. Physiol. Chem., 25, 165; 1898.

45. _____. and Kutscher, F.
Beitrage zur Kenntniss der Eiweisskorper.
Z. Physiol. Chem., 31, 165; 1900.

46. Kuiken, K. A., Norman, W. H., Lyman, C. M., and Hale, F.
Microbiological Determination of Amino Acids.
Science, 98, 266; 1943.

47. _____,
and Blotter, L.
The Microbiological Determination of Amino Acids.
I. Valine, Leucine and Isoleucine.
J. Biol. Chem., 151, 615; 1943.

48. Langheld, K.
Degradation of α-Amino Acids to Aliphatic Aldehydes by Means of Sodium Hypochlorite.
Ber., 42, 392; 1909.
Chem. Abstracts, 3, 1017; 1909.

49. _____.
Behaviour of α-Amino Acids Towards Sodium Hypochlorite.
Ber. 42, 2360; 1909.
Chem. Abstracts, 3, 2556; 1909.

50. Lewis, J. C., and Olcott, H. S.
A Lactobacillus Assay Method for l(+)-Glutamic Acid.
J. Biol. Chem., 157, 265; 1945.

51. Lieben, F., and Loo, Y. C.
On the Liberation of Free Amino Nitrogen from Proteins in the Van Slyke Apparatus.
J. Biol. Chem., 145, 223; 1942.

52. Lyman, C. M., Moseley, O., Wood, S. and Hale, F.
Note on the Use of Hydrogen Peroxide-Treated Peptone in Media for the Microbiological Determination of Amino Acids.
Arch. Biochem., 10, 427; 1946.

53. Macpherson, H. T.
The Basic Amino Acid Content of Proteins.
Biochem. J., 40, 470; 1946.

54. Mason, M. F.
The Manometric Determination of Amino Acids.
Biochem. J., 32, 719; 1938.

55. Millares, R., and Davis, S. G.
Determination of Tryptophane and Lysine in Microvolumes of Protein Hydrolysates.
Anal. Chem., 21, 414; 1949.

56. Naguchi, T.
The Separation of Hexone Bases by Electrolysis.
Bull. Inst. Phys. Chem. Res., Tokyo, 8, 152; 1929.
Chem. Abstracts, 23, 2372; 1929.

57. Nelson, J. A.
Studies on the Determination of Amino Acids in Protein Hydrolysates--A New Micro-colorimetric Method for the Determination of Lysine.
McGill University Thesis, 1945.

58. Neuberg, C.
Enzymes: ninhydrin.
Biochem. Z., 56, 495; 1914.
Chem. Abstracts, 8, 1131; 1914.

59. Neuberger, A.
The Lysine Content of Casein and Zein.
J. Biol. Chem., 158, 717; 1945.

60. Norman, M. F.
The Oxidation of Amino Acids by Hypochlorite.
Biochem. J., 30, 484; 1936.

61. Pennington, D., Snell, E. E., and Williams, R. J.
An Assay Method for Pantothenic Acid.
J. Biol. Chem., 135, 213; 1940.

62. Polonovski, M., and Moreno-Martin, F.
Use of the Ninhydrin Reaction in Determination of the Amino Acid Function.
Anales soc. espana fis quim., 33, 574; 1935.
Chem. Abstracts, 29, 7230; 1935.

63. Regnery, D. C.
A Leucineless Mutant Strain of Neurospora Crassa.
J. Biol. Chem., 154, 115; 1944.

64. Reisen, W. H., Schweigert, B. S., and Elvehjem, C. A.
Microbiological Determination of Methionine in Proteins and Foodstuffs.
J. Biol. Chem., 165, 347; 1946.

65. Rittenberg, D., and Foster, G. L.
A New Procedure for Quantitative Analysis by Isotope Dilution with Application to the Determination of Amino Acids and Fat Acids.
J. Biol. Chem., 133, 737; 1940.

66. Roche, J. and Mourgue, M.
Leucine and Valine Content of Proteins.
Compt. rend. soc. biol., 137, 766; 1943.
Chem. Abstracts, 39, 3313; 1945.

67. Rockland, L. B., and Dunn, M. S.
Growth Studies on Tetrahymena geleii H.
J. Biol. Chem., 179, 511; 1949.

68. Ruhemann, S.
Trikетohydrindene Hydrate. III. Its Relation to Alloxan.
J. Chem. Soc. (Trans.), 99, 1486; 1911.

69. Ryan, F. J., and Brand, E.
A Method for the Determination of Leucine in Protein Hydrolysates and in Foodstuffs by the Use of a Neurospora Mutant.
J. Biol. Chem., 154, 161; 1944.

70. Sauberlich, H. E., and Baumann, C. A.
The Effect of Dietary Protein upon Amino Acid Excretion by Rats and Mice.
J. Biol. Chem., 166, 417; 1946.

71. Schein, A. H., and Berg, C. P.
Destruction and Racemization of Basic Amino Acids on Autoclaving Casein with Sulfuric Acid.
Fed. Proc., 2, 69; 1943.

72. Shoenheimer, R., Ratner, S., and Rittenberg, D.
Studies in Protein Metabolism. The Metabolic Activity of Body Proteins Investigated with l(-)-Leucine Containing Two Isotopes.
J. Biol. Chem., 130, 703; 1939.

73. Shankman, S., Camien, M. N., and Dunn, M. S.
The Determination of Glycine in Protein Hydrolysates with Leuconostoc Mesenteroides P-60.
J. Biol. Chem., 168, 51; 1947.

74. Shemin, D.
Amino Acid Determinations on Crystalline Bovine and Human Serum Albumin by the Isotope Dilution Method.
J. Biol. Chem., 159, 439; 1945.

75. Simms, H. S.
The Nature of Ionizable Groups in Proteins.
J. Gen. Physiol., 11, 629; 1928.

76. Snell, E. E., and Guirard, B. M.
Some Interrelationships of Pyrridoxine, Alanine and Glycine in their Effect on Certain Lactic Acid Bacteria.
Proc. Nat. Acad. Sci., 29, 66; 1943.

77. _____, and Strong, F. M.
A Microbiological Assay for Riboflavin.
Ind. Eng. Chem., Anal. Ed., 11, 346; 1939.

78. _____, and Peterson, W. H.
Growth Factors for Bacteria.
Biochem. J., 31, 1789; 1937.

79. _____, and Wright, L. D.
A Microbiological Method for the Determination of Nicotinic Acid.
J. Biol. Chem., 139, 675; 1941.

80. Sperber, E.
Electrolytic Separation of Basic, Neutral and Acidic Amino Acids in Protein Hydrolysates.
J. Biol. Chem., 166, 75; 1946.

81. Sprince, H., and Woolley, D. W.
Relationship of a New Growth Factor Required by Certain Hemolytic Streptococci to Growth Phenomena in Other Bacteria.
J. Exptl. Med., 80, 213; 1944.

82. Sprince, H. and Woolley, D. W.
The Occurrence of the Growth Factor Strepogenin in Purified Proteins.
J. Am. Chem. Soc., 67, 1734; 1945.

83. Steele, B. F., Sauberlich, H. E., Reynolds, M. S. and Baumann, C. A.
Media for Leuconostoc Mesenteroides P-60 and Leuconostoc citravorum 8081.
J. Biol. Chem., 177, 533; 1949.

84. Stokes, J. L., Guness, M., Dwyer, I. M., and Caswell, M.
A Uniform Assay for the Ten Essential Amino Acids.
J. Biol. Chem., 160, 35; 1945.

85. Taylor, E. S., and Gale, E. F.
Studies on Bacterial Amino-Acid Decarboxylases.
6. Codecarboxylase Content and Action of Inhibitors.
Biochem. J., 39, 52; 1945.

86. Teruuchi, Y., and Okabe, L.
The Cystine Content of Several Kinds of Protein Determined by a Modification of Okuda's Method.
J. Biochem. (Japan) 8, 459; 1928.
Chem. Abstracts, 22, 2959; 1928.

87. Tillmans, J., Hirsch, P., and Stoppel, F.
A New Procedure for Determining Tryptophane and Tyrosine in Proteins.
Biochem. Z., 198, 379; 1928.
Chem. Abstracts, 23, 211; 1929.

88. Toennies, G.
The Oxidative Conversion of Casein into Protein Free of Methionine and Tryptophane.
J. Biol. Chem., 145, 667; 1942.

89. Totter, J. R., and King, M. E. M.
The Influence of Strepogenin Concentrate on the Metabolism of Glutamic Acid by Streptococcus Faecalis.
J. Biol. Chem., 165, 391; 1946.

90. Tristram, G. R.
The Basic Amino Acids of Leaf Proteins, with a Discussion of Various Methods of Analysis.
Biochem. J., 33, 1271; 1939.

91. Ussing, H. H.
Analysis of Proteins by Means of Deuterium-Containing Amino Acids.
Nature, 144, 977; 1939.

92. Van Slyke, D. D.
The Analysis of Proteins by Determination of the Chemical Groups Characteristic of the Different Amino Acids.
J. Biol. Chem., 10, 15; 1911-12.

93. _____, and Birchard, F. J.
The Nature of the Free Amino Groups in Proteins.
J. Biol. Chem., 16, 539; 1913-14.

94. _____, and Dillon, R. T.
Gasometric Determination of Carboxyl Groups in Amino Acids.
Comp. rend. Lab. Carlsberg, 22, 480; 1936.
Chem. Abstracts, 32, 7950; 1938.

95. _____, MacFadyen, D. A., and Hamilton, P.
Gasometric Determination of Carboxyl Groups in Free Amino Acids.
J. Biol. Chem., 141, 627; 1941.

96. _____, MacFadyen, D. A., and Hamilton, P.
Determination of Free Amino Acids by Titration of the Carbon Dioxide Formed in the Reaction with Ninhydrin.
J. Biol. Chem., 141, 671; 1941.

97. Vickery, H. B.
Amino Acid Composition of Zein.
Compt. rend. trav. lab. Carlsberg, Ser. chim., 22, 519; 1938.
Chem. Abstracts, 32, 6267; 1938.

98. _____, and Block, R. J.
The Basic Amino Acids of Silk Fibroin.
J. Biol. Chem., 93, 105; 1931.

99. _____, and Leavenworth, C. S.
On the Separation of Histidine and Arginine. II. The Separation of the Silver Compounds at pH 7.0.
J. Biol. Chem., 72, 403; 1927.

100. _____.
Modifications of the Method for the Determination of the Basic Amino Acids of Proteins. The Bases of Edestin.
J. Biol. Chem., 76, 707; 1928.

101. Virtanen, A. I., and Laine, T.
Decarboxylation of d-Lysine and l-Aspartic Acid.
Enzymologia, 3, 266; 1937.
Chem. Abstracts, 31, 8596; 1937.

102. Womack, M., and Rose, W. C.
Evidence for the Existance of an Unidentified Growth Stimulant in Proteins.
J. Biol. Chem., 162, 735; 1946.

103. Woolley, D. W.
The Position of Strepogenin in the Protein Molecule.
J. Biol. Chem., 171, 443; 1947.

104. _____.
Strepogenin Activity of Derivatives of Glutamic Acid.
J. Biol. Chem., 172, 71; 1948.

105. Wright, N. C.
The Action of Hypochlorites on Amino Acids and Proteins.
Biochem. J., 20, 524; 1926.

106. Zittle, C. A., and Eldred, N. R.
Determination of l-Lysine with a Specific Decarboxylase.
J. Biol. Chem., 156, 401; 1944.

Part I

Appendix

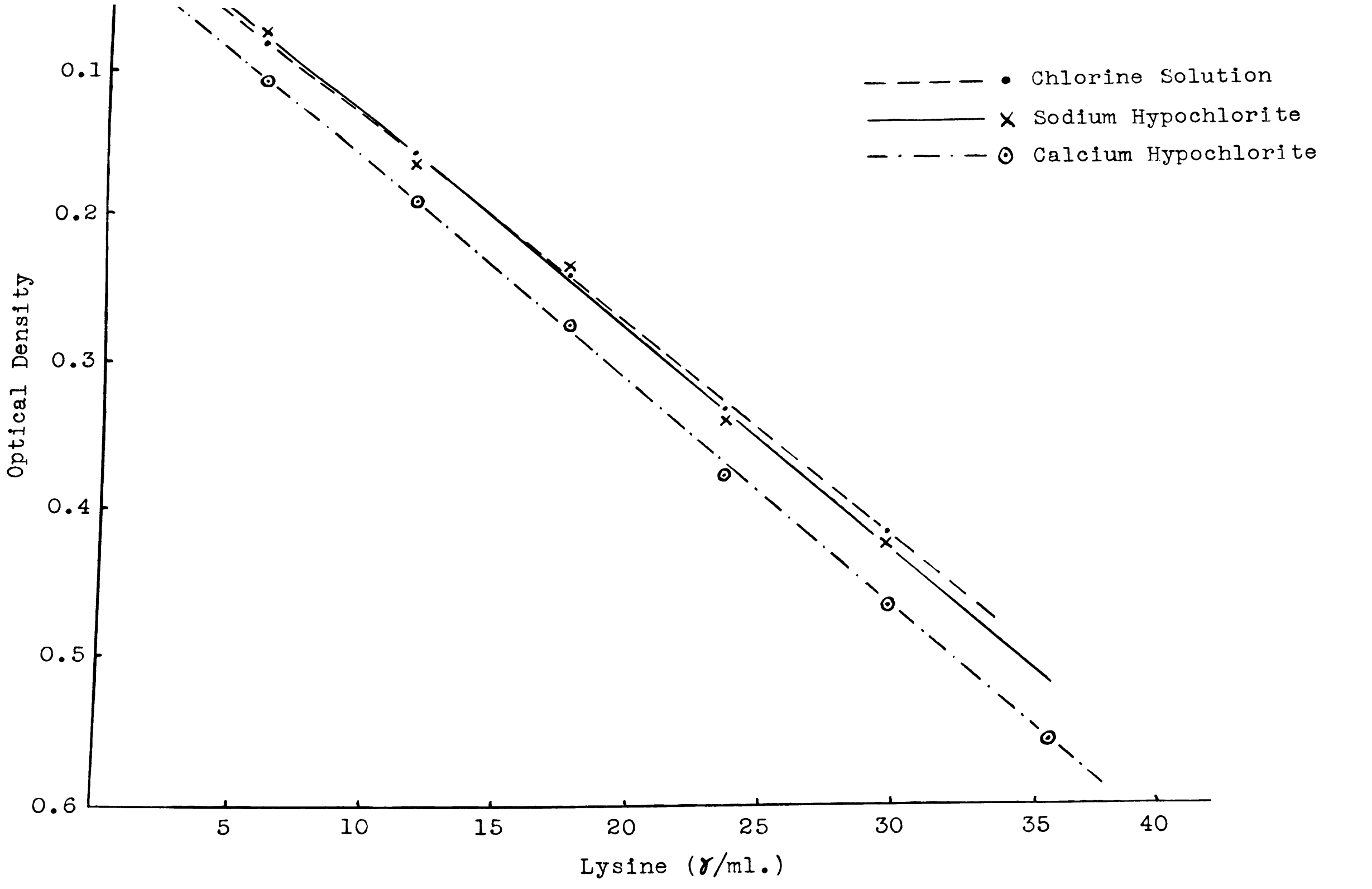

Chlorine Solution
Sodium Hypochlorite
Calcium Hypochlorite
Optical Density
0.1
0.2
0.3
0.4
0.5
0.6
5
10
15
20
25
30
35
40
Lysine (γ/ml.)

FIGURE 2.

Lysine Standard Curves Using Different Microorganisms

• Leuconostoc mesenteroides
× Streptococcus faecalis
⊙ Lactobacillus arabinosus

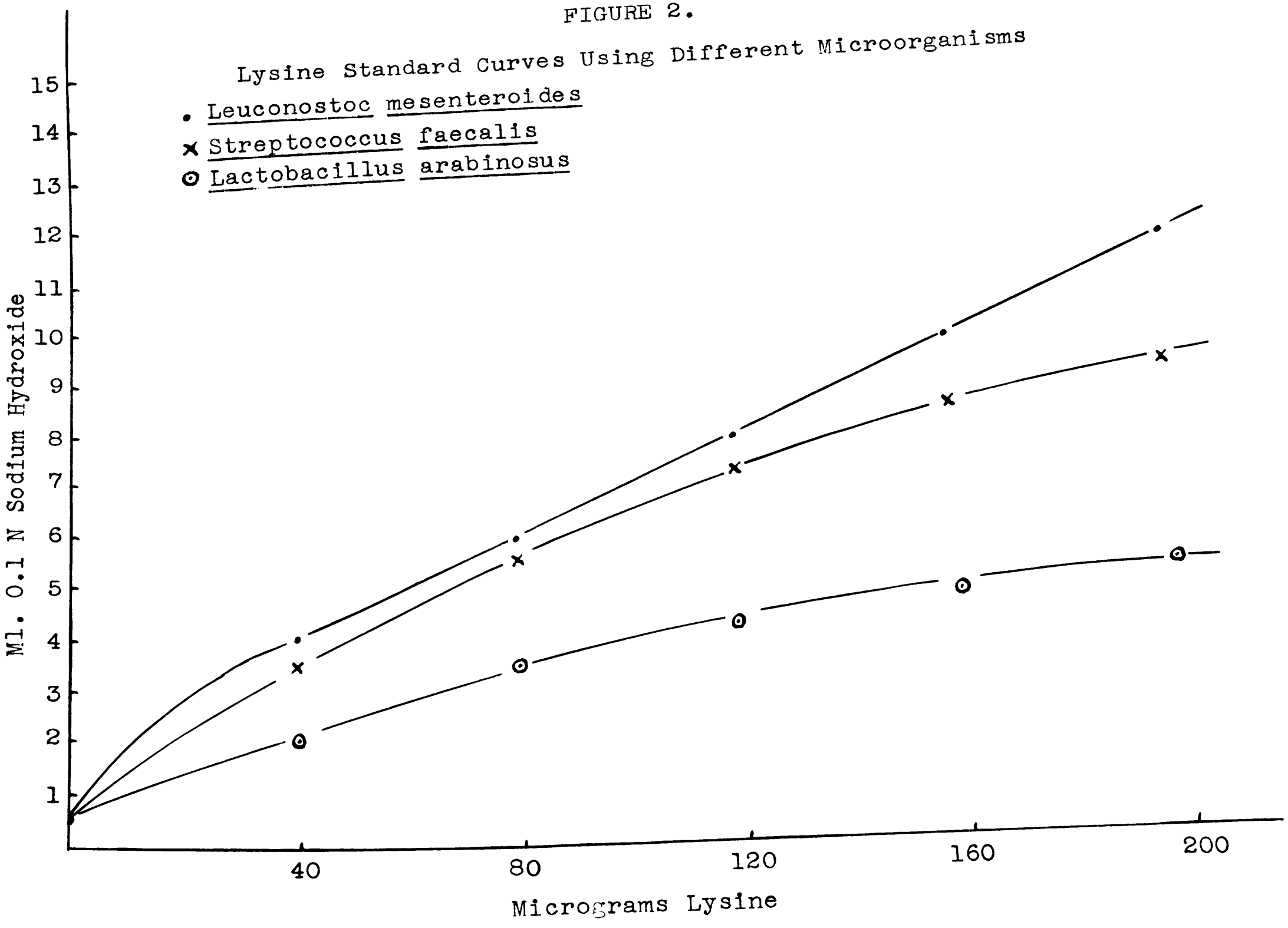


FIGURE 3.

Rack and Cover Used in Microbiological Assay of Lysine

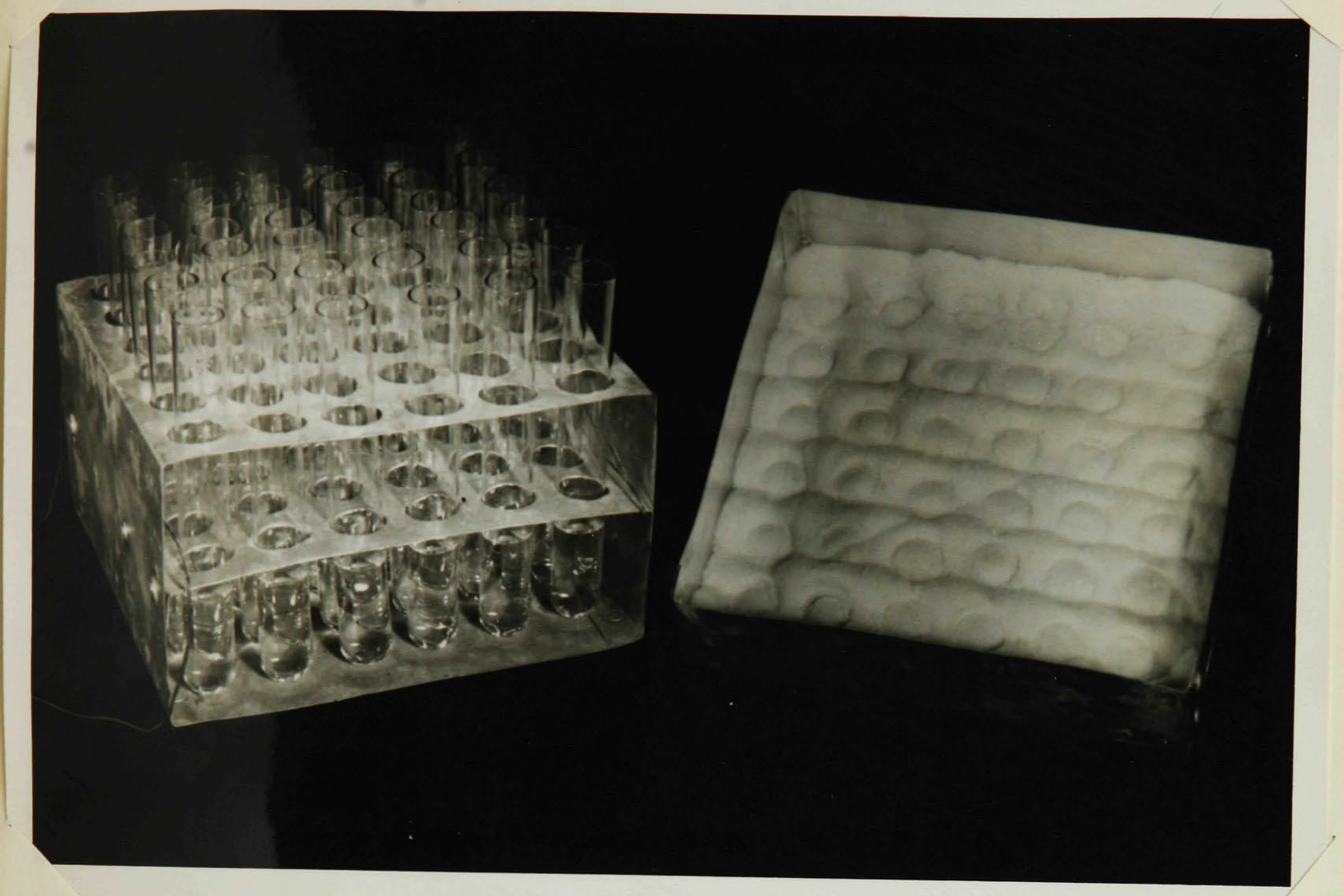


FIGURE 4.

Effect of Quantity of Zein Used on the Lysine Standard Curve

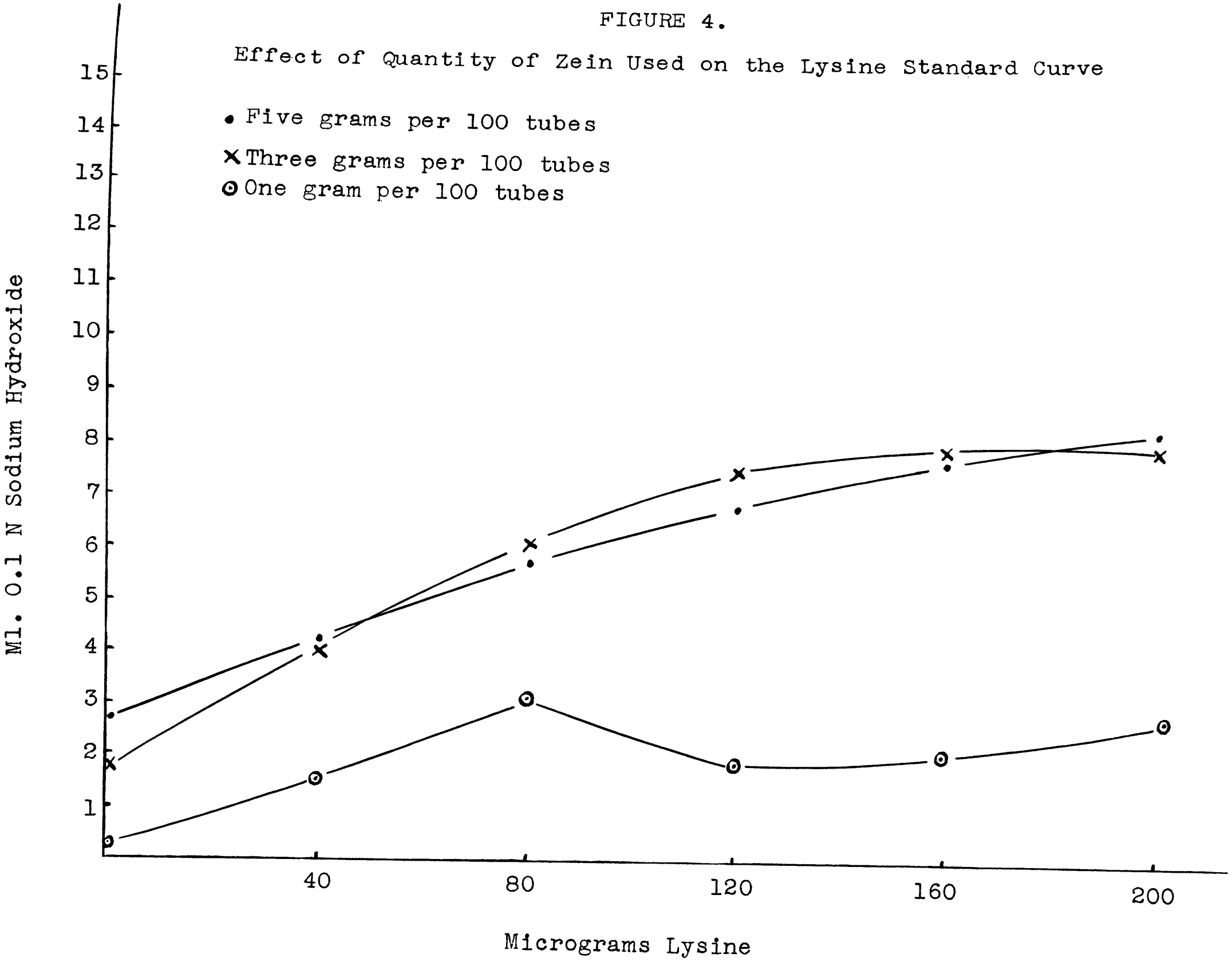


FIGURE 5.

Effect of Incomplete Hydrolysis of Zein on the Lysine Standard Curve

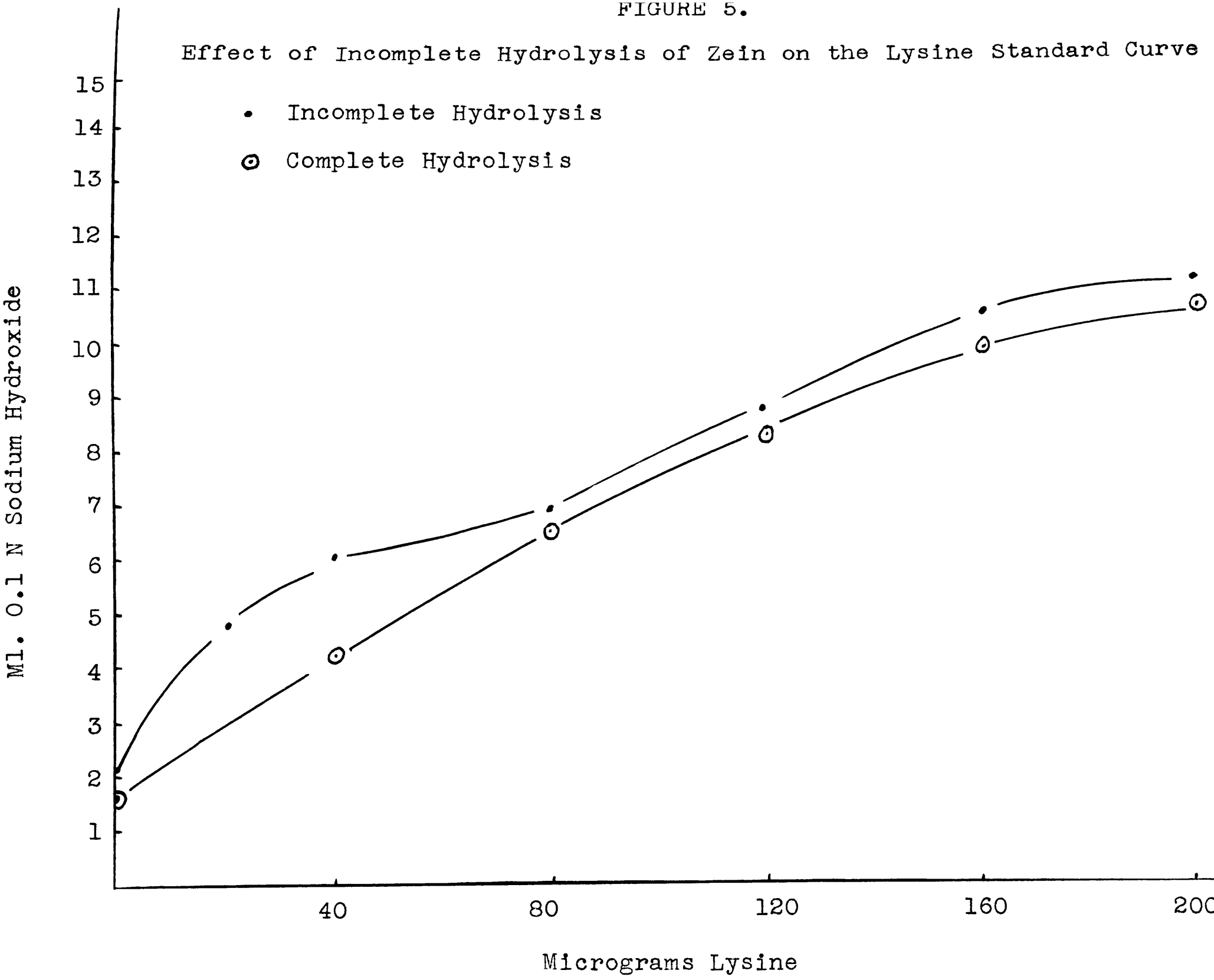


FIGURE 6.

Effect of Strength of Acid Used in Zein Hydrolysis on the Lysine Standard Curve

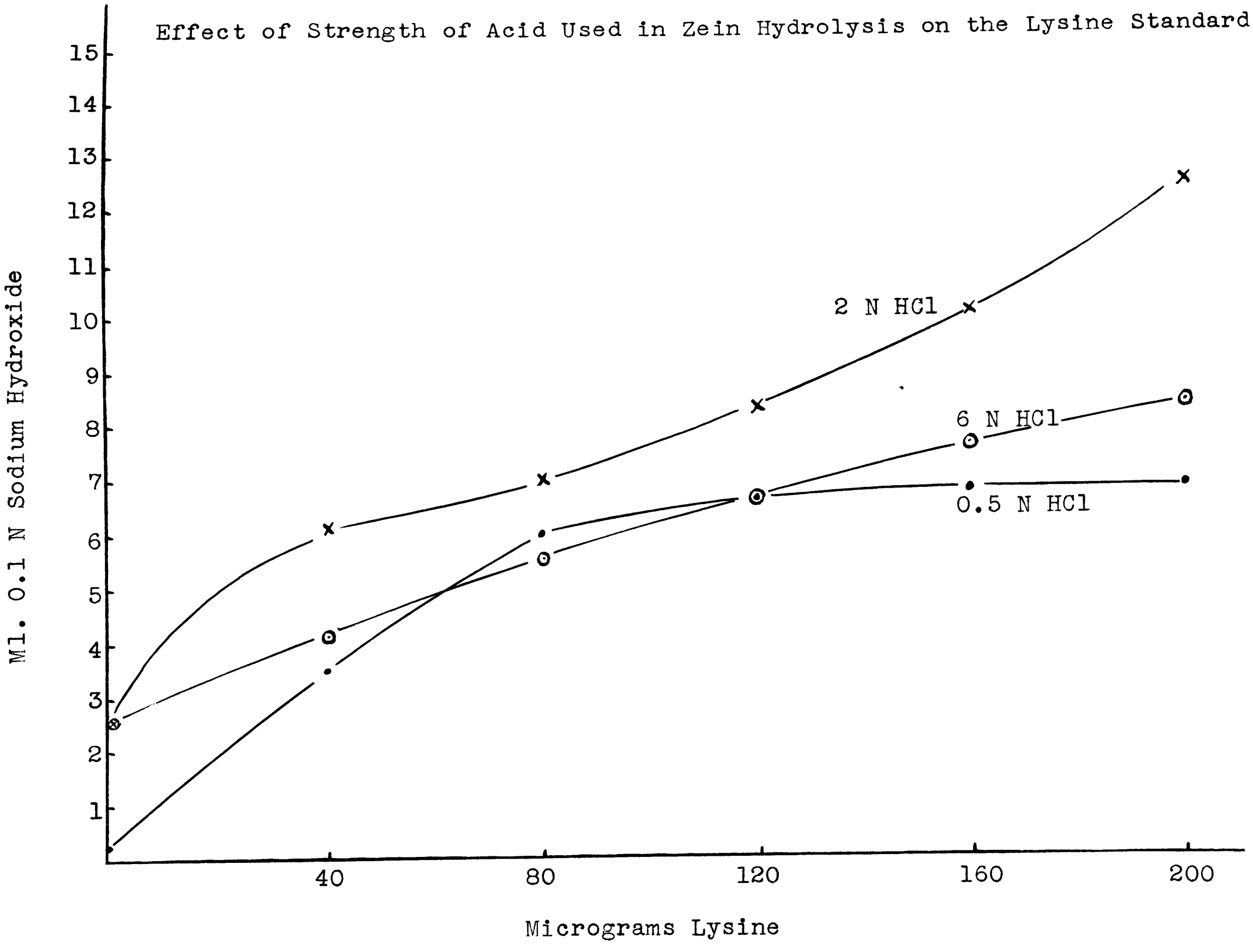


FIGURE 7.

Effect of Length of Hydrolysis of Zein on the Lysine Standard Curve

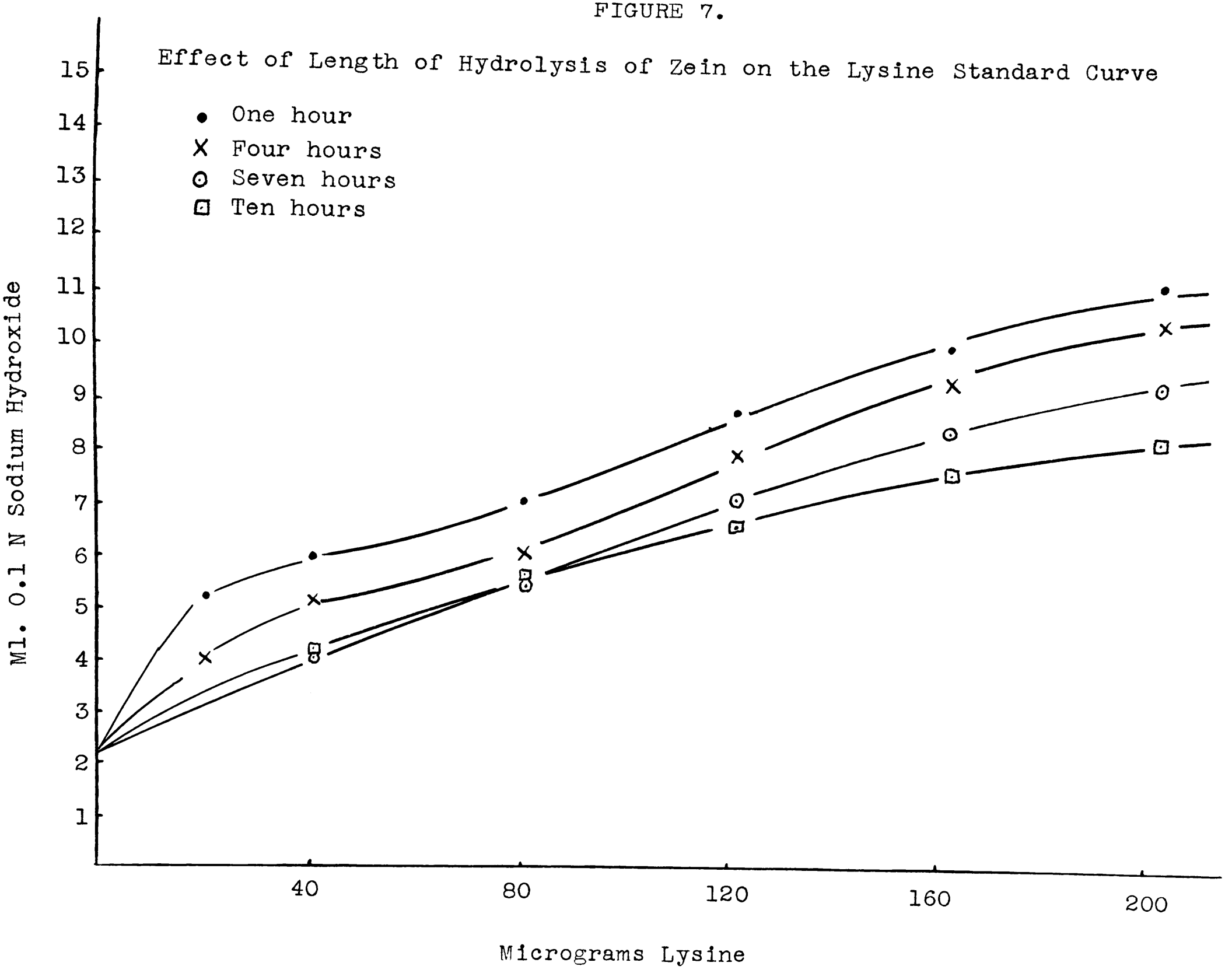


FIGURE 8.

Effect of Concentration of Acid used for the Hydrolysis of Zein on the Growth of Leuconostoc mesenteroides (Turbidity Measurements)

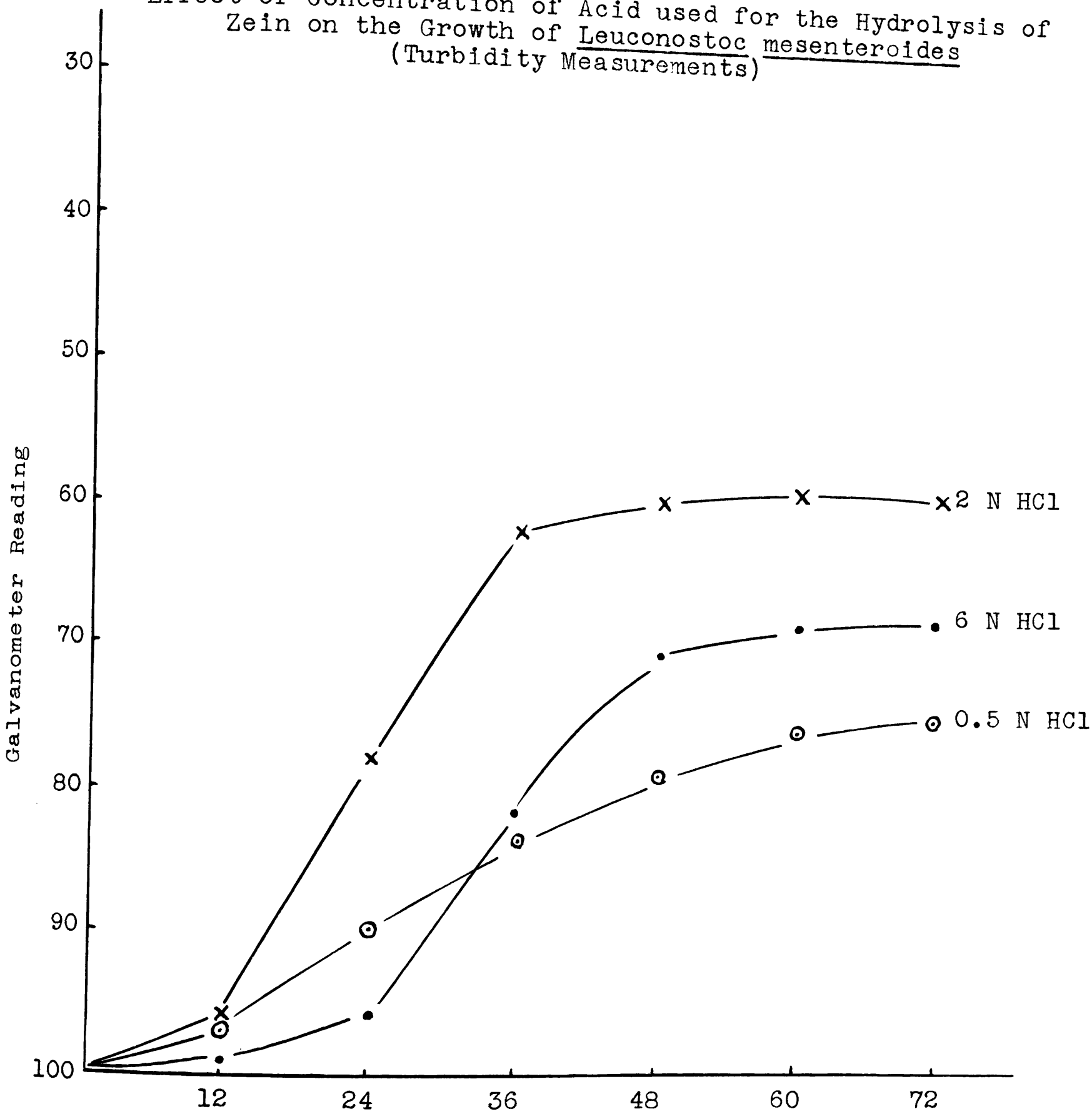


FIGURE 9.

Effect of Length of Hydrolysis of Zein on the Growth of Leuconostoc mesenteroides (Turbidity Measurements)

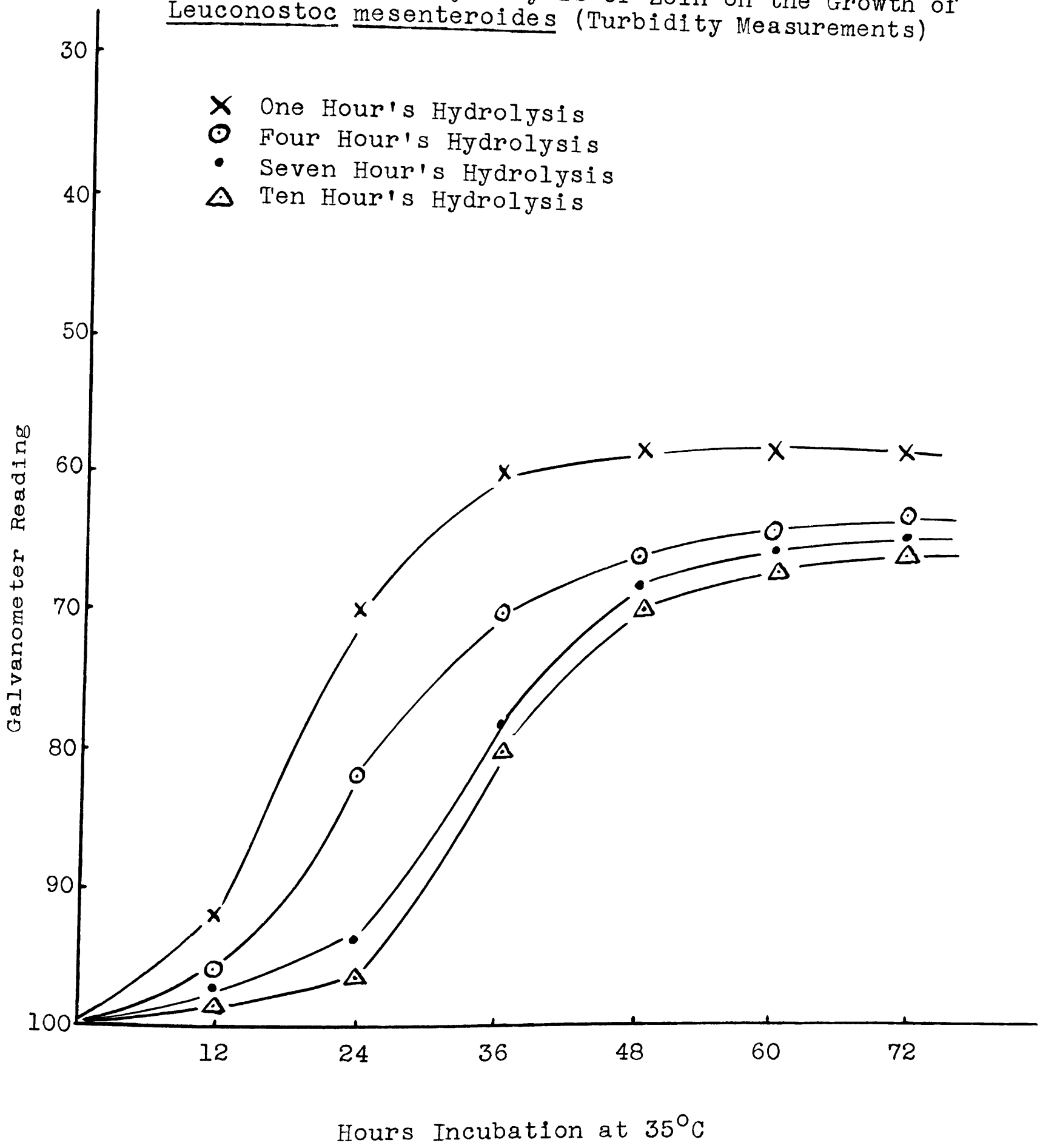


FIGURE 10.

Effect of Salt Concentration on the Lysine Standard Curve

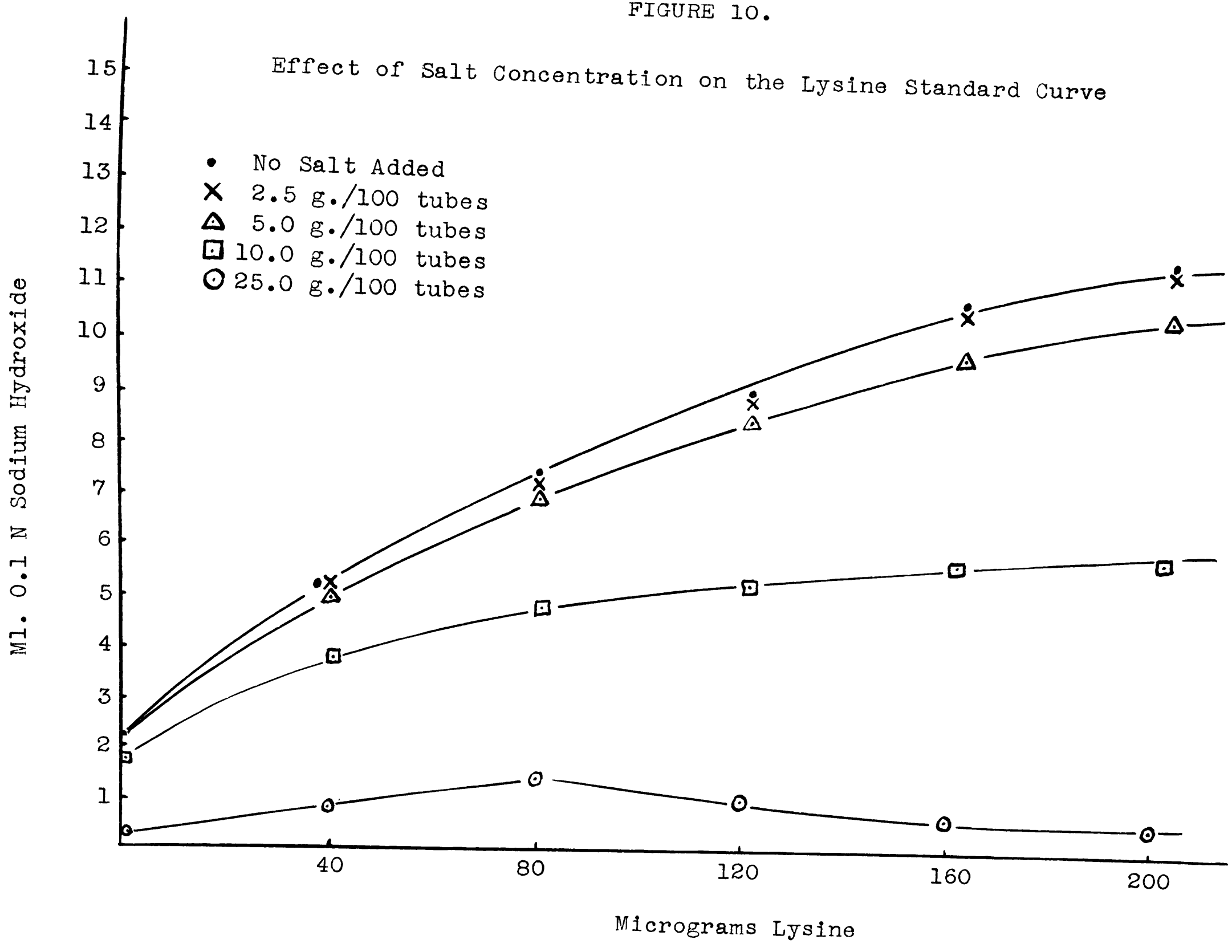


FIGURE 11.

Effect of Adding Those Amino Acids in which Zein is Deficient on the Lysine Standard Curve

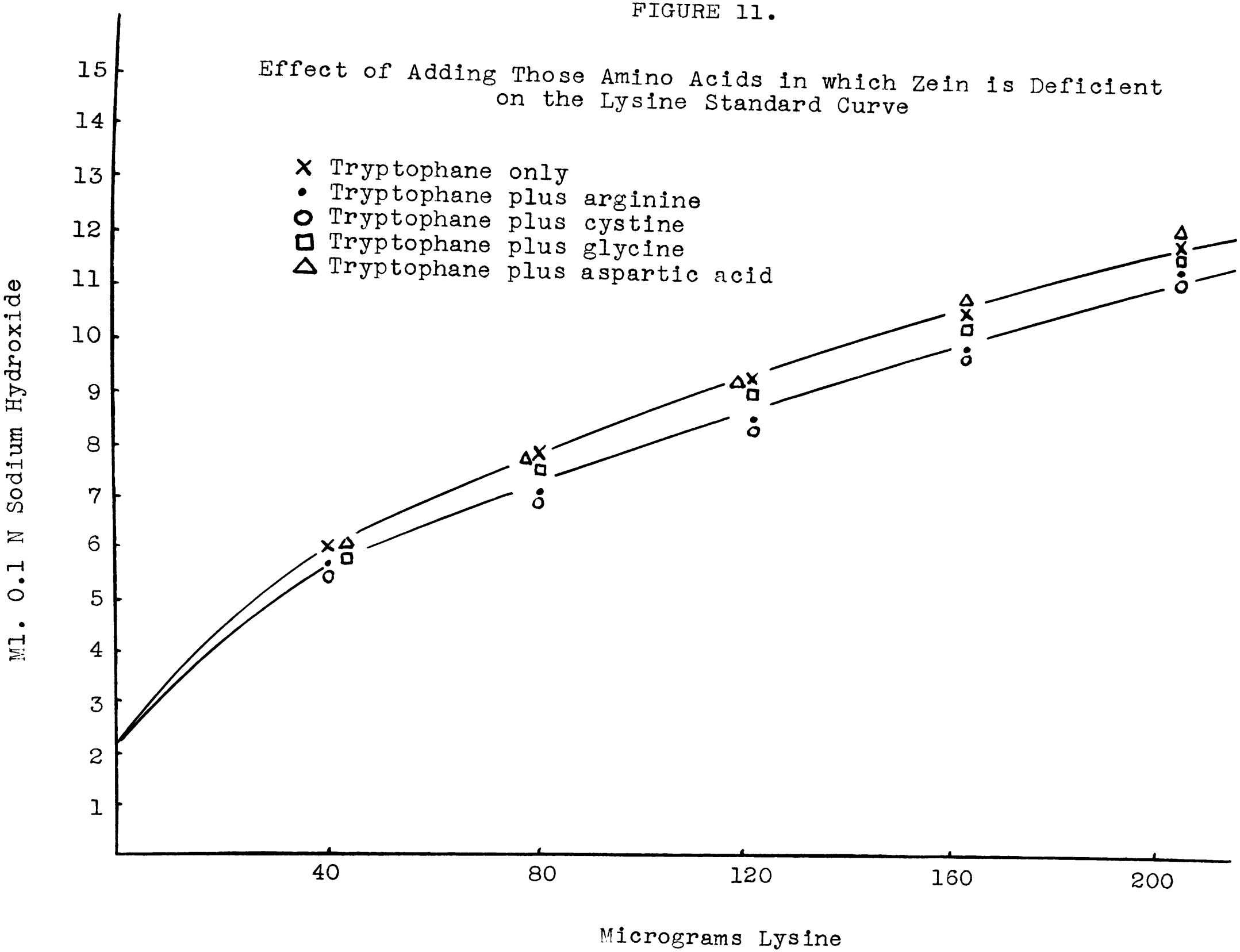


FIGURE 12.

Effect of Excess Glutamic Acid on the Lysine Standard Curve

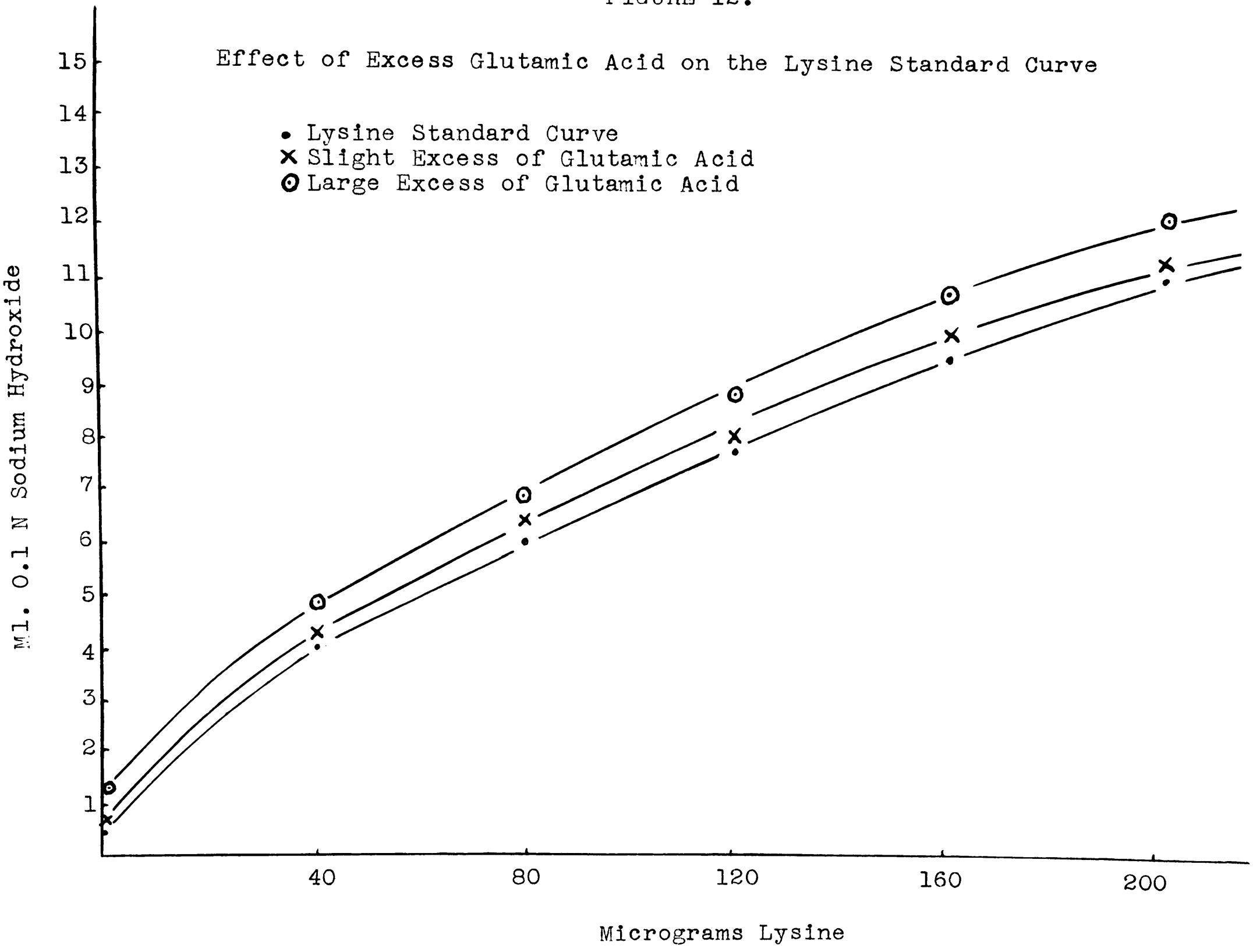


FIGURE 13.

Effect of Excess Leucine on Lysine Standard Curve

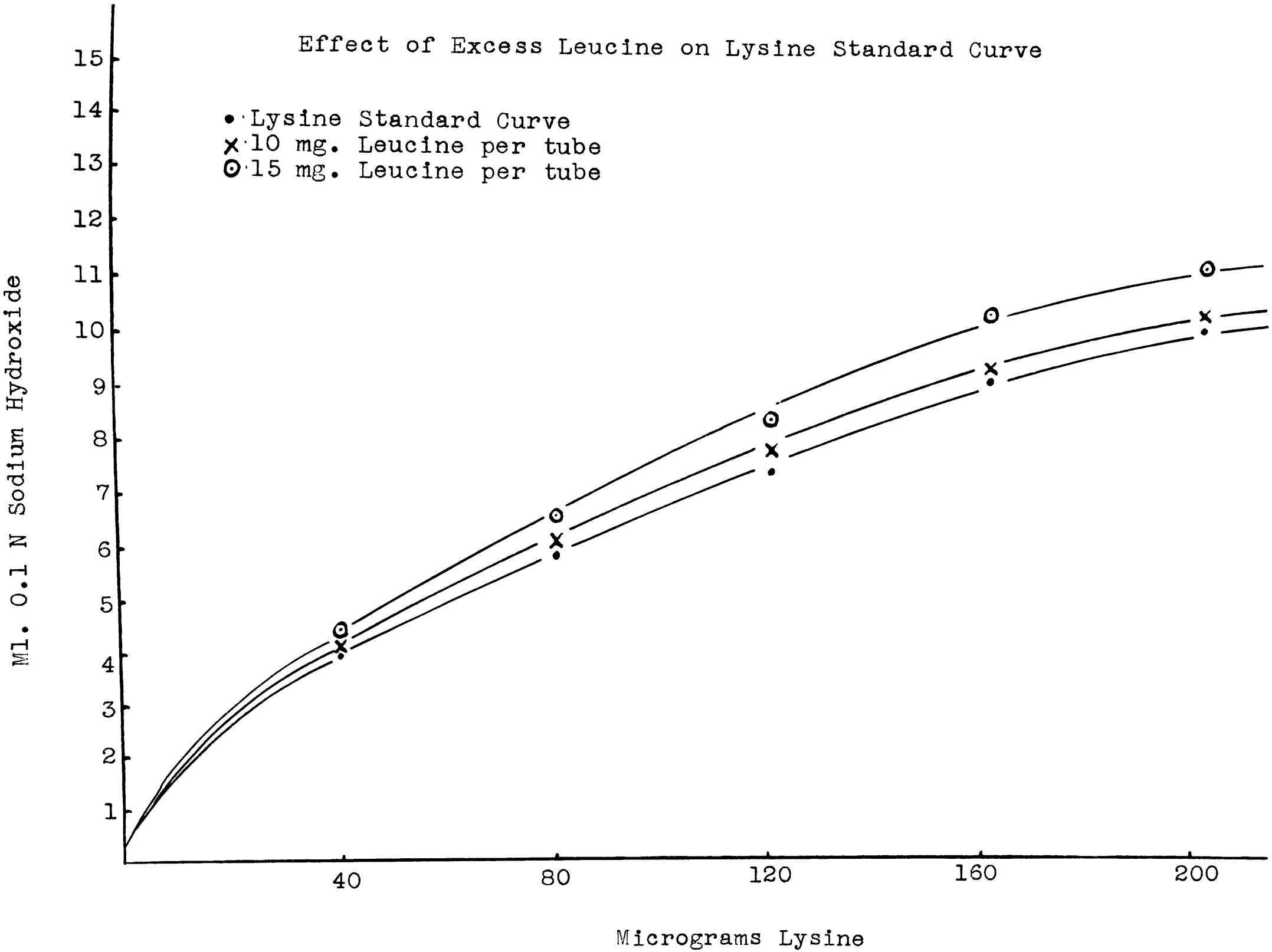


FIGURE 14.

Effect of Different Amounts of Histidine on the Lysine Standard Curve

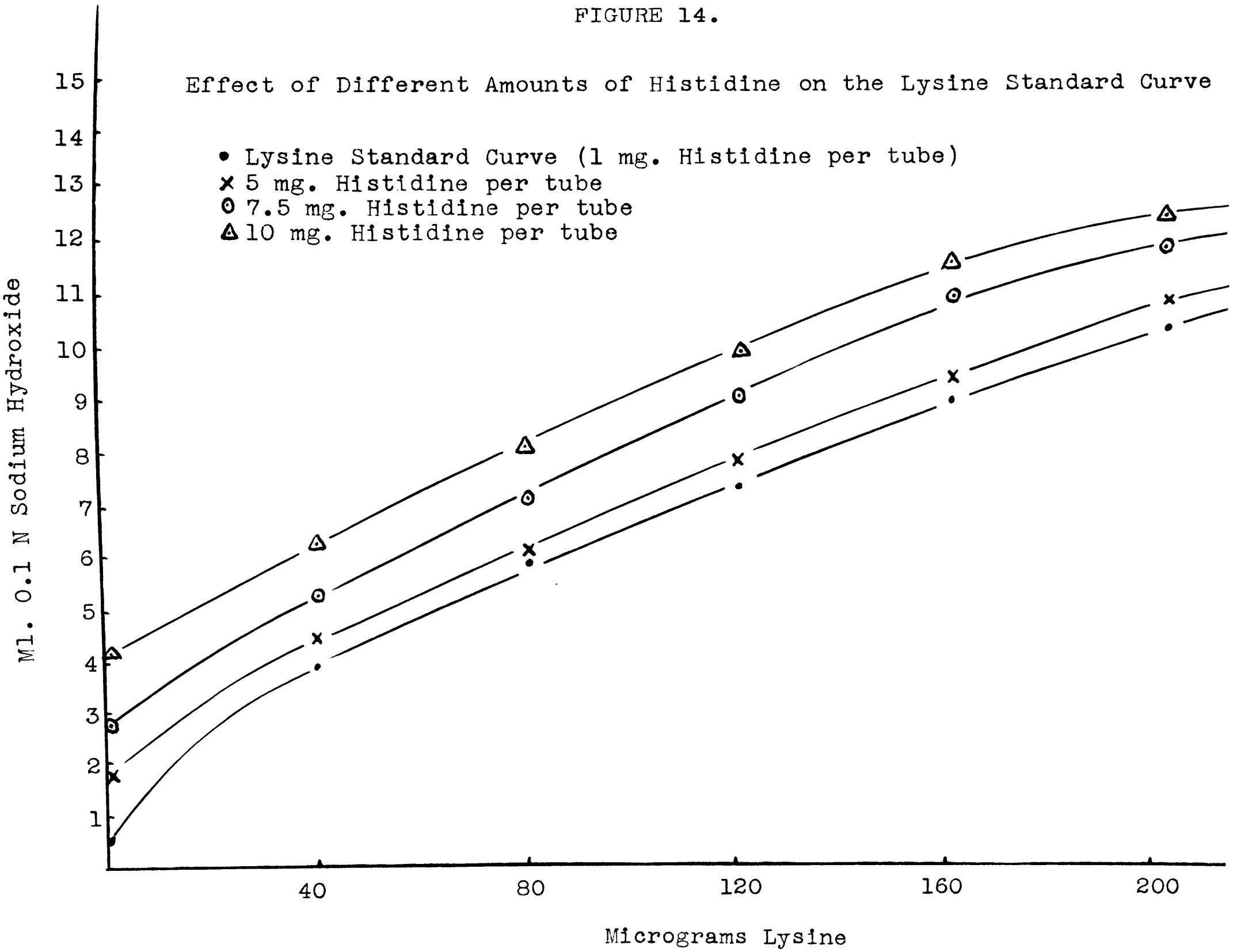


FIGURE 15.

Comparison of Lysine Standard Curves using Pure Amino Acids and Zein Hydrolysate

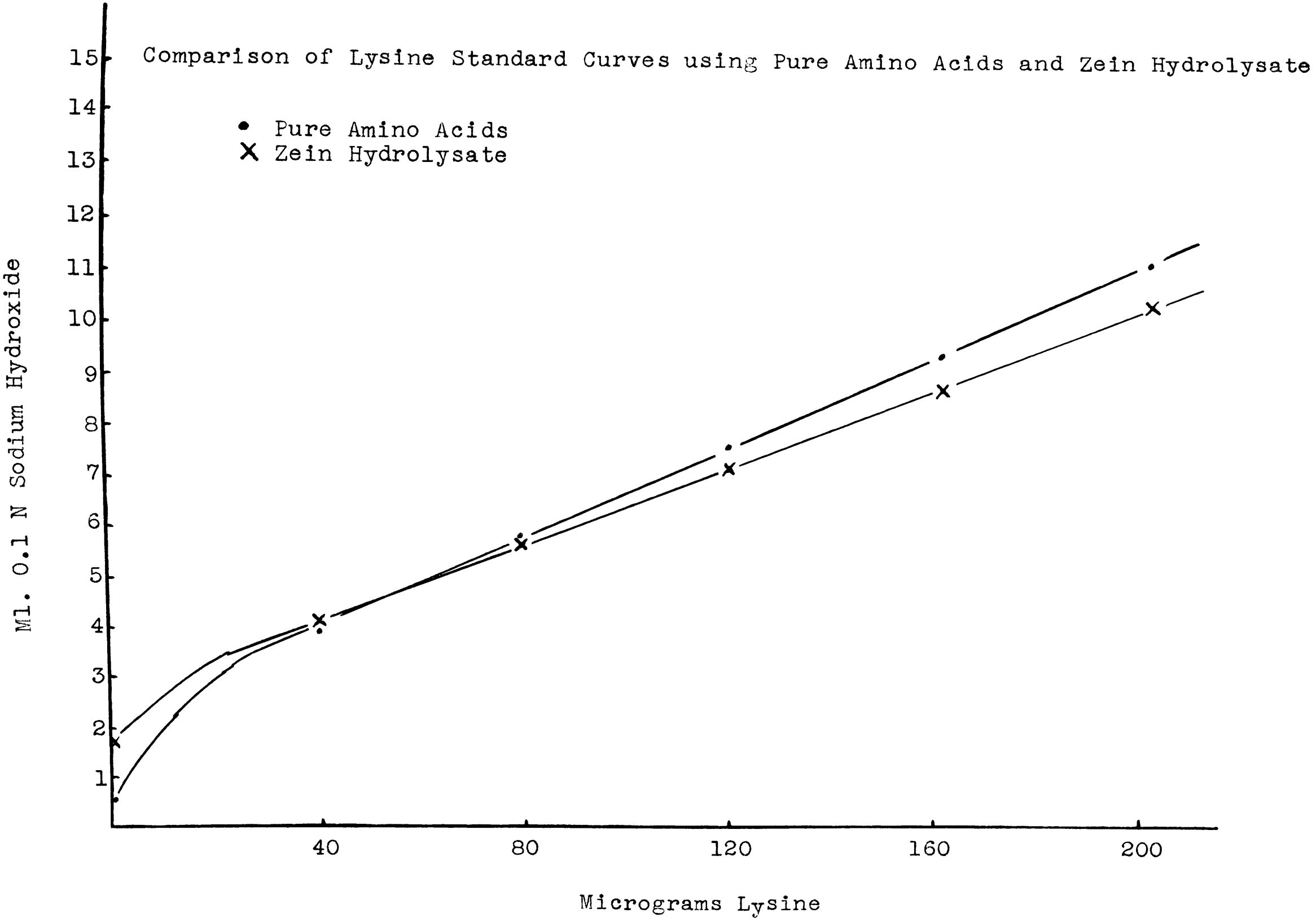


TABLE I

Effect of Concentration of Sodium Hypochlorite on the Colorimetric Determination of Lysine

Sodium Hypochlorite (% Available Chlorine)	Standard Solutions of Lysine γ/ml.	Galvanometer Reading	2-log G	Lysine Found γ/ml.
5.0	10	72.0	.143	10.05
2.5	"	71.0	.149	10.15
1.25	"	72.0	.143	10.05
0.675	"	72.0	.143	10.05
5.0	30	39.0	.409	29.00
2.5	"	38.5	.415	29.50
1.25	"	37.5	.428	30.13
0.675	"	38.0	.420	30.00

TABLE II

Basal Medium for Microbiological Assay

Amino Acids

dl-∝alanine.....	1,000 mg.
l-arginine HCl.....	250
dl-aspartic acid.....	800
dl-glutamic acid.....	1,000
glycine.....	100
l-histidine HCl.....	100
dl-isoleucine.....	200
l-leucine.....	100
dl-methionine.....	100
norleucine.....	100
norvaline.....	100
dl-phenylalanine.....	100
l-proline.....	200
dl-serine.....	100
dl-threonine.....	500
dl-valine.....	100

Dissolved in 100 ml. glass-distilled water

l-cystine.....	100 mg.
dl-tryptophan.....	100

Dissolved in 25 ml. 1N HCl

l-tyrosine.....	100 mg.

Dissolved in 25 ml. 0.1N NaOH

TABLE II (Continued)

Vitamins

thiamine·HCl.....	1 mg.
pyridoxine.....	1.6
nicotinic acid.....	2
riboflavin.....	2
calcium d-pantothenate....	1
para-aminobenzoic acid....	100 gamma
biotin.....	5
folic acid.....	1

Bases

adenine.....	12 mg.
guanine.....	12
uracil.....	12

Dissolved in 20 ml. 6N HCl

xanthine.....	12 mg.

Dissolved in 10 ml. 0.1N NaOH

Heat may be required to dissolve the bases

Other Materials

glucose.....	20 gm.
sodium acetate (anhydrous)	12
NH_4Cl.....	6
NaCl.....	5
*Salt solution A.....	5 ml.
*Salt solution B.....	5 ml.

Dissolved in 100 ml. glass-distilled water

* - See Barton-Wright (5)

TABLE III

Amino Acid Composition of Zein

Amino Acid	References 1	2	3	Others	Probable Average Values	Approximate wt. in 5 g. Zein	Quantity required per 100 tubes	Quantity Lacking in 5 g. Zein
	%	%	%	%	%	mg.	mg.	mg.
Glycine	0	0	0		0	0	100*	100
Alanine	9.8	9.9	9.9	8.9(33)	9.8	500	500	
Valine	1.9	3.0	2.4	2.2(66)	2.5	125	50	
Leucine)) Iso-) leucine)	25.0	25.0	23.7	27.2(66)	20.0	1,000	100	
		5.0	4.3		5.0	250	100	
Phenyl-alanine	7.6	6.6	6.4	5.03(42)	7.0	350	50	
Threonine		2.5	2.4		2.5	125	250***	125
Tyrosine	5.9	5.9	5.0	3.66(87)	5.0	250	100	
Trypto-phane	0.2	0.2	0.1	0.0(87)	0.0	0.0	50*	50
Glutamic Acid	31.3	35.6	35.6	24.8(50)	30.0	1,500	500	
B. Hydroxyglutamic Acid	2.5				2.5	125		

TABLE III (Continued)

Amino Acid Composition of Zein

Amino Acid	References			Others	Probable Average Values	Approximate wt. in 5 g. Zein	Quantity required per 100 tubes	Quantity Lacking in 5 g. Zein
	1	2	3					
	%	%	%	%	%	mg.	mg.	mg.
Aspartic Acid	1.8	3.4	3.4		2.5	125	400**	275
Proline	9.0	9.0	9.1	8.4(32)	9.0	450	100	
Serine	1.0				1.0	50	50	
Hydroxyvaline	1.5				1.5	75		
Cystine	0.8	1.0	0.8	0.58(86)	0.8	40	100	60
Methionine	2.3	2.5	2.0		2.3	115	50	
Arginine	1.8	1.6	1.6	1.99(97)	1.6	80	250**	170
Histidine	0.8	0.8	0.9	0.88(97)	0.9	45	100	
Lysine	0.0	0.0	0.0	0.06(106)	0.0	0	0	
Norvaline							50	50
Norleucine							50	50
Hydroxyproline			1.0	0.8(32)	0.8	40		

* - completely deficient
** - somewhat deficient
*** - may be adequate

1 - Schmidt C.L.A. The Chemistry of the Amino Acids and Proteins (Thomas) 2nd Ed. 1947. p. 217.
2 - Anson, M.L. and Edson, J.T. Advances in Protein Chemistry, Vol. II. (Academic) 1945.
3 - Block, R.J. and Bolling, B.S. The Amino Acid Composition of Proteins and Foods. (Thomas) 1945.

Microbiological Determination of Lysine in Casein Using Zein Hydrolysate as Source of Amino Acids in Medium

Casein per ml. γ	Titration Volume 0.1 N NaOH per tube ml.	Lysine Found per tube γ	Lysine Found per ml. γ	Lysine Found % of Casein
500	4.10	38	38	7.60
500	4.10	38	38	7.60
1,000	5.60	76	38	7.60
1,000	5.70	77	38.5	7.70
1,500	7.15	118	39.3	7.86
1,500	7.15	118	39.3	7.86
2,000	8.65	158	39.5	7.90
2,000	8.65	158	39.5	7.90
2,500	10.10	196	39.2	7.84
2,500	10.20	198	39.4	7.88
			Maximum Deviation 3.8%	Average 7.77 Moisture Free 7.85

TABLE V

Recovery of Added Lysine From Casein and Casein Hydrolysate Using Zein Hydrolysate

	Lysine Added per ml. γ	Lysine Found per ml. γ	Lysine in Casein per ml. γ	Recovered γ	Recovered %
Casein	--	39.4	39.4	--	--
Casein	20	57.8	39.4	18.4	92
Casein Hydrolysate	20	58.7	39.4	19.3	97

TABLE VI

Lysine in Stored Foods*

	Original		6 Months		12 Months		18 Months	
	Chemical Method	Microbiological Assay	Chemical Method	Microbiological Assay	Chemical Method	Microbiological Assay	Chemical Method	Microbiological Assay
Soybean Meal	2.3**	3.4	2.0	3.0	2.3	3.2	2.3	3.7
Meat Meal	0.8	1.3	1.0	1.6	1.0	1.0	1.7	1.3
Ground Oats	0.3	0.25	0.3	0.25	0.2	0.25	0.1	0.22
Dehydrated Potatoes	0.5	0.25	0.5	0.26	0.4	0.27	0.5	0.24

* - Stored at 37° and high humidity

** - Per cent of moisture-free material

TABLE VII

Comparison of Chemical and Microbiological Methods for Determination of Lysine Changes of Stored Milk Powders

	Original	Storage Temperature °C	6 Months		12 Months		18 Months	
			Modified Chemical Method					
			H	L	H	L	H	L
Drum-Dried	2.7*	27	2.5	2.7	2.2	2.7	1.9	2.6
Skim Milk		37	2.1	2.7	1.8	2.6	1.6	2.6
Spray-Dried	2.5	27	2.4	2.5	2.2	2.4	2.1	2.4
Skim Milk		37	2.15	2.4	1.9	2.4	1.6	2.4
			Modified Microbiological Method					
Drum-Dried	3.43	27	3.02	3.32	2.76	3.28	2.51	3.21
Skim Milk		37	2.54	3.30	1.93	3.29	1.54	3.24
Spray-Dried	3.07	27	2.94	3.10	2.82	2.91	2.74	2.82
Skim Milk		37	2.52	3.06	2.09	2.90	1.76	2.81

* - Values in % of Milk Powder (Moisture-free)
H - High Humidity
L - Low Humidity

Part II

Changes in Stored and Heated Foods

REVIEW OF LITERATURE

Fundamental Studies of the Browning Reaction.

The browning reaction.

It has been known for many years that carbohydrates and proteins react together under certain conditions to form brown or black complex materials, and the formation of such materials has been closely studied because of its significance in the "browning" of foods. The interaction of carbohydrates and proteins has also received attention in connection with its interference with various analytical procedures for the determination of carbohydrates and proteins or amino acids. Thus it is known that in the presence of amino acids, the reduction of Fehlings solution is inhibited (54, 34). Barfoed's reagent is unaffected by glucose in the presence of glycine (97). Polarimetric methods of estimating sugars have been shown to be influenced by the presence of amino acids (37, 40, 190) and conversely, the determination of amino nitrogen by the Van Slyke manometric procedure is affected by the presence of sugars (54, 34). The study of these interferences has contributed also to the knowledge of the browning reaction itself, although the interactions do not necessarily proceed to the stage of formation of brown materials.

In 1902 Samuely (147) first investigated the cause of the formation of dark substances during acid hydrolysis of proteins. He suggested that the initial reaction in the formation

of such substances was a condensation between a carbohydrate and the available amino groups of the protein or of the amino acids formed during hydrolysis. Samuely heated glucose with various nitrogen-containing compounds and noted the quantity of "melanoidin" (dark substances) formed during the reaction.

However, Maillard (106) may be regarded as the originator of detailed studies on the interaction of sugars and proteinaceous compounds. He observed that the s trength of the aldehydic function of the sugar involved, was closely related to the rate and intensity of the interaction. Any increase in aldehydic function greatly increased both rate and intensity of the reaction. Consequently, this sugar-protein or "browning" reaction has been referred to as the "Maillard Reaction". Numerous workers have extended Maillard's initial investigations and have studied various types of interacting systems, including mono-, di- and polysaccharides on one hand, and amines, amino acids, polypeptides and proteins on the other. Sprung (55) has reviewed the general reaction occurring between aldehydes and amines. The most usual type of reaction is one similar to the aldol condensation of aldehydes with the formation of Schiff bases.

$$R\text{-}CHO + R^1NH_2 \rightleftharpoons RCH(OH)\text{-}NHR^1 \rightarrow RCH = NR^1 + H_2O.$$

Primary amines apparently react more readily than secondary amines, while tertiary amines, unless part of a cyclic structure, do not enter into the reaction. It has been shown that many aldehyde-amine compounds polymerize, and it may well

be that polymerization is a step in the formation of the dark brown substance produced when sugars and nitrogenous compounds react.

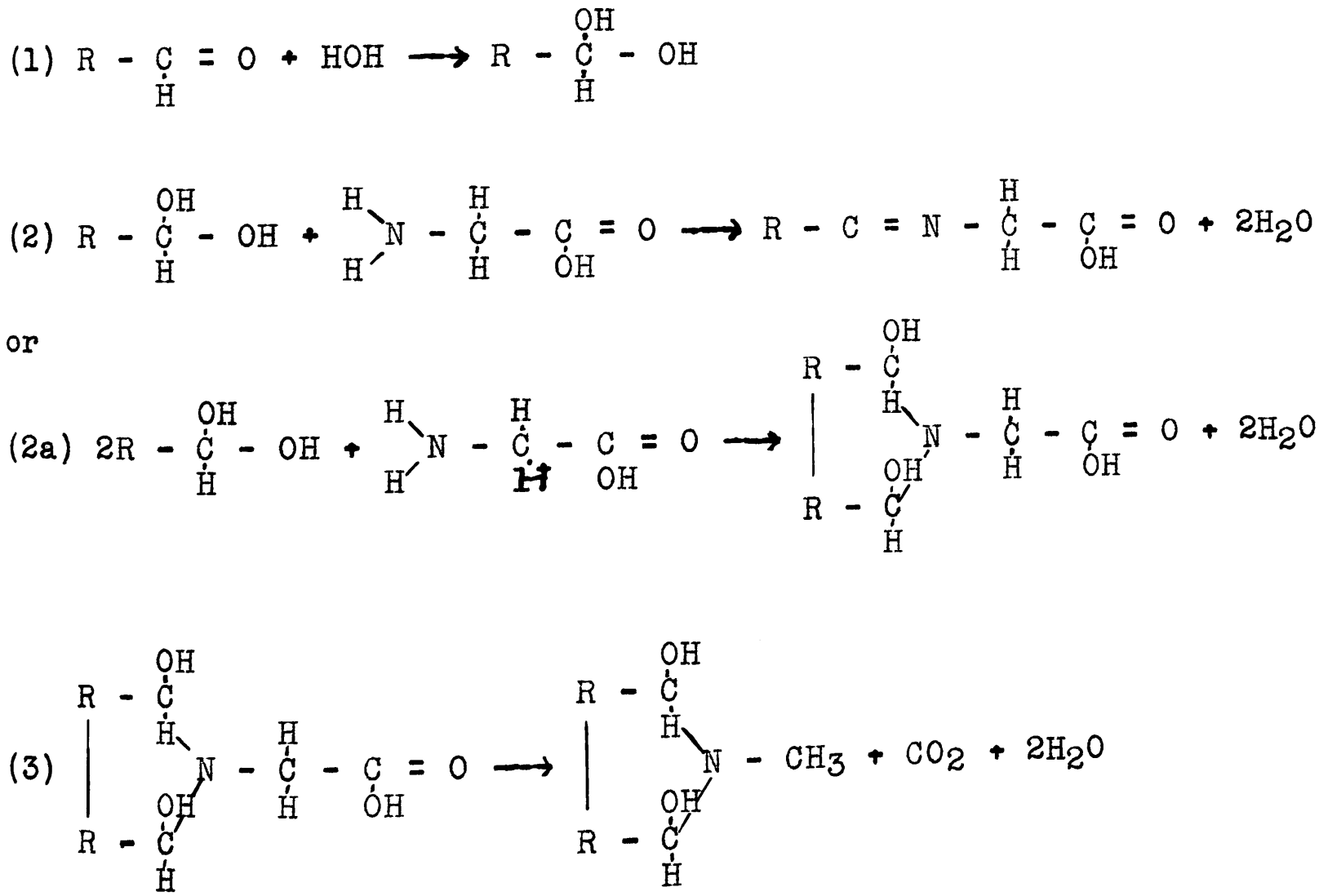
In a study on the interaction of glucose and glycine von Euler and his co-workers (41, 42) showed that the reaction proceeded faster in the alkaline pH range. Cryoscopic measurements showed that a decrease of the number of molecular species occurred, indicating definite chemical combination. Frankel and Katchalsky (51, 52), in a study of the interaction of amino acids and sugars in solution, noted a decrease of basicity under mild reaction conditions. They attributed this to the loss of the basic amino groups when these groups combined with the aldehydic group of a sugar. The same results were not observed when a non-aldehydic sugar was mixed with an amino acid. Maillard (106) showed that the sugar-amino acid reaction did not require atmospheric oxidation although carbon dioxide was formed in the process. He also demonstrated that during the period up to the completion of the evolution of carbon dioxide all the brown material produced was water-soluble. Water-insoluble products were formed until after all the carbon dioxide had been evolved. It was noted that the reaction between amino acids and sugars could be stopped at any stage by desiccation and started again by the addition of water.

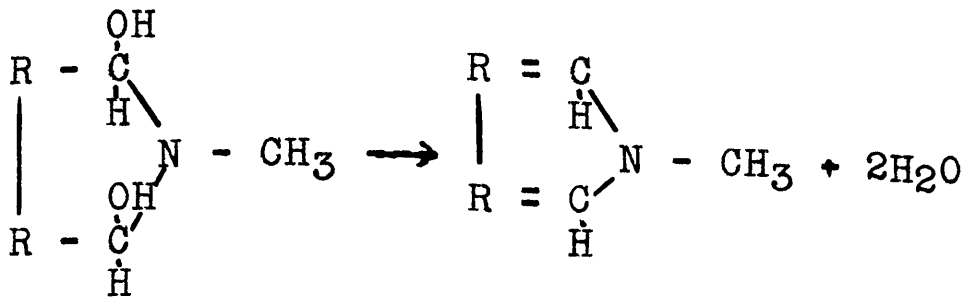
Grunhut and Weber (63) studied the course of the Maillard reaction by means of formol titrations to follow the decrease

in amino groups and Fehling titrations to follow the decline in reducing groups. These investigators suggested three distinguishable stages of the reaction:

1. Interaction to form a product still containing a primary amino group.
2. Loss of the amino group either by substitution or condensation.
3. A final stable state which is achieved by decarboxylation.

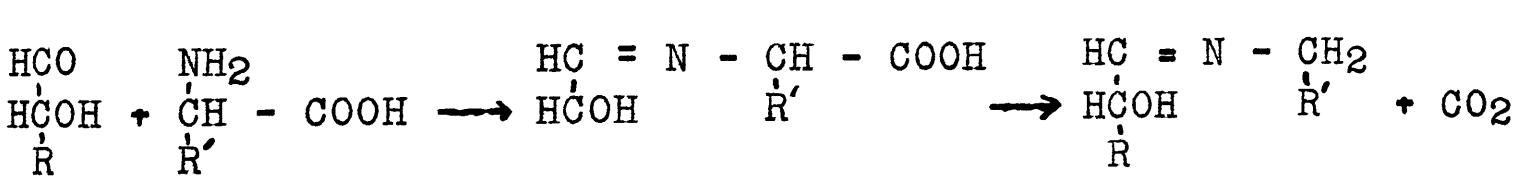
Enders (39) has reviewed the entire subject and suggests the following stages in the sugar-protein reaction:



(4) 

By this process, pyrrole compounds may be formed. These have been reported to be present during the deterioration of dried egg products (129A).

According to Maillard (106) the loss of one mole of carbon dioxide is accompanied by the loss of twelve moles of water. The initial stages of the reaction were postulated to be similar to the reaction between aldehydes and amines, i.e. a condensation leading to the production of Schiff bases followed by decarboxylation of the amino acid portion:



Du Toit and Page (35) have shown that the Maillard reaction may be inhibited by blocking the amino groups with formaldehyde. Stewart (162, 159) showed that complete removal of the reducing sugar from whole egg-white by fermentation or dialysis inhibited the deterioration (browning) of the product. Hurd and Kelso (80) have recently suggested that an α-hydroxy group is necessary for the browning reaction to occur. In support of their suggestion they showed that 3,4-didesoxy-aldopentose browned even more than glucose when heated with glycine, while 2,3,4-tridesoxyaldopentose gave only a slight discoloration.

Within the last few years attention has been focussed by several investigators on the possible role of 5-(hydroxymethyl)-2-furfuraldehyde in the browning reaction (148, 141, 153, 186). These authors have suggested that this substance is an intermediate product in the Maillard reaction. Seaver and Kertesz (151) attempted to show that uronic acids are intermediates in the browning reaction, as these substances browned at a greater rate than common sugars when heated with amino acids.

Thompson, Kocher and Fritzsche (169) have described reactions involving proteins and copper with the formation of coloured compounds and development of fluorescence. They showed that proteins can bind copper from copper oxide to form a complex which promotes browning to a high degree. Other investigators have shown that metals catalyse the browning reaction (175, 165). Thus it has been suggested (153) that the Maillard type of reaction may not be responsible for all of the browning of stored foods.

The role of lysine in browning.

It has been observed (56, 116) that heating dry casein at 140 - 200°C only slightly lessens the digestibility of the protein but considerably impairs the nutritional value for rats when fed at eighteen per cent or lower protein levels. It was found that supplementation of the diet with 0.2 per cent lysine overcame this deficiency and the authors attributed the

reduction in growth to injury of the lysine present. Similar observations were made by Waisman and Elvehjem (173) on autoclaved edestin and by Block et al (6) on a cake mixture. However, chemical analysis of heated casein (7, 191) detected little significant decrease in the amount of lysine present when estimated after acid hydrolysis of the casein. Eldred and Rodney (38) demonstrated that enzymatic digestion of heated casein differed only slightly from that of unheated casein, but that the available lysine, as determined by the specific enzyme, lysine decarboxylase, was appreciably less. The explanation for this phenomenon was that when casein was heated, the lysine formed a linkage which was unavailable for enzymatic digestion, but was susceptible to attack by strong acid. A similar conclusion was reached by Harris and Mattill (67) and by Patton et al (127). Neuberger and Sanger (122) had shown previously that ε-N-acetyllysine was nutritionally adequate for the rat but that ε-carbobenzoxylysine was not. Adamson (1) has also shown that heating of dl-lysine produced forty per cent dl-3-aminohomopiperidine, with a free ε-amino group available for linkage. Therefore it seems plausible that the ε-amino group of lysine enters readily at higher temperatures into linkages which are unavailable for digestion in vivo.

Stevens and McGinnis (158) studied the effect of autoclaving lysine in the presence of carbohydrates on its utilization by chicks. They showed that lysine, when autoclaved

by itself, was capable of supplementing an overheated soybean oil meal for chick growth as effectively as was the untreated lysine. However, when lysine was autoclaved with cerelose, it was either destroyed or otherwise rendered unavailable for chick growth. They suggested that the darkening of autoclaved soybeans could be due to a reaction between the carbohydrates and amino groups, but that a similar reaction does not occur on heating casein since casein contains little carbohydrate. Patton et al (127) refluxed casein with five per cent glucose solution and found that the lysine, arginine and tryptophane present lost about one third of their original activity as determined by microbiological assay. Evans and McGinnis (46) and Reisen et al (140) found that after autoclaving soybean oil meal only fifty to seventy per cent of the original lysine was available for growth of the microorganisms Streptococcus faecalis R and Leuconostoc mesenteroides P-60. The reason for this observation was not clear at the time because similar autoclaving of lysine by itself did not in any way impair its nutritional value. In the light of later work it is reasonable to assume that in this case autoclaving the soybean oil meal had brought about actual destruction of lysine.

It seems clear that two types of heat inactivation of lysine must take place, one being a destruction of the lysine, and the other a binding of the lysine in some form such that it is not liberated by digestion in vivo or by enzyme hydrolysis in vitro, but is liberated by acid hydrolysis. The

temperature and time of heating appear to be related to the amounts of lysine bound and destroyed. Evans and Butts (44) have confirmed the above conclusion by autoclaving soybean protein with and without added lysine and determining the amount of lysine destroyed or inactivated by acid and by enzymatic hydrolysis. Approximately sixty per cent less lysine was liberated by enzymic digestion *in vitro* from the autoclaved meal than from the unheated meal and approximately twenty per cent of the added lysine was converted to a form from which active lysine could be liverated by acid but not by enzyme hydrolysis *in vitro*.

Sucrose was apparently the cause of most of the lysine destruction which occurred when soybean oil meal was autoclaved. Very little loss of lysine occurred when soybean protein was autoclaved in the absence of this sugar, but approximately twenty-five per cent of the lysine was converted to a form from which biologically active lysine was liberated by acid but not by enzymic hydrolysis *in vitro*. The addition of twenty per cent sucrose to the soybean protein resulted in a fifty per cent destruction of the lysine by autoclaving. Dry heat treatment did not destroy or inactivate nearly as much lysine as did autoclaving at the same temperature for the same time.

A theory of Melnick, Oser and Weiss (112) has advanced our understanding of the role of lysine in digestibility studies. They suggested that the nutritional efficiency of

a protein depends not only upon its amino acid composition, but that the amino acids must be capable of liberation by enzymatic hydrolysis in vivo at rates permitting mutual supplementation. Pader, Melnick and Oser (126) showed that lysine is liberated only one quarter as rapidly from heated casein as from unheated casein by enzymatic hydrolysis in vitro. Therefore, if in vivo digestion proceeds at the same rate as in vitro digestion, the reason for an apparent lysine deficiency in autoclaved proteins is readily concluded.

The Browning Reaction in Foodstuffs.

Dairy and poultry products.

Milk and condensed milk:- Jensen and Plattner in 1905 (85) observed that the heating of milk to high temperatures caused the development of a brown discolouration. They attributed this to some reaction of the casein. Wright (188) determined the effect of heat on milk by separating the protein after heating. He found the separated product to be dark yellow brown, whereas the control remained perfectly white. He measured the change in the racemization of the protein after different heat treatments and showed that there was no appreciable change in the protein as judged by this criterion. He concluded that the protein had no part in the formation of the brown discolouration. When he heated casein and lactose together the brown colouration rapidly appeared. He attributed this result to caramelization of the lactose,

and considered it possible that the protein everted a catalytic action. Kometiani's (94) results substantiated this conclusion, as his data showed no change in the amino nitrogen content of heated milk and only a relatively slight rise in the formol titration values. Other workers have also ascribed the discolouration to caramelization (102, 154).

An alternative explanation of the origin of the brown colour was first suggested by Jensen and Plattner (85), who pointed out that solutions of either casein or lactose heated separately do not brown at all. Since a mixture of the two substances does discolour at elevated temperatures, these workers postulated an interaction of unspecified nature between casein and lactose as the cause of the browning.

Following studies by Englis and Dykins (40) who investigated the difference between glucose, a reducing sugar and fructose, a non-reducing sugar and their respective interactions with amino acids, Ramsey et al (139) proposed an aldo-amino group reaction. These authors stated that caramelization played no role in the discolouration of milk at elevated temperatures.

Webb (175) studied the effect of various amino acids and buffers at different pH ranges on solutions of pure lactose and found that the intensity of the colour formed on heating depends on the pH, and on the nature and concentration of the buffer. Phosphates appeared to exert a specific caramelizing effect. Webb concluded that a sugar-amino combination

accounts for a major portion of the colouration of autoclaved milk, although the contribution of the caramelizing effect of the milk phosphates was probably considerable. He also showed that the presence of copper or iron catalysed the browning reaction, whereas tin and sulfite retarded the production of colour.

Kass and Palmer (91) suggested that the brown discolouration of heated milk is by no means a simple stoichiometric reaction between lactose and amino groups, but that it is a "complex, and progressive formation of a melanoid". They attributed the origin and behaviour of the brown discolouration to the caramelization of the lactose by the casein and subsequent adsorption of the lacto-caramel by the colloidal caseinates.

Coulter and Combs (24) have studied synthetic systems containing varying proportions of all the chief components of milk including casein, serum proteins, lactose, and phosphate buffer. After discussing their own results and those of other workers, they suggested three main reactions as the cause of milk spoilage:

1. Interaction of protein and lactose - (139).
2. Lactose caramelization - (91).
3. Combination of 1. and 2. - (175).

In a study on the acid formation during heating of milk, Whittier and Benton (181) showed that acid is formed in direct proportion to the time and temperature of heating and of

lactose concentration. Coagulation of the casein had no effect on the rate of acid formation and the authors concluded that lactose is the principle source of the acid produced by heating milk.

Dried milks and eggs:- A common cause of spoilage of dried milks and especially of dried whole milk is oxidative change attributed to the fat components. Little or no browning occurs when this is the sole type of deterioration present. The cause of browning of dried milk has been attributed to the protein and carbohydrate compounds in the product (188). Flavour deterioration commonly accompanies browning. The resultant flavours having been defined by various workers (164, 79) as "stale", "musty" or "gluey". Lea et al (100) described powders stored at 47°C and 37°C for some time, as "heated", "burnt", "scorched" or "cooked". This flavour was considered to consist of two components (a) a "burnt" or "caramel" taste associated with the protein or carbohydrate, and (b) a "butter-toffee" taste associated with the fat. They noted loss of solubility of the milk powders after lengthy storage periods.

Little quantitative information on the browning of milk powders was available before 1942, when Doob, Willmann and Sharp (33) investigated the effect of moisture on the browning of dried whole milk and whey. They showed that browning was related to temperature, time of storage and moisture content. According to these authors, browning is markedly accelerated

at temperatures over 30°C. However, even at 50°C, discolouration could be inhibited by low moisture content. They stated that the pH of the stored milk and whey powders decreased as the browning increased. These results were substantiated by Tarassuk and Jack (165) who also added to the causes of browning such minor items as contamination with iron or copper and the presence of polyphenol substances added as anti-oxidants. They showed that browning is accompanied by (a) production of CO_2, (b) uptake of O_2 (c) increase in reducing groups, (d) marked decrease in solubility (e) increase in titratable acidity (f) development of caramelized flavours and (g) increase in the moisture content in the advanced stages of the reaction. Powders of less than four per cent moisture did not brown to any appreciable extent at 40°C storage temperatures. The development of stale or oxidized flavour stopped when browning commenced. Whether this is a masking or an inhibition effect was unknown.

Chapman and McFarlane (17) have developed a method for determining acid ferricyanide reducing substances in dry milk and showed that they increased during storage of dry milk in contact with the atmosphere. They showed (18) that these reducing substances inhibited the flavours due to oxidation of the fat in whole milk powders.

In 1945 Harland and Ashworth (66) suggested the use of thiamin di-sulfide for estimation of reducing power of milk. Apparently this reagent is a much weaker oxidant than

ferricyanide as it shows no reduction whatsoever by normal unheated milk, and even with heated milk, its sensitivity is not as great as that of ferricyanide. Consequently Chapman and McFarlane (17) expressed the opinion that these reagents must react with two different reducing systems. Lea (98) agreed with these workers as he has stated that the interaction of the proteins and lactose causes the increase of reducing substances. Moster (119) has substantiated the above conclusions by showing that the addition of tyrosine and other amino acids, with the exception of cysteine, to milk powders did not increase the acid ferricyanide reducing substances, although tyrosine by itself in alkaline solution is a strong reducing agent. Moster showed that the total reducing capacity of milk could be accounted for by the reducing substances formed from artificial mixtures of amino acids and lactose in proportions similar to those obtaining in milk. He also showed by dialysis experiments that the reducing substances formed in stale milk powder are associated with both the protein and sugar fractions. He concluded that the type of reaction producing both the reducing substances and browning, included degradation of lactose which is catalysed by the milk protein, and condensation of lactose or its decomposition products with the amino groups of the protein. This latter condensation is most probably through the ϵ-amino group of lysine, since this group forms the majority of free amino groups on the protein (171). Subsequently the availability

of lysine for digestion may be hindered, which evidence is in agreement with the results of numerous nutritional studies on the availability of lysine.

Modifications of the Chapman and McFarlane method have been advanced by Lea (98) and by Crowe et al (26). Holm et al (76) have shown that preheating milk prior to drying improved the keeping quality of the powder and explained this by suggesting that reducing substances were formed which acted as antioxidants in the whole milk powder.

Fluorescence production has been shown to be associated with deteriorative changes of certain food products. Workers at the National Research Council of Canada have shown that heat treatment and storage of dried egg and of reducing sugar-protein mixtures increase the blue fluorescence of a ten per cent potassium chloride extract of the material (134, 167, 178, 179, 180, 187, 131).

Numerous workers (23, 5, 69, 124, 132, 159, 36) postulated that fluorescence was the result of interaction between reducing sugars and proteins.

Dutton and Edwards (36) suggest that fluorescence of lipids of eggs may be caused by interaction of lipid amines with aldehydes. Pearce and Thistle (133) and Thistle et al (166) have suggested the use of fluorescence of the fat-free portion as a measure of the deterioration of dried egg products. However other workers (8, 50) indicated a closer relation between loss of palatability of stored egg products

and the increase of the fluorescence of the lipid fraction. Pearce (129) showed the development of fluorescence in many different types of food products.

Information on the fluorescence of milk products is not abundant. It is well known that milk emits a yellow fluorescence under ultra violet light (32, 138), and that this fluorescence is principally due to riboflavin. Radley and Grant (138) showed that heat treatment changed the yellow fluorescence to a blue colour and attributed this to riboflavin breakdown.

Pearce (130) found greater initial blue fluorescence in extracts from spray-dried whole milk than in those from roller-dried milks and both types of milk powders showed decided increases in fluorescence on storage. He could not show any correlation between loss of palatability and increase of fluorescence. He concluded that fluorescence is not a satisfactory index of palatability in milk powder.

Jenness and Coulter (84) reviewed the situation and studied the fluorescence of various fractions of milk. They showed that riboflavin, the lipids and the proteins all were partly responsible for fluorescence. The normal processing of dry whole milk appeared to be without effect on the fluorescence of these components.

Pearce (130) compared the suitability of a number of objective tests with subjective scores of palatability and concluded that none of the tests were as accurate as

palatability for the determination of deterioration. This is not at all surprising when the complexities of the system are considered and it is remembered that the "taste is always the final test". It is assumed that the nutritional qualities of dried milk are in general related to palatability. It is advisable to point out that this assumption is not as yet by any means a proven fact.

Coulter et al (25) have conducted a somewhat extensive study of the non-lipid changes in dried whole milk during storage (20°C, 37°C, 60°C) and found the following to occur: (a) development of a stale or "burnt feathers" flavour (b) production of acid ferricyanide reducing substances (c) production of carbon dioxide (d) loss of oxygen (e) increase of fluorescence (f) loss of lactose (g) increase of acidity (h) loss of protein solubility (i) increase in browning. The rate of change for the above variables increased logarithmically in the initial stages, with increase in the vapor pressure of the water in the system.

Several investigators have studied the decrease of solubility of dried milk products. Wright (189) and Howat and Wright (78) suggested two types of protein insolubility of milk powders takes place through the action of heat: (a) insolubility due to denaturation of the protein while still in solution (b) insolubility due to overheating of the powder. Lea (99) suggested that the milk sugars exert a marked influence on the protein insolubility by reason of the

reducing sugar-amino group reaction. He also suggested that non-reducing sugars may retard the development of insolubility.

Since the initiation of this present study the results of a most thorough coordinated investigation of the changes of skim milk powder during storage have been published by Henry and her co-workers (74). Milk powders of different moisture contents were stored at 20°C, 28.5°C and 37°C for a period of two years. Various chemical, physical and nutritional tests were carried out on the stored materials. The powders of the lowest moisture and at the lowest storage temperature showed little change. On the other hand, the high-moisture powders stored at 37°C rapidly became unpalatable, discoloured and insoluble. The pH, free amino nitrogen and lactose content of these powders decreased; oxygen was absorbed, carbon dioxide was produced and acid ferricyanide reducing substances increased.

The authors attributed the major cause of deterioration of the high-moisture powder to a reaction involving the free amino groups of the milk protein which consist very largely of the ϵ-amino groups of the lysine residues (171). The first stage of the reaction appeared to the authors to be a linkage between the protein amino groups and the aldehydic groups of the reducing sugar. The rate of the reaction was much greater at 7.6 per cent moisture than at five or three. The sugar-protein reaction was postulated to take place in at least two stages, the initial combination resulting in neither

discolouration nor loss of solubility, which characteristics follow only as a result of a secondary stage.

Off flavours ascribed by Henry et al (74) to stored milk powders, were "caramelized" in the case of inert gas-packed powders and as "stale" and "gluey" in the case of air-packed powders. Some off flavour was noticed in dry milks of a very low moisture content and these flavours were attributed to fat oxidation. This latter change was unnoticed in gas-packed powders.

Of the chemical tests conducted by these investigators, only the Chapman and McFarlane method for the determination of reducing substances showed promise when correlated with palatability changes.

The nutritional tests carried out by Henry et al (74) showed that as the browning of the milk progressed, the biological values similarily declined. Addition of 1.25 per cent lysine to badly-deteriorated samples restored the biological value to its original figure. The addition of histidine caused a slight improvement but added arginine yielded no increase in biological value of deteriorated milk powder.

Hodson and Krueger (75), by means of micro-biological assay, showed that in four-year-old samples of skim milk powder, twenty-two per cent lysine, nineteen per cent histidine, ten per cent arginine, nine per cent tryptophane and twelve per cent methionine were lost due to deterioration.

Cereals and cereal products.

Leavitt and Leclerc (101) in 1909, observed changes in the nitrogenous components of cereals in storage. They noted that solubility of nitrogen in water and alcohol decreased as the storage time of grains increased. During the next few decades several workers studied the biological values of the various cereal proteins but because of a lack of uniformity of methods the results are not comparable. Morgan and King (118) and Morgan (116) compared the relative gains in body weight of rats fed raw and heated proteins. They demonstrated that baking, toasting and "exploding" of cereals impaired the growth-promoting value of wheat proteins. Similarily Greaves and Hirst (55) showed that enzymes could affect the composition of flour on storage. Murlin et al (120), Mitchell and Block (115) and Jones et al (87) showed that the proteins of oats were definitely superior to the other grains with rye and rice close seconds. Corn, wheat and barley were poor substitutes for casein in rat feeding trials (87). Kuether and Myers (95) showed that the proteins of both rolled and exploded oats were equally well utilized as dietary nitrogen supplements. However these oat products were deficient in lysine. Stewart et al (161) and Stewart and Carroll (160) also noted lysine destruction in heated and exploded oats. Mitchell (114) showed that exploded wheat lost thirteen per cent of its protein value.

Most intensive studies on the changes in the protein of

wheat and corn during storage were carried out by Jones and Gersdorff in 1941 (89). These authors carefully examined the changes of various nitrogenous fractions of the proteins of wheat during storage. They noted a decrease of solubility, an increase of amino nitrogen and a decrease of true protein over a period of two years. They attributed these changes to the enzymes known to occur in wheat. Proteinases and oxidases were blamed but the details of the reactions were not stated.

In another paper on the storage of corn, Jones, Divine and Gersdorff (88) studied the change in the biological value of the corn proteins on storage. They noted that the rats on the feeding trial ate less of the stored feed than the fresh material, and also that the gain of body weight per gram of protein eaten decreased as the storage period of the corn increased. The authors suggest that the chemical changes, viz. decrease of solubility, increase in free amino nitrogen and a decrease in digestibility *in vitro* which they found occurring in stored grains, may also be the cause of the loss of biological value and perhaps of palatability.

Peters *et al* (135) have published some interesting results on the effect of heat on oat proteins. They showed by microbiological assay, that severe heating decreased the quantity of lysine in the oat proteins. By means of *in vitro* enzymatic studies they showed that heat treatment of insufficient severity to cause lysine destruction might nevertheless result in considerable unavailability of lysine to pancreatic

attack. These authors suggest that the damaging effect of heat processing on the growth-promoting efficiency of oat proteins may be accounted for principally by the changes in lysine content and lysine linkages.

Soybean oil meal.

With the development of solvent extraction for the removal of oil from soybeans, a protein product of high nutritional quality has been produced. However it has been shown by Osborne and Mendel (125) and by Robison (145) that feeding cooked soybean oil meal improved the growth of rats and swine respectively, over that obtained with the raw meal. Wilgus, Norris and Heuser (183) reported that a short heating time yielded a most nutritive feeding material, only slightly inferior to fish meal as a protein supplement. Klose et al (93) showed a deficiency of methionine, lysine and leucine in over-autoclaved soybean meal.

Several investigators (71, 72, 86) reported that heat made the nitrogen complex of the soybean more available for digestion but that the heat inactivated the cystine in the protein. An early explanation (70) for the marked improvement of soybean meal after heating, was that the raw protein was deficient in available cystine and that heating increased the available amount of this amino acid.

The discovery by Ham and Sandstedt (65) and by Bowman (12) of a definite trypsin inhibitor in soybean oil meal has partially corrected our misunderstanding of the effect of heat

on soybean proteins. Kunitz (96) showed this trypsin inhibitor to be of a proteinaceous nature and actually crystallysed it in pure form. Borchers et al (10) have devised a scheme whereby adequately autoclaved soybean oil meal may be identified in chick rations, by determining the quantity of trypsin inhibitor in the meal. Gerry et al (53) found that the addition of dl-methionine reduced the effect of the growth inhibitor in unheated soybean meal, but that this amino acid was not the only factor concerned. Other workers have advanced our knowledge concerning the trypsin inhibitor (9, 176, 177).

Several investigators have realized that the trypsin inhibitor does not account for all of the deficiencies of soybean oil meal as a protein supplement in livestock rations. De and Ganguly (28) separated glycinin, the soy protein from the whole bean and compared the protein efficiency of the whole soybean with that of its protein alone. These authors showed that heat is not essential for enhancing the nutritive value of the protein in raw soybean as glycinin was more effective in promoting growth than autoclaved soybean. Evans and McGinnis (48, 47, 111, 43) have conducted a thorough investigation of the effect of heat treatment of soybean meal on its nutritive value. These authors suggested that cystine and/or methionine are the limiting factors in heated soybean meal.

Reisen et al (140, 19, 20, 21) have studied the subject of amino acid impairment of heated soybean meal and have published several papers on the problem. They showed (1) that

the liberation of lysine, arginine and tryptophane was decreased, on hydrolysis, by long heating of the soybean and (2) that the amount of each of the essential amino acids liberated by pancreatic hydrolysis was increased by a four minute autoclaving period but was decreased by a four hour period. Supplementation of over-heated soybean meal with lysine and methionine gave improved chick growth, comparable to that of the adequately-heated meal. The theory postulated by Melnick, Oser and Weiss (112) that the rate of hydrolysis of amino acids by enzymes in vivo, permitting mutual supplementation of the amino acids, should be considered here. Although nothing has been reported concerning the rates of liberation of amino acids from soy protein, the results given by Reisen et al indicate that lysine, arginine and tryptophane may not be released as fast as the other amino acids. Consequently the ability of these three amino acids to mutually supplement the other amino acids in vivo is probably restricted.

Further studies by Evans and Butts (45) on the hydrolysis of the proteins in vitro and by the availability of amino acids for growth of Leuconostoc mesenteroides P-60, have yielded further positive results. These authors have shown that autoclaving soybean oil meal with sucrose, does not destroy methionine, but merely binds it in a form in which it is unavailable for use by the microorganism. Lysine was shown by the same technique to be partially destroyed and partially inactivated when autoclaved with protein only. The authors

concluded that carbohydrates were responsible for the binding and destruction of lysine.

Dehydrated potatoes and other vegetables.

In an early paper on the study of dehydrated vegetables, Mangels and Gore (108) reported dried onions, turnips, celery, tomatoes and cabbage as being very easily injured by heat treatment, whereas potatoes (white), carrots, string beans and sweet corn were fairly resistant to heat. Since that time many workers have investigated the keeping qualities of dehydrated vegetables.

Ross (145) has recently reviewed the literature on the deterioration of dehydrated potatoes. The most important type of spoilage of potatoes in storage, is caused by the formation of a reddish-brown discolouration accompanied by a scorched or caramelized flavour. The badly-browned potato powder does not reconstitute as readily as the freshly-dehydrated potatoes and is definitely of inferior quality. Burton (13) analysed samples of badly-browned mashed potato powder and reported a slight loss of sucrose, hexose sugars and amino nitrogen compared to the fresh material. In a later report Burton (14) suggested that three reactions may occur in stored potato powder: (1) anaerobic development of a brown colour accompanied by the evolution of carbon dioxide (2) absorption of oxygen accompanied by the evolution of carbon dioxide (3) absorption of oxygen unaccompanied by evolution of carbon dioxide but resulting in an "off " flavour. This

latter reaction was thought to occur only at low moisture levels. He could show no definite relationship between the loss of sucrose and browning (as determined by Lovibond colour units) but did show a correlation between the loss of glucose and the development of brown colour. He demonstrated that a considerable quantity of sucrose was hydrolysed to hexose sugars and he presumed these hexose sugars then could take part in the browning reaction. Burton therefore concluded that the browning of dried potatoes was probably brought about by a caramelization of hexose sugars and that the Maillard reaction might occur only as a secondary step in the reaction. There appeared to be no direct correlation of browning and a loss of amino nitrogen. Burton however analysed for amino nitrogen by means of the formol titration and it has been shown since then that this method is not as satisfactory as the Van Slyke procedure for following the course of the browning reaction (119, 137).

Patton and Pyke (128) have indicated that both amino acids and reducing sugars are involved in the browning of potatoes. Caldwell et al (15) indicated that reducing sugars and amino acids are always present in potatoes and are enormously concentrated on dehydration. Denny and Thornton (31) on the other hand achieved browning by heating potato starch and glucose in hot fat. Ross (145) is of the opinion from his own observations, that some constituent, other than reducing sugars is concerned in the browning of potatoes. When

potatoes were leached with sugar solutions, the rehydrated product did not brown at the rate anticipated from their reducing sugar content. The author thus concluded that both amino compounds and reducing sugars were responsible for potato browning.

Browning of dehydrated potatoes has been reported to be accompanied by an increase in ultraviolet fluorescence (128) and a decrease in pH (13).

The knowledge of the proteins of potatoes is extremely limited. Groot and his associates (59, 60, 61, 62) have reported that potatoes contain two types of protein, tuberin and tuberinin, the former, of as high amino acid content as casein with the exception of lysine. Investigations concerning the nitrogenous composition of the potato have disclosed that only one half of the nitrogen is supplied by proteins (122) the remainder being chiefly in the form of amino acids and amides. These authors stated that histidine, cystine and lysine appeared to be lacking in potatoes. Despite these shortcomings in composition, the proteins of potatoes are comparable to cereal proteins and casein in biological value (60, 62). No studies have been observed concerning the nutritive value of stored dehydrated potatoes.

Little information concerning the browning of other dehydrated vegetables has been published. Evans and St. John (49) showed that the protein quality index as proposed by Almquist et al (2) for animal proteins could not be applied

satisfactorily to overcooked vegetable proteins. Legault et al (103) studied the development of browning of dehydrated carrots, potatoes, sweet potatoes, onions and cabbage during storage. These authors demonstrated that browning proceeded in a linear fashion up to the limit of palatability. The effect of oxygen on the process was relatively small for samples of moisture content within commercial storage levels. The browning rates varied exponentially with the reciprocal of the absolute temperature and with the moisture content. The temperature coefficient of browning of the dehydrated vegetables was relatively high, the Q_{10} value ranging from 5.0 to 8.4. The authors showed that the browning rates of carrots, white potatoes and sweet potatoes respectively were in the ratio of 27:3.3:1. Although the carrots browned almost eight times as fast as white potatoes, their storage lives under similar conditions were approximately the same. The natural flavours and pigments of the carrots apparently increased the tolerance of the product to the effects of browning.

Fruits and fruit products.

Most fruit products readily undergo a darkening of colour during heating and processing, and means to control this change have been under study for many years. However as yet, our knowledge of the fundamental reaction causing the darkening is obscure. Three theories concerning this deterioration in fruit products have been set forth (156): (1) Maillard

reaction--a condensation of amino acids with reducing sugars (2) ascorbic acid theory--which applied to citrus fruits--involves the oxidation of ascorbic acid and similar compounds which in turn react with nitrogenous material to form dark pigmented products (3) the "active aldehyde" theory. This latter theory postulates the decomposition of sugars and sugar acids to furfuraldehydes or similar compounds characterized by having an active carbonyl group, and these products condense with nitrogenous materials to form brown coloured substances. Stadtman (156) stated that it was possible for all three types of browning to occur simultaneously.

Temperature has been shown to be the most important factor in the storage of fruit products (73, 123) the optimum temperature being determined for each individual fruit product. Moisture content of stored fruits has received considerable attention (157, 73). Other workers have shown that influence of moisture content is related to the presence or absence of oxygen. As oxygen is increased the quantity of moisture in the product must be decreased to ensure optimal storage quality (157).

Several workers have studied the chemical changes which occur during browning of fruit products, and have shown that carbon dioxide is produced throughout the deterioration process (27, 64). The quantity of carbon dioxide produced is markedly increased with an increase of temperature (157).

The suggestion that the darkening of fruit might be

the result of a Maillard-type reaction was proposed by Hall (64) in 1927. This author showed that the amino nitrogen content of fruit concentrates decreased during storage. Other workers have noted a decline of amino nitrogen values during storage (174).

Joslyn and Marsh (90) have isolated from darkened orange juice a pigment strikingly high in nitrogen. These workers could find no change in the amino nitrogen content of orange juice during storage, although a marked increase in browning was noted when amino acids and other amines were added to orange juice. Similar results have been reported on sugar syrup and grape juice concentrate (142).

Hall (64) reported a slight loss of reducing sugar by orange concentrate during storage. This has been confirmed by Curl et al (27) and these authors have demonstrated that the loss of reducing sugar roughly paralleled the increase of the dark substances. Stadtman et al (157) removed the sugar from apricot syrups by fermentation. The rate of browning of the sugar-free product was less than that of the control but did not decrease to the extent which had been expected. When fructose or glucose was added to the fermented syrup the browning was restored to its original rate. The results indicated that only part of the browning in apricots involved sugar reactions. These authors investigated the extracts of darkened apricots by spectrophotometric analysis, and showed the development of a characteristic absorption

band having a maximum at 285 mu and a minimum at 245 mμ. Hydroxy-methylfurfural was positively identified as a constituent of the extract (172). Ethyl acetate extracts of browned apricots were found to contain both furfural and hydroxymethylfurfural. Since it has been shown that furfural will react with amino acids (34) the plausibility of furfural compounds as intermediates in the browning of fruit products deserves due consideration.

Fish and meat products.

The literature on the browning of fish products is limited. It has been pointed out that high temperatures have a detrimental effect on the nutritive value of fish proteins and considerable browning of the flesh occurs, (82, 110). Maynard et al (109) demonstrated that fish dried under vacuum was superior in growth promoting properties to that dried by direct flame. Schneider (150) attributed this to the higher digestibility of absorbed nutrients of the vacuum-dried product. In 1935 Wilgus et al (184) explained the loss of nutritive value on the basis of the Vitamin B_2 content and stated that the type of heating had no practical significance on the protein efficiency of dried fish.

Deas and Tarr (29, 30) have investigated the browning of fish products by means of microbiological assay procedures. They showed that fish proteins were relatively high in lysine and in reducing sugars or allied compounds. They suggested that fish browning was caused by a Maillard type of reaction

between the amino acids and glucose or other aldehydic compounds.

Although most meat products are naturally of a dark brown colour and subsequent colour changes cannot be observed, there is reason to believe that a deterioration of the proteins similar to that observed in many other foods may occur. Morgan and Kern (117) showed that the biological value of meat proteins decreased after heating. Shigeki (152) demonstrated a decrease of pH, an increase of ammonia and amino nitrogen and a decrease of protein nitrogen of meat on storage. Wilder and Kraybill (182) reported a loss of lysine in cured-cooked luncheon meat but no loss of lysine when the meat was only cooked. No difference could be found in the feeding trials between cooked, cooked-cured and fresh luncheon meat. Grunhut et al (57) showed a slight loss of tryptophane during heat processing of meat, but suggested that this might be due mainly to protein denaturation. Millares and Fellers (113) substantiated these results in an investigation on chicken-meat products. Further work on the effect of proteins of meat in spoilage is apparently lacking from the published literature. Lyman and Kuiken (105) have shown recently that meat is an excellent source of lysine, so it is reasonable to suggest that this amino acid may be a factor in meat spoilage.

MATERIALS AND METHODS

Preparation of Food Samples

Six food products of different composition were chosen for this study and comprised the following:

1. Roller drum-dried skim milk powder.
2. Spray-dried skim milk powder.
3. Defatted soybean meal.
4. Meat meal.
5. Ground oats.
6. Dehydrated potatoes.

The drum-dried powder was obtained from the Champlain Milk Products Company, Limited, Stanbridge, Quebec, and the spray-dried milk from the Borden Company, Limited, Toronto. Dr. W. D. McFarlane, of Victory Mills, Toronto, kindly supplied the defatted soybean meal; City Renderers, Limited, Montreal contributed the meat meal, while the dehydrated potatoes were obtained in three-pound tins from Gordon Beardmore and Company, Limited, Oakville, Ontario. The oats were selected from the feed supply at the Macdonald College granary.

The oats and potatoes were ground to a fine powder in a Wiley Mill, but the other materials were in a fine enough state to be used without grinding.

A proximate analysis was carried out on each food material and results are shown in Table VIII. Crude fibre was not determined. The methods employed for the individual

determinations were as follows:

Moisture - Samples were heated for five hours at 105°C. Moisture was determined by difference.

Fat - Samples were extracted in a Soxhlet for sixteen hours using petrol ether as a solvent. Fat was determined by difference.

Ash - Samples were heated for two hours at 600°C in a muffle furnace, and weighed for ash content.

Protein - The usual Kjeldahl procedure was used to determine nitrogen. Factors used to convert nitrogen to crude protein for each food product were as follows:

Milk powders - N x 6.38 (144)

Soybean meal - N x 5.7 (77)

Potatoes* - N x 6.25 (81)

Meat meal - N x 6.25

Oats - N x 6.25

Storage of Food Samples

Samples of the six different food products were placed in tightly-sealed pint jars and three jars of each food or feed were stored in both 27°C and 37°C rooms. A fifty ml. Erlenmeyer flask, containing a humidity-controlling material was added to each of the three samples (at both temperatures) in order to regulate the relative humidity of the atmosphere above the food material. Humidities desired were eighty

---

* Although it is common convention in agricultural practice to calculate the protein value of the potato as that obtained by adding together the true protein value and one-half of the value obtained by multiplying the non-protein nitrogen by 6.25, in this investigation the term protein, in the case of the potato samples, refers to the figure obtained by multiplying the total nitrogen by 6.25 (i.e. to "crude protein" as understood in agricultural analysis)

per cent, fifty per cent and zero per cent. Henceforth these shall be designated as high, medium and low humidities respectively. Table IX shows the substances used at the different temperatures for the degree of humidity required (83).

Analyses

Analyses of the different food products were made at the time they were first put in storage and at periodic intervals up to eighteen months.

Various analyses were carried out with the aim of ascertaining which analysis might afford a measure of the degree of browning (and/or deterioration). Throughout this work, special care was taken to carry out the analyses under the same conditions, so that the only variable would be the time interval between tests. The determinations were carried out in duplicate so far as was possible, and results given are averages of closely-agreeing values.

Visual observations of the products were carried out at regular intervals. Flavour changes were measured on the stored milk powders by a taste panel of seven persons, at six, twelve and eighteen months. The flavour tests were made directly on the milk powder, as it was considered that the insolubility of some of the badly-deteriorated samples would affect the tasters' opinions of the "reconstituted" milk.

Colorimetric procedure.

Preliminary experiments showed that water extracts of

the food materials were invariably turbid even after considerable centrifugation. The same was true for low concentrations of ethanol in water. It was not until seventy-five per cent ethanol was used that a clear filtrate appeared in the case of milk powders. The pH of series of samples of each of the clear filtrates was adjusted slightly on either side of their normal value and the light transmission determined on these samples. It was found, using a 420 mμ filter in the Evelyn colorimeter, that the normal pH of the filtrate gave optimum values for light transmission. Consequently no pH adjustments were made in the ensuing tests.

One gram of each food product was shaken on a mechanical shaker for one hour with 20 ml. of seventy-five per cent ethyl alcohol. The mixture was filtered through a Whatman #40 filter paper, 10 ml. of filtrate was added to a colorimeter tube, and the galvanometer deflection on the Evelyn colorimeter was determined using a 420 mμ filter. The instrument was standardized to read 100 with a solution of seventy-five per cent alcohol.

Fluorescence Procedure.

The method of Pearce (129A) was used on all food products. A 2.5 gram sample of the material was defatted with three 25 ml. portions of chloroform and filtered each time through a Whatman #1 filter paper. The material was allowed to dry for one hour at room temperature. One gram of the

defatted material was placed in a 250 ml. Erlenmeyer flask and 100 ml. of ten per cent sodium chloride added. The mixture was shaken for thirty minutes on a mechanical shaker and then filtered through a Whatman #1 filter paper. Fifteen ml. of the supernatant was added to a cuvette and the fluorescence determined on the Coleman photofluorimeter using a B1 filter. The instrument was set at fifty using a standard solution of 0.2 micrograms of quinine sulfate per ml.

pH.

It was observed that pH changes were proceeding in all six food products. Consequently an arbitrary method of determining the pH was carried out as follows:

One gram of the food product was added to 10 ml. of water, the suspension well mixed and the pH was determined on the mixture using a Beckman pH meter with a glass electrode.

Solubility of nitrogen.

As a measure of the degree of denaturation of the food proteins, the solubility of nitrogen in three per cent sodium chloride was determined for all the food products at intervals during the storage period following the method of Jones and Gersdorff (89). In order that all extractions of the fresh and stored samples should be conducted to give comparable results, care was taken to carry them out under as uniform conditions as possible.

The extractions were performed in a temperature-controlled

room at 27°C. One gram of sample was shaken at a uniform rate with 20 ml. of three per cent sodium chloride for one hour in a mechanical shaker. The mixture was centrifuged for exactly fifteen minutes and the extract was transferred to a 25 ml. graduated flask and made up to 20 ml. Total nitrogen was determined using the microkjeldahl method on one ml. aliquots of the solution. The method of Pregl (136) was modified to employ the digestion mixture of Campbell and Hannah (16) and the boric acid solution as used by Stover and Sandin (164).

Reducing groups.

The method of Chapman and McFarlane (17) was used on all food materials. One hundred mg. of the food was placed in a test tube (22 x 150) and five ml. distilled water at 70°C, 5 ml. of potassium acid phthalate, sodium hydroxide buffer (pH 5.0) and five ml. of potassium ferricyanide were added. A blank was also prepared. The tubes were placed in a water bath at 70°C for twenty minutes and then cooled to 25°C in an ice bath. Five ml. of ten per cent trichloracetic acid was then added and the mixture filtered through a Whatman #40 filter paper. Five ml. of the filtrate was added to a colorimeter tube, 5 ml. of distilled water and 1 ml. of 0.1 per cent solution of ferric chloride were added. The tubes were shaken, allowed to stand for ten minutes and the intensity measured on the Evelyn colorimeter, using a 660 mμ filter.

A calibration curve was prepared using cysteine hydrochloride. This amino acid rapidly reduced potassium

ferricyanide under the conditions of the test. The cysteine solution was prepared in freshly boiled distilled water, saturated with carbon dioxide. The calibration curve was prepared by subjecting varying concentrations of cysteine hydrochloride to the entire procedure and expressing the galvanometer readings as moles x 10^{-5} of cysteine, or as reducing groups, since cysteine has one SH group per mole. Owing to the fact that ferric ferrocyanide was not in true solution, but was present as a highly dispersed suspensoid sol, the values did not follow Beer's law. Thus it was necessary to refer to the calibration curve in all calculations.

During the initial stages of the storage period it was found that the 5:5 dilution of the filtrate obtained as described in the Chapman and McFarlane procedure, gave very low galvanometer readings. It was therefore difficult to estimate the reducing groups of the foods from the calibration curve. A dilution of 1:9 resulted in high galvanometer values whereas a 2:8 dilution gave suitable intermediate readings. Consequently this dilution was used throughout the investigation.

Lactose in milk powders.

This procedure was carried out only on the eighteen month samples. Comparative analyses were made at the same time on fresh samples procured from the original sources.

Two grams of milk powder was reconstituted with water, diluted to about 300 ml. and thoroughly mixed. Two ml. of ten per cent acetic acid were added and the mixture stirred

until a precipitate formed. The samples then were heated to boiling for thirty minutes, cooled, filtered and the precipitate thoroughly washed with hot water. The combined filtrate and washings were concentrated to approximately 150 ml., cooled and made up to 200 ml. with water. The amount of this solution required to reduce 25 ml. of Benedict's quantitative reagent was determined. Sixty-seven mg. of lactose completely decolourizes 25 ml. of Benedict's solution (68).

Benedict's reagent was prepared as follows:

A. Copper Sulfate - 9 gms. Dissolve in 50 ml. water.

B. Sodium carbonate - 100 gms.
Potassium citrate - 100 gms.
Potassium thiocyanate - 62.5 gms.
) Dissolve in 400 ml. water by heating. Filter.

Stir solutions A and B together, add 2.5 ml. of five per cent potassium ferracyanide solution and dilute to 500 ml.

Amino nitrogen.

Jones and Gersdorff (89) investigated the amino nitrogen of the proteins of stored grain by applying the Van Slyke nitrous acid method (170) to a three per cent sodium chloride extract of the grain. This procedure was carried out in the present project, but was found to be unsuitable for the purpose in hand. The fraction of the total protein soluble in three per cent sodium chloride declines as denaturation proceeds during storage, although the insoluble portion may still retain appreciable free amino nitrogen. As a consequence, the amino nitrogen value of three per cent sodium chloride extracts

will not afford a true measure of the total decline of free amino nitrogen.

Amino nitrogen determinations were carried out directly on the whole material of the stored samples by the Van Slyke procedure (170) in order to obtain more reliable values for total amino nitrogen. Great difficulty was experienced in the case of these samples owing to foaming in the Van Slyke apparatus. Addition of caprylic alcohol did not completely abolish foaming. It was found that heating the materials (in solution) in a boiling water bath for ten minutes decreased the foaming. The method used was as follows:

One gram of the food product was mixed well with 5 ml. water in a test tube and the test tube inserted in a boiling water bath for ten minutes, during which time the sample was stirred constantly. The sample was cooled, washed into the Van Slyke apparatus and made up to 10 ml. with water in the measuring cylinder. The shakingwas carried out for thirty minutes to give most of the free amino nitrogen (which includes the ϵ-amino group of lysine) time to react. This measurement was carried out only at six month intervals on the soybean meal, meat meal, oats and potatoes, and at two, four, twelve and eighteen months on the milk powders.

Hydrolysis of food proteins.

It has been postulated that the first stage of the sugar-protein reaction includes a loose linkage between the carbohydrate and the amino group, such linkage being stable to

enzymatic attack but not to strong acids. Acid hydrolysis should release the total amino acids from any such linkages. Enzymatic hydrolysis however would not release amino groups bound in this way. A comparison of the decline in amino groups liberated by acid hydrolysis with the decline in amino groups liberated by enzymatic hydrolysis might, therefore, give some information as to the extent of the formation of such loose linkages.

Acid hydrolysis:- Acid hydrolysis was performed by subjecting five gram samples of the food products to 50 ml. of 6 N sulfuric acid, and heating the mixture at 105°C in a glycerine bath for a period of twenty hours. The mixture was constantly stirred by means of a mercury-sealed stirrer. Aliquots of the mixture were taken at intervals during the hydrolysis and amino nitrogen and total nitrogen determinations were carried out on each aliquot, in order to determine degree of protein hydrolysis.

Enzymatic hydrolysis:- Enzymatic hydrolysis of the foods was carried out on the fresh materials and at six month intervals. The procedure used was as follows:

To fifteen grams of the food material, 250 ml. of 0.2 N disodium phosphate buffer (pH 8.3) and one gram of pancreatin* were added in a 500 ml. Erlenmeyer flask. Toluene was added in sufficient quantity to form a thin layer on top of the liquid. The samples were incubated at 37°C and amino nitrogen was determined daily on 5 ml. aliquots in duplicate by the

---

* Merck, U. S. P.

method of Van Slyke (170).

In addition, experiments were carried out at the commencement of this project to determine the effect of a more severe heat treatment on the course of the *in vitro* digestion of the original material. The fresh material was heated to 80°C for forty-eight hours in an air oven and the procedure described above was applied to the treated samples.

It was originally intended to relate the enzymatic breakdown of the protein of the foods *in vitro*, with the nutritive properties of the different foods by means of actual feeding trials. However, it did not prove feasible to carry out such nutritional tests.

Titration curves.

Titration curves have been used to follow changes in the ionizing groups of proteins (22). It was considered useful, therefore, to investigate the changes in titration curves of the foods as effected by different periods of storage under different conditions of temperature and humidity.

The method for obtaining the titration curves was as follows:

One gram of the food product (two grams in the case of oats and potatoes) was mixed with 50 ml. of water. 5.85 g. sodium chloride was added (in all cases except meat meal) and dissolved. The volume was then made up to 100 ml. The pH was adjusted to six using 0.1 N hydrochloric acid, successive aliquots of 0.01 N sodium hydroxide were added and the

pH determined by means of the Beckman instrument after each addition. A blank was carried out using a 100 ml. sample of 1 N sodium chloride. The volume of alkali required to bring the blank to a particular pH was subtracted from the volume of alkali required to bring the food product mixture to the same pH.

To investigate the possible change in titration curves due to severe heating of the foods, a portion of each of the original products was heated for sixteen hours at 80°C in an air oven. Titration curves were then carried out as described above.

Lysine determination.

The method used for lysine was a microbiological assay developed by Barton-Wright (4) as discussed in the first part of this study, modified by the use of hydrolysed zein as a source of the amino acids required for the medium. The colorimetric procedure suggested by Boulet (11) was also applied. The comparison of the two methods is discussed in Part I.

The above methods included acid hydrolysis of the products prior to lysine determination. In order to examine the effect of storage of milk powders on the liberation of lysine by enzymes in vitro, microbiological assay of lysine was also carried out on pancreatic hydrolysates of the drum- and spray-dried milks.

RESULTS AND DISCUSSION

Changes in the Properties of Stored Foods

The effect of the humidity-controlling materials on the foodstuffs separated the products into high-, medium- and low-moisture levels. The process was slower than desired, however, and in some cases the separation was not completely satisfactory at the earlier stages of the study. The moisture levels observed for the milk powders and potatoes were approximately nine, five and one per cent while slightly higher values were observed for the other food products. The method used, i.e. that of storing all the material in one container, suffered from the disadvantage that the jars had to be opened each time a sample was required, thus admitting atmospheric conditions to the container. Nevertheless, since most of the products under study have been produced commercially with moisture contents varying from two to ten per cent, the conditions attained should give results of practical value.

Flavour of milk powders.

The flavour of the milk powders became flat and powdery at the high humidity regardless of the temperature. Different persons described the poor taste as being "musty", "stale", "slightly rancid", and "flat". It was assumed that the "rancid" flavour originated from the deterioration of the fat present in the skim milk powder.

The results of the taste panel are shown in Table X

for six, twelve and eighteen months storage. The value of ten was assigned to a fresh, high quality powder and zero to an inedible, highly unsatisfactory product. As may be observed, the high humidity and high temperature markedly decreased the flavour and quality of the milk powders. Even at the higher temperature, a low moisture content kept the quality of the product relatively high, with a value of nine for both milk powders at eighteen months storage.

The effect of temperature and humidity on the rates of flavour decrease is shown in Table XI. It will be observed that the rate of flavour deterioration was more than eight times as high at 37° (high humidity) than that of the samples at the low humidity (both temperatures). These figures are in agreement with the results of other workers (74).

Only a slight difference in keeping quality was observed between the two types of milk powder, and this at 27°C (high humidity). The spray-dried milk had a slight edge on the drum-dried material, which may have been the result of the difference in the manufacturing processes of the two powders. It is known that drum-dried milk powder, especially when not vacuum-dried, undergoes a more rigorous heat treatment than the spray-dried powder, which may cause a greater susceptibility to chemical deterioration than its spray-dried counterpart. On the other hand it has been reported (92) that roller-dried milk powder consists of platelets of irregular shape which contain few air cells, whereas the spray-dried

powder is in the form of spherical particles which always contain some air cells. It is difficult to estimate whether the presence of air cells in the spray-dried milk had counteracted the more severe heat treatment of the roller-dried milk, with the result that both powders underwent spoilage to an approximately equal extent.

Colour changes.

Colour changes were noted in all of the products under investigation. The meat meal became paler in colour during storage, all samples regardless of temperature or humidity, showing an equal degree of change. A very slight darkening of shade was noticed in the ground oats at the higher temperature and humidity conditions but the difference from the original product was not sufficient to be visible when photographed.

The colour photograph (Figure 16) shows the colour changes in the other four products under storage after a period of eighteen months. The greatest increase in colour is noticed at the extremes of temperature and humidity (Column C) for the milk powders and the dehydrated potatoes which changed from a yellow-white colour to an orange-brown in the case of the milk powders, and to a dark brown in the case of the potatoes.

A peculiar fact was observed upon examination of the "browned" samples of dehydrated potatoes. The majority of the potato particles were dark brown in colour, but white

granules, apparently little changed from the original product, could be detected. This phenomenon could be explained by the lack of one of the precursors of the browning reaction in that particular portion of the potato, or to failure of such granules to absorb moisture uniformly with the main bulk of the sample. Ross et al (146) made similar observations on dried potatoes and found that such uncoloured particles were much lower in reducing sugar content than the browned particles.

In all cases, the samples of the products stored at 27° and at the lowest humidity (column B) were similar to samples of the fresh products. Increased colour was observed for the high humidity samples at 27° (column A) and a very slight increase in the samples at low humidity and 37° (column D). These results are in parallel with the palatability results on the milk powders which would indicate that the colour-forming reaction is also responsible for the food spoilage.

As may be seen from the photograph (Figure 16), the texture of the materials stored at the high humidity and temperature was considerably affected. The milk powders, besides undergoing the colour change, became lumpy and more "crystalline" in texture, in all probability due to the crystallization of α-lactose monohydrate, which is formed in milk powder at high relative humidities (169). No quantitative work was carried out on this chemical change. Slight "packing" or lumpiness was observed in the stored soybean meal, but little or no change was observed in the other

products. The reason for the texture change in the soybean meal is obscure.

A reliable chemical method for determining the colour increases of food products during storage is highly desired. The method used in this investigation was satisfactory on a comparison basis but was subject to various limitations. The loss of solubility, for instance, perceivably would influence the accuracy of the method as well as numerous chemical changes presumably occurring in the foods.

Little or no colour changes were detected by this method in the case of soybean meal, oats or meat meal, indicating the lack of sensitivity of the procedure, for a slight but definite change in colour was observed in the case of both the soybean meal and the oats, and a slight paling in the case of the meat meal.

Figure 17 shows the results given by this method on the two types of milk powders and the dehydrated potatoes. It is of interest to note that these results are roughly parallel with the visual colour observations, an indication that the coloured substances are water-alcohol-soluble.

It is significant that those products containing an appreciable quantity of reducing sugar (i.e. milk powders and potatoes) were the only ones to show a rapid increase of colour. Other products, such as the soybean meal or the oats, containing considerable amounts of sugar, but mainly as non-reducing sugar, did not increase in colour to the extent of

the other products. Thus it seems significant, that for appreciable browning to occur in food products, the carbohydrates of the foods should be in the aldose form.

High moisture content and high storage temperature were factors which rapidly accelerated the discolouration of the milk powders and potatoes, the rate being approximately ten times faster at the higher temperature. The products stored at medium or low humidity browned at much slower rates than the high-humidity materials.

Fluorescence.

No changes in the amount of fluorescing substances of either the soybean meal or the ground oats were observed after lengthy storage periods. The fluorescent substances of meat meal increased noticeably but all samples, regardless of temperature or humidity, increased to the same extent. Due to this fact it was concluded that the fluorescence of the meat meal was produced by a different reaction than that causing fluorescence in milk powders and potatoes.

The results of the increase of fluorescing materials in the dried milks and potatoes are shown in Figure 18. In the case of the milk powders, it is apparent that the moisture content of the samples had considerably more effect on the production of fluorescing substances than had temperature. At both 27°C and 37°C and under high humidity conditions both types of milk powders showed a considerable increase of fluorescence over the original powders. However, little change

whatsoever was observed for any of the milk powder samples under the low humidity conditions.

Different results were observed in the case of the dehydrated potatoes. At 37°C and under high humidity conditions a marked increase of fluorescent substances occurred, while the similar sample at 27°C showed only a slight increase of fluorescence. It would appear from these results that the reaction producing fluorescent substances in potatoes may differ considerably from the reaction producing fluorescence in milk powders.

pH.

The results of the pH changes of the products during storage are shown in Table XII. A decrease in pH of all the materials was observed.

Meat meal showed little regular pH change, an indication that the amount of bone normally found in tankage appreciably affects the pH. In this experiment, the small pieces of bone in the meal were not pulverized prior to storage, consequently duplicate samples would not contain similar quantities of the bone constituents. This factor markedly affected the ability for uniform sampling of the meat meal and results consistently varied. The pH of meat meal during storage however, showed a slight downward trend, with no differences attributable to differences in storage temperature or humidity.

The figures of Table XIII have been calculated from the pH values of the foods after different storage periods and

conditions. It is noticeable that temperature had a marked effect on the decrease of pH, but only when the humidity was high. With a low humidity, the samples seldom produced more acidity by increasing the storage temperature by 10°C. In several cases the figures were actually lower, which would serve to emphasize the importance of moisture in the deterioration of the foods.

An exception to the above phenomenon was noted in the case of the ground oats. The humidity had a partial effect on the pH change but a greater decrease was observed at 37°C with low humidity, than at 27°C with high humidity, after both twelve and eighteen month's storage. A reasonable explanation may be found in the action of the normally-accurring enzymes of the oats. It is known that the enzymes break down the grain components with the production of acid, and from the results of Table XIII, do so at a faster rate at 37°C, the optimum enzyme temperature, than at 27°C. Apparently the enzymes were slightly affected by the amount of moisture in the sample, but were not completely inhibited by the very low moisture conditions.

Solubility of nitrogen.

Results of the nitrogen solubility determinations are illustrated in Figure 19. Only the results of the high humidity conditions are reported as slight turbidity in the filtrates of the samples stored at low humidity, gave erroneous results.

No change was observed for either the meat meal or the dehydrated potatoes while decreases in solubility appeared in all four other food products after storage.

Reducing substances.

The results of the Chapman and McFarlane (17) procedure are shown in Table XIV. It was observed in the early part of the storage that the only products showing a regular trend were the milk powders. Consequently the study was carried through on these products only.

As in several of the other properties studied, the formation of reducing substances in dried milk was greatly accelerated by high humidity and temperature conditions. This conclusion is similar to that of other workers (74, 119). The drum-dried powder showed considerably higher amounts of reducing groups than the spray-dried product, and during storage continued to exhibit a higher content of reducing substances. This difference may be attributed to two factors: 1. The higher initial moisture content of the drum-dried products and 2. The more drastic treatment which the roller-dried product undergoes during manufacture. To state any definite conclusions on the differences however, would be unjustified.

Reducing sugar change in milk powders.

It was unfortunate that more complete results on the change of the reducing sugar in the stored foods could not

be obtained. The results which were obtained are shown in Table XV, from which it is evident that temperature and humidity during storage of the milk powders had a considerable effect. Greatest loss of sugar occurred under the high temperature and high humidity conditions. A striking feature of the results was that the low humidity did not decrease the amount of reducing sugar loss to the same extent as it reduced some of the properties considered previously. Although the loss of sugar at the low humidity level was not as great as under the high humidity conditions, in three out of four instances at both 27° and 37°, an appreciable change in the reducing sugar content was noted. Possibly a reaction, independent of the moisture content, was taking place. This might be a degradation of the sugar molecules not involving interaction with nitrogenous compounds. Numerous workers have suggested that the browning reaction of foods is the result of caramelization of the sugars, and that the proteins may be involved only as catalysts. It is probable that more than the one reaction is occurring simultaneously in foods during storage, and the sugar losses obtained here are most likely a summation of the losses due to these different reactions.

Amino nitrogen.

Several workers (119, 137, 74) have reported that the formol titration does not give an accurate measure of the losses of amino nitrogen of proteins during storage. Results of amino nitrogen determinations in the present investigation,

by both formol titration and the Van Slyke nitrous acid method, are shown in Table XVI, and fully confirm the foregoing reports. As suggested previously, the reaction in foods whereby amino nitrogen is lost in the presence of aldehydes may include the simple formation of imino compounds. These would react in the formol titration method (104) but not in the Van Slyke procedure, so that the former method will give erroneous results for loss of amino nitrogen. Consequently in the present study only the Van Slyke values have been considered to give a true picture of the amino nitrogen losses.

From Table XVI it is clear that the loss of amino nitrogen of the milk powders is related to both temperature and humidity of storage. An increase of ten degrees greatly decreased the amino nitrogen value of the food, especially under high humidity conditions. At low humidity, however, a ten degree rise in storage temperature brought about very little decrease in amino nitrogen. These results support the contention that the amino groups were taking part in the browning reaction. As suggested before, this reaction is almost completely prevented by very low moisture conditions.

A somewhat similar reaction appeared to have occurred in the case of the stored potatoes, greatest loss of amino nitrogen being at the higher temperatures and humidity. No loss was observed at the low humidity level, which demonstrates the necessity of a certain quantity of moisture for the browning reaction to occur.

The results with the other food products showed opposite trends for the amino nitrogen values, in that all samples showed a definite increase. This may be attributed to proteolysis in the case of these products, brought about perhaps by the increasing acidity of the foods.

Acid and enzymatic hydrolysis of stored foods and feeds.

The results shown in Figure 20 reveal that storage under various conditions had little to do with the release of amino nitrogen from proteins by acid hydrolysis of the foods. In all cases differences of length of storage or severity of temperature or humidity were of negligible consequence for the course of the acid hydrolysis curves.

The final degree of hydrolysis (amino nitrogen/total nitrogen) of the potato samples was considerably lower than for the other foods. It has been mentioned previously that only about fifty per cent of the total nitrogen of the potato is present as protein nitrogen. Consequently, with a high non-protein nitrogen fraction, less amino nitrogen was released by acid hydrolysis of potatoes than of the other products.

In contrast to the results with acid hydrolysis, the enzymatic hydrolysis of most of the food products was influenced markedly by differences in storage conditions. Meat meal showed only small differences in its enzymatic hydrolysis curves, these slight differences in all probability being caused by the differences of solubility of the meal. Similar

slight changes were observed for the soybean meal. Only twenty per cent or less hydrolysis by the enzymatic method was registered. However when the sample was heated to 80°C for forty-eight hours the procedure yielded hydrolysis of over thirty per cent. This difference was attributed to the destruction of the trypsin inhibitor, which has been discussed previously and no further work was done on it.

Figure 21 reveals the effect of eighteen month's storage at 37°C under different humidity conditions, and also the effect of severe heating of the foods. Both types of milk powder gave similar results (Figure 21A and 21B). The product stored at 37°C and under high humidity conditions, which had shown the most discolouration and least palatability, also showed a smaller digestibility in vitro than did the fresh powder. Similarily, the milk powders stored at the same humidity but at the lower temperature gave less hydrolysis than the original, the difference being appreciably more in the case of the spray-dried product. A reason for this difference has not been apparent to the investigator.

The most significant result of the enzymatic hydrolysis of the milk powders was the greatly increased hydrolysis of the low-humidity storage samples over that of the fresh powder. It would appear from the values obtained that the conditions of storage at low humidity levels improved the susceptibility of the milk proteins to enzymatic attack. It may also be observed that greater hydrolysis resulted in the

case of the milk powders stored at 27°C than at 37°C. These results add weight to the evidence that the Maillard reaction in dried milk powders was responsible for the decrease of nutritive value which has been shown to accompany stored and heated foods (56, 116, 173). In the milk powders of high moisture content (i.e. high humidity), the free amino groups (probably the ϵ-amino group of lysine) may have formed a linkage, possibly with the reducing sugars, or with other compounds present. This linkage may not have been destroyed by enzymatic attack, but was probably susceptible to strong acids. These results would offer strong evidence for the necessity of a minimum quantity of moisture for nutritional deterioration to occur, since the low-moisture samples showed such marked improvement of enzymatic hydrolysis *in vitro*. If nutritional deterioration and browning are brought on by similar reactions (i.e. amino group-reducing sugar interaction) as is suggested, then the presence of moisture appears to be essential for browning.

In comparing drum-dried skim milk with the spray-dried powder (Figures 21A and 21B) there appeared to be little difference in general between the two, with the exception that the changes brought about in the former material were somewhat more exaggerated. It is suggested that the spray powder was the more digestible of the two types of powders because of its less rigorous heat treatment during manufacture. However, the relative nutritive values of the two

case of the milk powders stored at 27°C than at 37°C. These results add weight to the evidence that the Maillard reaction in dried milk powders was responsible for the decrease of nutritive value which has been shown to accompany stored and heated foods (56, 116, 173). In the milk powders of high moisture content (i.e. high humidity), the free amino groups (probably the ϵ-amino group of lysine) may have formed a linkage, possibly with the reducing sugars, or with other compounds present. This linkage may not have been destroyed by enzymatic attack, but was probably susceptible to strong acids. These results would offer strong evidence for the necessity of a minimum quantity of moisture for nutritional deterioration to occur, since the low-moisture samples showed such marked improvement of enzymatic hydrolysis in vitro. If nutritional deterioration and browning are brought on by similar reactions (i.e. amino group-reducing sugar interaction) as is suggested, then the presence of moisture appears to be essential for browning.

In comparing drum-dried skim milk with the spray-dried powder (Figures 21A and 21B) there appeared to be little difference in general between the two, with the exception that the changes brought about in the former material were somewhat more exaggerated. It is suggested that the spray powder was the more digestible of the two types of powders because of its less rigorous heat treatment during manufacture. However, the relative nutritive values of the two

forms of milk powder is still controversial.

Heating for two days at 80°C affected both milk powders to about the same extent. The degree of hydrolysis and to some extent the rate of hydrolysis was decreased in both samples. It is difficult to estimate the amount of browning reaction by this criterion as the heated powders were much browner than the stored samples. It is suggested that the high temperature browning was more concerned with a caramelization of the sugar rather than an interaction of the sugar and amino groups, for the decrease of hydrolysis did not appear to be proportional to the much greater increase in the brown discolouration.

Results of a similar nature to those of the milk powders were obtained for the dehydrated potatoes (Figure 21C), but the samples under the more severe humidity conditions did not decrease in the degree of hydrolysis to the same extent as the dried milks. In fact a slight increase was observed for the potato sample stored at 27°C under low humidity conditions over the hydrolysis of the fresh product. A similar reaction to that suggested for the deterioration of the milk powders is believed to occur in dehydrated potatoes during storage, but it must be kept in mind that the type and quantity of proteins in potatoes is considerably different to those of milk products. The heating of dehydrated potatoes at 80°C for two days caused a brown colour to form rapidly, but apparently the effect of proteolytic enzymes on the potato protein is

not affected as much as in the milk powders.

The type of reaction observed in milk powders and dehydrated potatoes was definitely not observed for oats after storage. It will be observed from Figure 21D that all of the stored oats samples showed increased hydrolysis after eighteen months over that of the fresh product. On the other hand, the severely heated oats showed a significant decrease in hydrolysis. There appeared to be only one explanation for the above results, namely the naturally-occurring enzymes. The effect of storage was to increase the enzymic activity within the oats, and it is evident that the higher temperature (37°C) was more favourable for this activity than was the 27°C condition. Humidity had some effect on the enzymes of the oats, because at both temperatures the greater hydrolysis occurred at the highest extreme of humidity. By heating the oats to 80°C for two days, the effect of the naturally-occurring enzymes is apparently destroyed, resulting in the lower hydrolysis curve as shown in the diagram.

Titration curves.

The titration curves of the food products after storage are illustrated in Figure 22. As the curves of the oats, soybean meal and meat meal did not reveal any significant changes, the results for these products were omitted. It is of interest to note that only those products (milk powders and potatoes) containing appreciable quantities of reducing sugars showed appreciable changes in their titration curves

after lengthy storage periods.

An examination of the curves of the milk powders (Figures 22A and 22B) reveals that both heating and lengthy storage of the materials have affected various portions of the curves. The 80°C heat on the powders had little effect in the range pH six to pH eight, but caused considerable shifting above this range. It will be observed that above pH nine, more groups were titrated in the heated sample than in the fresh material. This might indicate that the severe heat caused some hydrolysis of the protein and so released titratable groups, but it also reveals that the groups present did not become bound to an appreciable extent. This evidence supports the theory that the milk proteins were not involved in the reaction. Since the milk powders were badly browned after the severe heat treatment it is thus suggested that caramelization of the sugars was chiefly responsible for the deterioration in this case.

On the other hand the slope of curves of the stored milk powders, above pH ten, does not reveal as great an extent of titratable groups as in the fresh or heated samples. This is an indication that some of the basic groups have become unavailable for titration within this pH range. Since lysine has a pK^1 value of 10.53 (149) it is reasonable to suggest that this amino acid is the source of the change in the titration curves.

It would appear from the above results that heat-spoilage

and long term storage-spoilage of milk powders are the outcome of different chemical or physical reactions. The more drastic heat treatment might have caused more insolubility of the proteins, and so have affected the curves. However, the solubility of the milk powders was almost nil after eighteen months storage at 37°C (high humidity) (Figure 19), so that this factor alone should not produce the differences observed. It is obvious that the stored powders, both drum-dried and spray-dried, have lost some basic groups within the range pH 7 to pH 9.5. An explanation for difference observed over this range is not forthcoming. Histidine has a pK^1 value of 6.1 (58) and might have been lost in a chemical reaction, however, this pK^1 value lies slightly outside the range in question. A more satisfactory answer may be advanced on the grounds of the work of Balson and Lawson (3), who suggested a reaction between amino acids and aldehydes which resulted in the formation of imino compounds. If a similar reaction were to occur in food products between aldoses and proteins the imino materials would be expected to ionize at a lower pH and results as obtained here might be expected. Definite conclusions as to the cause of the discrepancy of the titration curves however, cannot be advanced at the present time.

A comparison of the titration curves of the two types of milk powders (Figure 22C) shows negligible differences between the products. It is evident that similar reactions

have occurred in both powders and to almost the same extent.

The titration curves of the dehydrated potatoes (Figure 22D) showed a noticeable difference to those observed for the milk powders in the range pH six to pH nine. In the case of the milk powders, no differences in the curves were observed between the fresh product and the severely heated powder up to pH 8.5. In the case of the potatoes however, the heated sample was similar in nature to the stored sample up to pH 9.5, both showing a decrease of groups titrated in this range. Above pH 9.5 results were similar in nature to those observed for the milk powders and similar reactions may be involved. However, as for the milk powders, an explanation for the discrepancy observed at the lower pH values cannot be advanced without further investigation.

Lysine.

The results of lysine determinations on the stored foods have been shown in Part I, Tables VI and VII. The values obtained by the chemical procedure were generally lower than those obtained by microbiological assay. The latter procedure has been used therefore, as the criterion for lysine changes in the stored products. However, the chemical method has been shown to give parallel relative values for lysine during storage of the food products. An examination of the rates of loss of lysine from the milk powders, as determined by both methods, substantiated these views. For both methods the rate of loss of lysine was approximately

six times as rapid at 37°C (high humidity) than at 27°C (low humidity). This figure is in approximate agreement with the value obtained from the taste panel (Table XI) where the rate of flavour deterioration was about eight times as great from the highest to lowest extremes of temperature and humidity.

It is evident (Table VII) that the humidity of the atmosphere or its resultant moisture content of the milk powders was of primary importance in determining the loss of lysine. At the higher temperature, when the humidity was low, the loss of lysine was approximately the same as at 27°C. Moisture was apparently an essential for the reaction in which lysine is lost.

Table XVII shows a comparison of differences between the loss of lysine as determined (a) on acid hydrolysates and (b) enzymatic hydrolysates of the milk powders after eighteen months storage. The relative loss of lysine liberated by enzymic hydrolysis was much greater than the relative loss of lysine liberated by acid hydrolysis. This strongly suggests that lysine has reacted with other compounds during storage to form secondary products. Whether these are intermediate or final products in browning is unproven. However, apparently these new materials are of three different types: (a) those not susceptible to acid hydrolysis, i.e. l-lysine cannot be recovered as such from the combination by acid hydrolysis; (b) those susceptible to acid hydrolysis, i.e. the lysine has formed some linkage which may be broken by acid to release

lysine; and (c) those susceptible to enzymatic attack, i.e. lysine has formed a loose linkage (or has not formed any linkage whatsoever), giving a combination from which lysine may be released by enzymes.

If these three types of compound be postulated, then it is possible to develop a theory of the browning reaction in foods: under optimum conditions (high humidity or moisture and relatively high storage temperature) the lysine of the food proteins forms a very loose linkage with the food carbohydrates. At higher extremes of storage (longer time, higher temperature or higher humidity) this linkage becomes stronger to the extent that enzymes cannot break it, but strong acids can. Finally, under more severe storage conditions, the intermediate compound changes in some way, perhaps by degradation or polymerization to a product from which the original lysine as such cannot be released. From the results shown in Table XVII, it is apparent that at eighteen months approximately forty to fifty per cent of the lysine is in this latter form at the high temperature and humidity, while approximately seventy per cent of the lysine is in the form which cannot be attacked by enzymes. Assuming that the acid-proof portion is included in the seventy per cent figure, about twenty to thirty per cent of the lysine after eighteen months storage has formed the intermediate stage mentioned previously and has yet to be converted to the final form. These figures are somewhat similar to results reported by Henry et al (74). These authors

found that a high-moisture skim milk powder after ninety-five days storage at 37°C showed approximately twenty-three per cent loss of lysine after acid hydrolysis and sixty-three per cent after enzymatic hydrolysis. The difference in storage time (about fifteen months) would probably account for much of the difference between the foregoing results and those obtained in the present study.

Milk powders.

Palatability relationships:- The judgement of taste panels is the ultimate means of evaluating palatability. It is not yet possible to replace such judgements by chemical or physical measurements except to a limited extent and for certain foodstuffs (133). Nevertheless, a study of correlations between such measurements and the results of organoleptic tests offers the possibility of detecting the chemical and physical changes responsible for deterioration in palatability. Accordingly, the changes studied in the present project have been brought into relation with changes in palatability, and the relevant results for the dried skim milk are shown in Figure 23.

It is evident from Figure 23A that a considerable deterioration on palatability took place before there was appreciable development of colour. Thus the colour measurements were an unreliable guide to palatability in the earlier stages of deterioration. In the later stages of deterioration increasing colour was associated with loss of palatability. It is evident, therefore, that a sample with appreciable colour will have very low palatability but that a sample devoid of appreciable colour may or may not have deteriorated in taste. It may be that the technique employed was insufficient to detect colour changes in the earlier stages, or it

may be that the actual substances responsible for poor flavour are devoid of colour.

Figure 23B shows some degree of correlation between the increase of the reducing substances as determined by the Chapman and McFarlane ferricyanide procedure and the decrease of palatability. Towards completion of the interaction a rise in the number of reducing groups with a similar decrease of the rate of deterioration (as determined by palatability) leads one to the belief that further degradation products of the sugar-protein reaction are produced. This is a belief held by many investigators, since there is evidence to suggest (17, 98) that the production of reducing substances during browning is not solely a consequence of simple denaturation of the protein with consequent exposure of reducing groups. It is probable, therefore, that several reactions contribute to the production of reducing substances, and that these may occur simultaneously. A more detailed study of this phase of the reaction should be carried out to ascertain the various types of reducing substances produced during food storage.

On first glance Figure 23C would appear to show no correlation whatsoever between palatability and solubility of the milk powders, since the points are spread over the entire graph. However, those points in the upper left portion of the Figure represent samples held at low humidity condition for eighteen months storage. If these points are neglected,

then the other points appear in general to follow the semi-circular line. From these results it would appear that the loss of solubility of nitrogenous materials was of two types. One type was related to the browning reaction and required a certain amount of moisture, and the other, which may be a straight denaturation, was brought about by the general storage conditions, and did not manifest itself until some time after the insolubility of the first type had appeared. It is difficult to estimate the extent to which these two types of insolubility are linked with the increased rate of formation of reducing substances, but the possibility of a relationship cannot be overlooked.

The relation between loss of palatability and loss of lysine is depicted in Figure 23D. The slope of the line of best fit is approximately 45°, and it is evident that a close relationship existed between the disappearance of lysine and the increase of the disagreeable flavour. A similar relationship existed between the loss of lysine and the loss of reducing sugar (Figure 24F) and also between the loss of amino nitrogen and the decrease of reducing sugars (Figure 24E). Since lysine supplies the greater part of the free amino nitrogen of milk proteins (171) the latter two relationships are not surprising. Such linear relationships suggest that the interaction between lysine and reducing sugar may be largely responsible for the decrease of the palatability of the powders.

It may be remarked that the slope of the curve (Figure 23D) representing the drum-dried milk powder is slightly steeper than in the case of the spray-dried powder. This indicates that for a given loss of lysine, the spray-dried powder deteriorated in flavour to a greater extent than did the drum-dried powder.

A correlation existed (Figure 23F) between the decrease of amino nitrogen and palatability. The curve showing the relationship indicates that considerable loss of amino nitrogen occurred prior to a decrease of palatability. This is in contrast to the curve previously considered (Figure 23D) where palatability and lysine apparently decrease at about the same rate even during the initial stages. Since lysine provides the majority of the free amino nitrogen of milk proteins, there is an apparent discrepancy between the two sets of results. The discrepancy is explainable, however, from a consideration of the methods of analysis for both the amino nitrogen and lysine. It has been suggested that in the "browning reaction" lysine initially forms loose linkages with the aldehydic groups of carbohydrates, the compound so formed subsequently being changed or degraded in such a way that the original form of lysine is destroyed. If the rate of the initial reaction is faster than the second reaction then much more of the intermediate product will be formed at any given time than of the final product, the initial reactants being in sufficient excess to keep the reaction

going. Lysine was determined on the stored food after acid hydrolysis, so that any lysine decrease thus determined will be a measure of the formation of the final product. Strong acid would release any bound lysine (i.e. such as would exist in the intermediate product) to its original form. On the other hand, the Van Slyke procedure for determining free amino groups of proteins would not measure the bound ϵ-amino groups of the proteins, of either the final or the intermediate product. Consequently, an "apparent" loss of free amino groups occurs in the initial stages of the reaction, but this "apparent" loss is not reflected in the lysine measurement after hydrolysis with strong acid.

Several investigators have observed that the pH of stored foods decreases with time. It has been suggested that if a "browning" type of reaction had occurred, the free amino groups of the food proteins would be bound by the carbohydrates, thus decreasing the basicity of the protein with a consequent increase of acidity. Such an explanation might be used for the results of the relationship between palatability of the milk powders and the pH of the system (Figure 23E), since a logarithmic curve is obtained. However, a definite conclusion regarding the acid-producing mechanism in stored foods cannot be advanced.

Amino nitrogen relationships:- The relationships between the loss of free amino nitrogen of the milk proteins and other properties are shown in Figures 24, A, B, C, D

and E. Here again the results of the solubility are obscured by the values of the low-humidity samples after eighteen months storage, indicating two different reactions causing insolubility of milk powders. It is known that the end products of the sugar-protein reaction are generally water soluble, so that if one of the methods producing insolubility is straight protein denaturation, then it may be that the intermediate product of the "browning reaction" is also insoluble. Since little insolubility was produced at the onset of the storage period, this explanation must be carefully considered.

Somewhat similar curves are observed in the correlation of amino nitrogen loss of the milk proteins with (a) colour (Figure 24A), (b) reducing substances (Figure 24B), and (c) pH, (Figure 24C). An initial period exists during which little change in these latter properties occurs while a large decrease of amino nitrogen is observed. This is similar to the correlation curve between palatability and loss of amino nitrogen (Figure 23F) and similar explanation may be applicable to the case of the above properties. In other words, changes of colour, of pH, and of one form of insolubility do not occur until formation of the intermediate product of the browning reaction has proceeded to a considerable extent. It is not as likely that the formation of reducing substances falls into the same category as the other properties just mentioned, viz. colour, pH, solubility, since

the reducing substances increase from the beginning of the reaction, although at a slower rate. These results support the view that the production of reducing substances is connected with the formation of the intermediate product of the sugar-protein reaction.

Dehydrated potatoes.

It has been found during this study that certain of the chemical changes occurring in dehydrated potatoes resemble the changes observed in the stored milk powders, while others do not. The increase of colour (Figure 17), although not as great as that of the milk powders, is similar in nature. Greatest increase in colour occurs at the higher storage temperature and at both temperatures the effect of high storage humidity is readily apparent.

The pH of stored potatoes decreased in much the same way as in the milk powders, but the effect of temperature was not as clearly defined (Table XIII. Amino nitrogen (Table XVI) showed a definite decrease after twelve and eighteen months storage but in this respect the storage temperature had a more marked effect on the results than had the humidity. At the low humidity levels little change in amino nitrogen was detected.

The other characteristics of stored dehydrated potatoes which bear similar relationships to those of dried milk powder are found in Figures 20 and 21C. Acid hydrolysis had little effect on the amount of amino nitrogen liberated from

stored potatoes, but enzyme hydrolysis yielded results strikingly similar in nature to the results obtained for the dried milks. Little change was observed for lysine (Table VI) although the sensitivity of the method may not have been great enough to detect the lysine changes in the size of the sample used. Similar conclusions may be reached concerning the lack of change of solubility of the potato proteins.

The results of the titration curves for stored potatoes (Figure 22D) differed considerably from those obtained with the milk powders. The most obvious change was the fact that the high temperature heating caused the disappearance of titratable groups in the range of pH six to pH nine. This was not observed for heated milk powders, while eighteen months storage of the two types of products had approximately the same effect on the titration curves. It is thus obvious that while two types of reactions may cause the different curves for the stored milk powders, it may be that only one reaction occurs to cause the observed change of both stored and heated potatoes. The only amino acids of the protein which could be blocked within the range of pH six to pH nine are histidine (pK^1 = 6.1) and cystine (pK^1 = 7.48). These amino acids were not investigated, so that definite conclusions cannot be drawn. It is reasonable to suppose, however, that the amino acids causing browning of dehydrated potatoes differ from those responsible for browning of dried milk powder. It has been suggested that the

lysine content of potato proteins is relatively low (121) so that the supposition may have some merit.

A reasonable conclusion on the browning of foods, is that the presence of reducing sugar in the natural food product greatly enhances the rate of browning. The only products in this study containing appreciable quantities of reducing sugar were the three products already discussed, namely the two milk powders and the dehydrated potatoes. This latter product was found to contain about eight per cent reducing sugar at the beginning of storage, although measurable losses could not be observed during storage. It is suggested that although the presence of reducing sugars accelerates the browning of foods, the presence of starch or other sugars can cause a sugar-protein reaction to occur. It may be that during storage some of the starch or sugar is hydrolysed with the formation of reducing sugars which may react with the amino groups of the proteins.

Other foods and feeds.

The soybean meal was the only other food product investigated which showed appreciable colour change in storage. This colour change (Figure 16) was observed chiefly under conditions of high temperature and high humidity. No differences could be observed in any of the other properties studied for soybean meal in storage, with the exception of solubility. This latter change could be attributed to denaturation, since the protein content of the soybean meal

was relatively high. The increase in colour could be ascribed to a browning reaction of the meal in storage and it is probable that some hydrolysis of the sucrose present occurred. If this were true, then the discolouration of soybean meal in storage would be related to the rate of breakdown of the sugar. As a consequence the "browning reaction" would be much slower in soybean meal than in other foods, such as milk powders and potatoes, where the reducing sugar is initially present.

It was difficult to associate storage deterioration of the meat meal with any measurements of changes in the proteins. This difficulty was probably due to the high fat and mineral content of the meal. A decrease of solubility provided the sole indication in this study that the proteins of the meat meal were undergoing any type of chemical change.

Enzymes are responsible to a large degree for the changes which proceed in stored grains. In the present investigation, the ground oats showed changes in pH, protein solubility and degree of enzymatic hydrolysis. No concurrent changes were observed in any other properties studied. Table XIII shows that the greatest effect on change in pH after twelve and eighteen months storage was that due to increase in temperature. Increased humidity slightly increased the change in pH. It seems almost certain that the enzymes present were chiefly responsible for the changes observed in the case of the ground oats, and that the increase of enzymatic

action was brought about by higher temperatures and humidity. A similar explanation might be given for the results of the enzymatic hydrolysis of the oats (Figure 21D) where it may be observed that the higher storage temperature and humidity resulted in greater hydrolysis of the oat proteins.

SUMMARY

1. Samples of drum-dried skim milk powder, spray-dried skim milk powder, defatted soybean meal, dehydrated potatoes, ground oats and meat meal (tankage) have been stored for eighteen months under different conditions of temperature and humidity. Changes in the materials have been followed throughout the storage period by means of chemical and physical tests.
2. The chemical and physical tests comprised observations on the following:
   (a) Flavour
   (b) Colour
   (c) Fluorescence of potassium chloride extracts
   (d) pH
   (e) Solubility of nitrogenous constituents
   (f) Reducing substances
   (g) Reducing sugar
   (h) Free amino nitrogen of the materials
   (i) Acid hydrolysis of the materials
   (j) Enzymatic hydrolysis of the materials
   (k) Titration curves on suspensions of the materials
   (l) Lysine content
3. The greatest degree of change attributable to changes in protein constituents was observed in the cases of the two types of milk powder and the dehydrated potatoes.

The changes observed in these materials were greatest under storage conditions of high temperature and high humidity.

4. The changes in the samples of milk powder and dehydrated potatoes appeared to be in large part a consequence of reaction between the protein and the reducing sugar components. It has been shown that the progress of deterioration of the milk powders was associated with changes in the lysine content of the proteins similar to those which are known to characterize the so-called "browning reaction".
5. A comparison of the changes in the two milk powders studied did not reveal consistent differences which could be attributed to the different methods of drying.
6. The samples of soybean meal, meat meal and ground oats changed in colour, acidity and solubility. The changes in the other properties and constituents studied were relatively slight. These observations suggest that the "browning reaction" did not play a major role in the deterioration of these products.
7. It has been shown that the changes which took place in short term accelerated tests were not necessarily the same in nature as those which occurred during long term storage tests. Accelerated tests, therefore, may give a measure of relative stability, but are unreliable as a means of ascertaining the nature of the changes which proceed during long term storage.

CLAIMS TO ORIGINAL RESEARCH

1. Changes in the proteins of five different foodstuffs and one feedstuff have been followed over a period of eighteen months under different conditions of temperature and humidity. It has been shown that the proteins of those materials containing an appreciable amount of reducing sugar underwent considerable changes. Materials containing little or no reducing sugar did not undergo such changes to a comparable degree.
2. Fresh evidence is submitted in support of the theory that lysine plays a major role in the browning of dried skim milk powders. The rate of browning of dried skim milk is shown to bear a close relation to the rate of disappearance of lysine.
3. It is shown that the browning of dehydrated potatoes during storage closely resembles browning of dried milk powders in most respects.
4. Evidence has been presented which suggests that high temperature browning of milk powders is brought about by a reaction different from that responsible for browning in long term storage tests.
5. The changes in the proteins of roller drum-dried and of spray-dried skim milk powders have been compared.
6. A modification of the colorimetric method of Boulet, Nelson and McFarlane for the determination of lysine has

been presented.

7. Hydrolysed zein has been used successfully as a source of amino acids in the basal medium for the microbiological assay of lysine.

8. Partially hydrolysed zein has been shown to contain materials which bring about increased growth response of Leuconostoc mesenteroides P-60 as compared with the growth response on completely hydrolysed zein.

9. The values for lysine content of the stored materials, secured by colorimetric and microbiological methods, have been compared. The colorimetric method yielded consistently lower values.

REFERENCES

1. Adamson, D. W.
   The Anhydrides of Basic Amino Acids.
   J. Chem. Soc., 39; 1943.

2. Almquist, H. J., Stokstad, E. L. R., and Halbrook, E. R.
   Concentrates in Chick Rations.
   J. Nutr., 10, 193; 1935.

3. Balson, E. W., and Lawson, A.
   The Potentiometric Determination of Polypeptides and Amino Acids. II. The Formaldehyde Titration.
   Biochem. J., 30, 1257; 1936.

4. Barton-Wright, E. C.
   The Microbiological Assay of Amino Acids.
   Analyst, 71, 267; 1946.

5. Bate-Smith, E. C., and Hawthorne, J. R.
   Dried Egg. X. The Nature of the Reactions Leading to Loss of Solubility of Dried Egg Products.
   J. Soc. Chem. Ind., 64, 297; 1945.

6. Block, R. J., Cannon, P. R., Wissler, R. W., Steffee, C. H., Straube, R. L., Frazier, L. E., and Woolridge, R. L.
   The Effects of Baking and Toasting on the Nutritional Value of Proteins.
   Arch. Biochem., 10, 295; 1946.

7. Block, R. J., Jones, D. B., and Gersdorff, C. E. F.
   The Effect of Dry Heat and Dilute Alkali on the Lysine Content of Casein.
   J. Biol. Chem., 105, 667; 1934.

8. Boggs, M. M., Dutton, H. J., Edwards, B. G. and Fevold, H. L.
   Dehydrated Egg Powders. Relation of Lipide and Salt-water Fluorescence Values to Palatability.
   Ind. Eng. Chem., 38, 1082; 1946.

9. Borchers, R., Ackerson, C. W., and Kimmett, L.
   Trypsin Inhibitor. IV. Occurrence in Seeds of the Leguminosae and Other Seeds.
   Arch. Biochem., 13, 291; 1947.

10. _____, and Mussehl, F. E.
    Trypsin Inhibitor. VI. Effect of Various Heating Periods on the Growth Promoting Value of Soybean Oil Meal for Chicks.
    Poultry Science, 27, 601; 1948.

11. Boulet, M. A.
A Modified Method for the Determination of Lysine and Its Application to Reactions between Reducing Sugars and Amino Acids.
Ph.D. Thesis, McGill University.

12. Bowman, D. E.
Fractions Derived from Soybeans and Navy Beans which Retard Tryptic Digestion of Casein.
Proc. Soc. Expt. Biol. and Med., 57, 139; 1944.

13. Burton, W. G.
The Storage Life of a Sample of Potato Flour Produced from Potato Slices Dried in a Sugar Beet Factory.
J. Soc. Chem. Ind., 64, 85; 1945.

14. _____.
Mashed Potato Powder. III. High Temperature Browning of Mashed Potato Powder.
J. Soc. Chem. Ind., 64, 215; 1945.

15. Caldwell, J. S., Brunstetter, B. C., Culpepper, C. W., and Ezell, B. D.
Causes and Control of Discoloration in Dehydration of White Potatoes.
The Canner, 100, (13, 14, 15); 1945.

16. Campbell, W. R., and Hanna, M. I.
The Determination of Nitrogen by Modified Kjeldahl Methods.
J. Biol. Chem., 119, 1; 1937.

17. Chapman, R. A., and McFarlane, W. D.
A Colorimetric Method for the Estimation of Reducing Groups in Milk Powders.
Can. J. Res., 23B, 91; 1945.

18. _____.
Wheat Germ Oil Antioxidants and Natural Reducing Substances on the Stability of Whole Milk Powder.
Can. J. Res., 24F, 47; 1946.

19. Clandinin, D. R., Cravens, W. W., Elvehjem, C. A. and Halpin, J. G.
Deficiencies in Overheated Soybean Oil Meal.
Poultry Sci., 25, 399; 1946.

20. _____.
Deficiencies in Overheated Soybean Oil Meal.
Poultry Sci., 26, 150; 1947.

21. Clandinin, D. R., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G.
The Relationship between Time and Temperature to the Nutritive Value of Soybean Oil Meal.
Poultry Sci., 27, 1370; 1948.

22. Cohn, E. J.
The Physical Chemistry of the Proteins.
Physiol. Rev., 5, 349; 1925.

23. Combs, W. B., and Coulter, S. T.
The Role of Oxygen Level on the Keeping Quality of Powdered Whole Milk.
Quartermaster Food and Container Institute Report No. 3, 1946.

24. Coulter, S. T., and Combs, W. B.
The Mechanism of Deteriorature Changes in Milk Products.
Quartermaster Food and Container Institute Report, 1947.

25. _____, Jenness, R., and Crowe, L. K.
Some Changes in Dry Whole Milk During Storage.
J. Dairy Science, 31, 986; 1948.

26. Crowe, L. K., Jenness, R., and Coulter, S. T.
The Reducing Capacity of Milk and Milk Products as Measured by a Modified Ferricyanide Method.
J. Dairy Science, 31, 595; 1948.

27. Curl, L. C., Moore, E. L., Weiderhold, E., and Veldhuis, M. K.
Concentrated Orange Juice Studies.
Fruit Products J., 26, 101; 1946.

28. De, S. S. and Ganguly, J.
Heat Treatment and the Biological Value of Soybean Protein.
Nature, 159, 341; 1947.

29. Deas, C. P., and Tarr, H. L. A.
Amino Acids in Fishery Products.
Fisheries Res. Bd. Canada.
Progress Rep. Pacific Coast Stas., 73, 50; 1947.

30. _____, Ney, P. W., and Tarr, H. L. A.
Amino Acids in Fishery Products. II.
Fishery Res. Bd. Canada.
Progress Rep. Pacific Coast Stas., 77, 97; 1948.

31. Denny, F. E., and Thornton, N. C.
Factors for Color in the Production of Potato Chips.
Contrib. Boyce Thompson Inst., 11, 291; 1940.

32. Déribéré, M.
La Fluorescence du Lait.
Lait 22, 122; 1942.
Chem. Abstracts, 37, 5501; 1943.

33. Doob, H., Willmann, A., and Sharp, P. F.
Influence of Moisture on Browning of Dried Whey and Skim Milk.
Ind. Eng. Chem., 34, 1460; 1942.

34. Dowell, C. T., and Menaul, P.
The Action of Furfurol and Dextrose on Amino Acids and Protein Hydrolysates.
J. Biol. Chem., 40, 131; 1919.

35. Du Toit, M. S., and Page, H. J.
Studies on the Carbon and Nitrogen Cycles in the Soil.
IV. Natural and Artificial Humic Acids.
J. Agr. Sci., 22, 115; 1932.

36. Dutton, H. J., and Edwards, B. G.
Spectrophotometric and Fluorometric Measurement of Changes in Lipides.
Ind. Eng. Chem., 37, 1123; 1945.

37. Dykins, F. A., and Englis, D. T.
Determination of Glucose in Presence of Fructose and Glycine by Iodimetric Method.
Ind. Eng. Chem., Anal. Ed., 3, 21; 1931.

38. Eldred, N. R., and Rodney, G.
The Effect of Proteolytic Enzymes on Raw and Heated Casein.
J. Biol. Chem., 162, 261; 1946.

39. Enders, C.
Zur Kenntnis der Melanoidine.
Kolloid Z., 85, 74; 1938.

40. Englis, D. T., and Dykins, F. A.
Effect of Amino Acids upon Rotation of Glucose and Fructose and Its Significance to Determination of Sucrose by Double Polarization Method.
Ind. Eng. Chem., Anal. Ed., 3, 17; 1931.

41. Euler, H. von, Brunius, E., and Josephson, K.
Uber Reactionen Zurischen Zuckerarten und Aminen.
II. Weitere Versuche mit Glucose und Aminosaure.
Z. Physiol. Chem., 155, 259; 1926.

42. Euler, H. von., and Josephson, K.
Zur Kenntnis der Aciditatsbedingung der Enzymatisehen Rohrzuckerspaltung.
Z. Physiol. Chem., 153, 1; 1926.

43. Evans, R. J.
Hydrolysis of Soybean Oil Meal Proteins by some Proteolytic Enzymes.
Arch. Biochem., 11, 15; 1946.

44. _____, and Butts, H. A.
Studies on the Heat Inactivation of Lysine in Soy Bean Oil Meal.
J. Biol. Chem., 175, 15; 1948.

45. _____.
Studies on the Heat Inactivation of Methionine in Soy Bean Oil Meal.
J. Biol. Chem., 178, 543; 1949.

46. _____, and McGinnis, J.
The Influence of Autoclaving Soybean Oil Meal on the Availability of Cystine and Methionine for the Chick.
J. Nutr., 31, 449; 1946.

47. _____.
Cystine and Methionine Metabolism by Chicks Receiving Raw or Autoclaved Soybean Oil Meal.
J. Nutr., 35, 477; 1948.

48. _____, and St. John, J. L.
The Influence of Autoclaving Soybean Oil Meal on the Digestibility of the Proteins.
J. Nutr., 33, 661; 1947.

49. _____, and St. John, J. L.
Estimation of the Relative Nutritive Value of Vegetable Proteins by Two Chemical Methods.
J. Nutr., 30, 209; 1945.

50. Fevold, H. L., Edwards, B. G., Dimick, A. L., and Boggs, M. M.
Dehydrated Egg Powders. Sources of Off-flavours Developed During Storage.
Ind. Eng. Chem., 38, 1079; 1946.

51. Frankel, M., and Katchalsky, A.
The Interaction of α-Amino Acids and Peptides with Sugars in Aqueous Solution.
Biochem. J., 31, 1595; 1937.

52. Frankel, M., and Katchalsky, A.
The Time Factor in the Interaction of Amino Acids with Sugars.
Biochem. J., 32, 1904; 1938.

53. Gerry, R. W., Carrick, W. C., and Hauge, S. M.
Untoasted Soybean Oil Meal in Simplified Chick Rations.
Poultry Science, 27, 621; 1948.

54. Gortner, R. A.
The Hydrolysis of Proteins in the Presence of Extraneous Materials and the Origin and Nature of the "Humin" of a Protein Hydrolysate.
Science, 48, 122; 1919.

55. Greaves, J. E., and Hirst, C. F.
Influence of Storage on the Composition of Flour.
Utah Agr. Expt. Sta. Tech. Bull. 194; 1925.

56. Greaves, E. O., Morgan, A. F., and Loveen, M. N.
The Affect of Amino Acid Supplements and of the Variations in Temperature and Duration of Heating upon the Biological Value of Heated Casein.
J. Nutr., 16, 115; 1938.

57. Greenhut, I. T., Potter, R. L., and Elvehjem, C. A.
Phenylalanine and Tryptophane Content of Meats.
Arch. Biochem., 15, 459; 1947.

58. Greenstein, J. P.
Studies of the Peptides of Trivalent Amino Acids.
J. Biol. Chem., 93, 479; 1931.

59. Groot, E. H.
Composition of Protein, Especially Potato Protein and Nutritive Value.
Voeding, 6, 153; 1945.
Chem. Abstracts, 42, 2033; 1948.

60. _____.
Investigation into the Biologically Important Amino Acids in Potato Protein in Connection with its Nutritive Value.
Arch. neerland. physiol., 28, 277; 1946.
Chem. Abstracts, 42, 6018; 1948.

61. _____, Jansen, L. W., Kentie, A., Oosterhuis, H. K., and Trap, H. J. L.
A New Protein in Potatoes.
Voeding, 6, 214; 1946.
Chem. Abstracts, 42, 2032; 1948.

62. Groot, E. H., and van der Linden, A. C.
The Nutritive Value of Potato Protein.
Voeding, 10, 18; 1949.
Chem. Abstracts, 43, 1877; 1949.

63. Grunhut, L., and Weber, J.
Quantitative Studien uber die Einwirkung von Aminosauren auf Zuckerarten.
Biochem. Z., 121, 109; 1921.

64. Hall, J. A.
Summary of the Orange Juice Problem.
Research Report, Calif. Fruit Growers Exchange, 1927.

65. Ham, W. E. and Sandstedt, R. M.
A Proteolytic Inhibiting Substance in the Extract from Unheated Soy Bean Meal.
J. Biol. Chem., 154, 505; 1944.

66. Harland, H. A., and Ashworth, V. S.
The Use of Thiamine Disulfide for the Estimation of Reducing Substances in Processed Milk.
J. Dairy Science, 28, 15; 1945.

67. Harris, R. L., and Mattill, H. A.
The Effect of Hot Alcohol on Purified Animal Proteins.
J. Biol. Chem., 132, 477; 1940.

68. Hawk, P. B., Oser, B. L., and Summerson, W. H.
Practical Physiological Chemistry.
Blakiston, Toronto, 12th Ed., 1947, p. 215.

69. Hawthorne, J. R., and Brooks, J.
Dried Egg. VIII. Removal of the Sugar of Egg Pulp before Drying. A Method of Improving the Storage Life of Spray-Dried Whole Egg.
J. Soc. Chem. Ind., 63, 232; 1944.

70. Hayward, J. W., and Hafner, F. H.
The Supplementary Effect of Cystine and Methionine upon the Protein of Raw and Cooked Soybeans as Determined with Chicks and Rats.
Poultry Science, 20, 139; 1941.

71. _____, Halpin, J. G., Holmes, C. E., Bohstedt, G., and Hart, E. B.
Soybean Oil Meal Prepared at Different Temperatures as a Feed for Poultry.
Poultry Science, 16, 3; 1937.

72. Hayward, J. W., Steenbock, H., and Bohstedt, G.
The Effect of Heat as Used in the Extraction of Soybean Oil upon the Nutritive Value of the Proteins of Soybean.
J. Nutr., 11, 219; 1936.

73. Heberlein, D. G., and Clifcorn, L. C.
Vitamin Content of Dehydrated Foods. Effect of Packing and Storage.
Ind. Eng. Chem., 36, 912; 1944.

74. Henry, K. M., Kon, S. K., Lea, C. H., and White, J. C. D.
Deterioration on Storage of Dried Skim Milk.
J. Dairy Res., 15, 292; 1948.

75. Hodson, A. Z., and Krueger, G. M.
Essential Amino Acid Content of Casein and Fresh and Processed Cow's Milk as Determined Microbiologically on Hydrolysates.
Arch. Biochem., 10, 55; 1946.

76. Holm, G. E., Greenbank, G. R., and Deysher, E. F.
Results of Preliminary Experiments upon the Effect of Separating or Clarifying, and Pasteurizing of a Milk upon the Keeping Quality of its Milk Powder.
J. Dairy Science, 9, 512; 1926.

77. Horvath, A. A.
The Soybean Industry, p. 141.
Chemical Pub. Co., New York, 2nd Ed., 1939.

78. Howat, G. R., and Wright, N. C.
Factors Affecting the Solubility of Milk Powders.
II. Influence of the Temperature of Reconstitution on Protein Solubility.
J. Dairy Res., 4, 265; 1933.

79. Hunziker, O. F.
Condensed Milk and Milk Powder.
(Hunziker, La Grange, Ill.) 5th Ed., 1935.

80. Hurd, C. D., and Kelso, C. D.
Hydroxylation of 2,3-Dihydropyran and the Application of Desoxyaldopentoses in the Browning Reaction.
J. Am. Chem. Soc., 70, 1484; 1948.

81. Hutchinson, J. C. D., Bacon, J. S. D., Macrae, T. F. and Worden, A. N.
The Nutritive Value of Potato Protein for the Pig.
Biochem. J., 37, 550; 1943.

62. Groot, E. H., and van der Linden, A. C.
The Nutritive Value of Potato Protein.
Voeding, 10, 18; 1949.
Chem. Abstracts, 43, 1877; 1949.

63. Grunhut, L., and Weber, J.
Quantitative Studien uber die Einwirkung von Aminosauren auf Zuckerarten.
Biochem. Z., 121, 109; 1921.

64. Hall, J. A.
Summary of the Orange Juice Problem.
Research Report, Calif. Fruit Growers Exchange, 1927.

65. Ham, W. E. and Sandstedt, R. M.
A Proteolytic Inhibiting Substance in the Extract from Unheated Soy Bean Meal.
J. Biol. Chem., 154, 505; 1944.

66. Harland, H. A., and Ashworth, V. S.
The Use of Thiamine Disulfide for the Estimation of Reducing Substances in Processed Milk.
J. Dairy Science, 28, 15; 1945.

67. Harris, R. L., and Mattill, H. A.
The Effect of Hot Alcohol on Purified Animal Proteins.
J. Biol. Chem., 132, 477; 1940.

68. Hawk, P. B., Oser, B. L., and Summerson, W. H.
Practical Physiological Chemistry.
Blakiston, Toronto, 12th Ed., 1947, p. 215.

69. Hawthorne, J. R., and Brooks, J.
Dried Egg. VIII. Removal of the Sugar of Egg Pulp before Drying. A Method of Improving the Storage Life of Spray-Dried Whole Egg.
J. Soc. Chem. Ind., 63, 232; 1944.

70. Hayward, J. W., and Hafner, F. H.
The Supplementary Effect of Cystine and Methionine upon the Protein of Raw and Cooked Soybeans as Determined with Chicks and Rats.
Poultry Science, 20, 139; 1941.

71. _____, Halpin, J. G., Holmes, C. E., Bohstedt, G., and Hart, E. B.
Soybean Oil Meal Prepared at Different Temperatures as a Feed for Poultry.
Poultry Science, 16, 3; 1937.

72. Hayward, J. W., Steenbock, H., and Bohstedt, G.
The Effect of Heat as Used in the Extraction of Soybean Oil upon the Nutritive Value of the Proteins of Soybean.
J. Nutr., 11, 219; 1936.

73. Heberlein, D. G., and Clifcorn, L. C.
Vitamin Content of Dehydrated Foods. Effect of Packing and Storage.
Ind. Eng. Chem., 36, 912; 1944.

74. Henry, K. M., Kon, S. K., Lea, C. H., and White, J. C. D.
Deterioration on Storage of Dried Skim Milk.
J. Dairy Res., 15, 292; 1948.

75. Hodson, A. Z., and Krueger, G. M.
Essential Amino Acid Content of Casein and Fresh and Processed Cow's Milk as Determined Microbiologically on Hydrolysates.
Arch. Biochem., 10, 55; 1946.

76. Holm, G. E., Greenbank, G. R., and Deysher, E. F.
Results of Preliminary Experiments upon the Effect of Separating or Clarifying, and Pasteurizing of a Milk upon the Keeping Quality of its Milk Powder.
J. Dairy Science, 9, 512; 1926.

77. Horvath, A. A.
The Soybean Industry, p. 141.
Chemical Pub. Co., New York, 2nd Ed., 1939.

78. Howat, G. R., and Wright, N. C.
Factors Affecting the Solubility of Milk Powders.
II. Influence of the Temperature of Reconstitution on Protein Solubility.
J. Dairy Res., 4, 265; 1933.

79. Hunziker, O. F.
Condensed Milk and Milk Powder.
(Hunziker, La Grange, Ill.) 5th Ed., 1935.

80. Hurd, C. D., and Kelso, C. D.
Hydroxylation of 2,3-Dihydropyran and the Application of Desoxyaldopentoses in the Browning Reaction.
J. Am. Chem. Soc., 70, 1484; 1948.

81. Hutchinson, J. C. D., Bacon, J. S. D., Macrae, T. F. and Worden, A. N.
The Nutritive Value of Potato Protein for the Pig.
Biochem. J., 37, 550; 1943.

82. Ingvaldsen, T.
Fish Meals. I. Effect of High Temperature Employed for Drying on the Nitrogen Partition.
Can. Chem. and Met., 13, 97; 1929.

83. International Critical Tables.
1st Edition.
Vol. 1, p. 67 (1926).

84. Jenness, R., and Coulter, S. T.
Measurement of Fluorescent Materials in Milk and Milk Products.
J. Dairy Science, 31, 369; 1948.

85. Jensen, O. W. and Plattner, E.
The Cause of Discoloration of Milk on Heating.
Rev.-gen.-lait., 4, 361, 388, 419; 1905.

86. Johnson, L. M., Parsons, H. T., and Steenbock, H.
The Effect of Heat and Solvents on the Nutritive Value of Soybean Protein.
J. Nutr., 18, 423; 1939.

87. Jones, D. B., Caldwell, A., and Widness, K. D.
Comparative Growth Promoting Values of the Proteins of Cereal Grains.
J. Nutr., 35, 639; 1948.

88. _____, Divine, J. P., and Gersdorff, C. E. F.
The Effect of Storage of Corn on the Chemical Properties of its Proteins and on its Growth-Promoting Value.
Cereal Chem., 19, 819; 1942.

89. _____, and Gersdorff, C. E. F.
The Effect of Storage on the Protein of Wheat, White Flour, and Whole Wheat Flour.
Cereal Chem., 18, 417; 1941.

90. Joslyn, M. A., and Marsh, G. L.
Browning of Orange Juice.
Ind. Eng. Chem., 27, 186; 1935.

91. Kass, J. P., and Palmer, L. S.
Browning of Autoclaved Milk.
Ind. Eng. Chem., 32, 1360; 1940.

92. King, N.
The Microscopal Examination of Milk Products.
Netherlands Milk and Dairy J., 2, 137; 1948.
Chem. Abstracts, 43, 1874; 1949.

93. Klose, A. A., Hill, B., and Fevold, H. L.
Food Value of Soybean Protein as Related to Processing.
Food Technology, 2, 201: 1948.

94. Kometiani, P. A.
Veranderungen einiger Milchbestandteil durch Erhitzen.
Milch. Forsch., 12, 433; 1931.
Chem. Abstracts, 26, 1987; 1932.

95. Kuether, C. A., and Myers, V. C.
The Nutritive Value of Cereal Proteins in Human Subjects.
J. Nutr., 35, 651; 1948.

96. Kunitz, M.
Crystallization of a Trypsin Inhibitor from Soybean.
Science, 101, 668; 1945.

97. Kuzin, A. M., and Makaeva, Z.
Reaction of Simple Sugars in the Presence of Glycine.
Biokhimiya, 4, 367; 1939.
Chem. Abstracts, 34, 1693; 1940.

98. Lea, C. H.
A Note on the Chapman and McFarlane Method for the Estimation of Reducing Groups in Milk Powder.
Analyst, 72, 336; 1947.

99. _____.
The Reaction Between Milk Protein and Reducing Sugar in the Dry State.
J. Dairy Res., 15, 369; 1948.

100. _____, Moran, T., and Smith, J. A. B.
The Gas Packing and Storage of Milk Powder.
J. Dairy Research, 13, 162; 1943.

101. Leavitt, S., and Leclerc, J. A.
Change in the Composition of Unground Cereals During Storage.
J. Ind. Eng. Chem., 1, 299; 1909.

102. Leeds, A. R.
The Chemical and Physical Changes Attendant upon the Sterilization of Milk.
J. Am. Chem. Soc., 13, 34; 1891.

103. Legault, R. R., Talburt, W. F., Mylne, A. M., and Bryan, L. A.
Browning of Dehydrated Vegetables during Storage.
Ind. Eng. Chem., 39, 1294; 1947.

104. Levy, M., and Silberman, D. E.
The Reaction of Amino and Imino Acids with Formaldehyde.
J. Bio. Chem., 118, 723; 1937.

105. Lyman, C. M., and Kuiken, K. A.
The Amino Acid Composition of Meat and Other Foods. I. Arginine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophane and Valine.
Texas Agr. Expt. Sta. Bull., 708, 5; 1949.

106. Maillard, L. C.
Synthèse des Matières Humiques par Action des Acides Amines sur les Sucres Reducteurs.
Ann. Chim., IX, 5, 258; 1916.
Chem. Abstracts, 10, 2577; 1916.

107. _____.
Identity of Synthetic with Natural Humic Substances.
Ann. Chim., 7, 113; 1917.
Chem. Abstracts, 11, 2010; 1917.

108. Mangels, C. E., and Gore, H. C.
Effect of Heat on Different Dehydrated Vegetables.
Ind. Eng. Chem., 13, 525; 1921.

109. Maynard, L. A., Binder, R. C., and McCay, C. M.
Vitamin A and Protein Content of Various Fish Meals.
J. Agr. Res., 44, 591; 1932.

110. _____, and Tunison, A. V.
Influence of Drying Temperature upon Digestibility and Biological Value of Fish Proteins.
Ind. Eng. Chem., 24, 1168; 1932.

111. McGinnis, J., and Evans, R. J.
Amino Acid Deficiencies of Raw and Overheated Soybean Oil Meal for Chicks.
J. Nutr., 34, 725; 1947.

112. Melnick, D., Oser, B. L., and Weiss, S.
Rate of Enzymic Digestion of Proteins as a Factor in Nutrition.
Science, 103, 326; 1946.

113. Millares, R., and Fellers, C. R.
Vitamin and Amino Acid Composition of Processed Chicken Meat Products.
Food Research, 14, 131; 1949.

114. Mitchell, H. H.
Determination of the Nutritive Value of the Proteins of Food Products.
Ind. Eng. Chem., Anal. Ed., 16, 696; 1944.

115. _____, and Block, R. J.
Some Relationships between the Amino Acid Content of Proteins and their Nutritive Value for the Rat.
J. Biol. Chem., 163, 599; 1946.

116. Morgan, A. F.
The Effect of Heat upon the Biological Value of Cereal Proteins and Casein.
J. Biol. Chem., 90, 771; 1931.

117. _____, and Kern, L. E.
The Effect of Heat Upon the Biological Value of Cereal Proteins and Casein.
J. Nutr., 7, 367; 1934.

118. _____, and King, F. B.
Change of Biological Value of Cereal Proteins due to Heat Treatment.
Proc. Soc. Expt. Biol. and Med., 23, 353; 1926.

119. Moster, J. B.
Studies on Chemical Methods of Measuring Changes in the Protein Fraction During the Storage of Foods.
McGill University Thesis, 1947.

120. Murlin, J. R., Nasset, E. E., and Marsh, M. E.
The Egg-Replacement Value of the Proteins of Cereal Breakfast Foods with Consideration of Heat Injury.
J. Nutr., 16, 249; 1938.

121. Neuberger, A., and Sanger, F.
The Nitrogen of the Potato.
Biochem. J., 36, 662; 1942.

122. _____.
The Availability of the Acetyl Derivatives of Lysine for Growth.
Biochem. J., 37, 515; 1943.

123. Nicholls, P. F., and Reed, H. M.
What Happens to Fruit Products in the Tropics.
Western Canner and Packer, 23, 11; 1931.

124. Olcott, V. S., and Dutton, H. J.
Changes in Stored Dried Eggs.
Ind. Eng. Chem., 37, 1119, 1123; 1945.

125. Osborne, T. B., and Mendel, L. B.
The Use of Soy Bean as Food.
J. Biol. Chem., 32, 369; 1917.

126. Pader, M., Melnick, D., and Oser, B. L.
Factors Affecting the Availability of Lysine in Heat-Processed Casein.
J. Biol. Chem., 172, 763; 1948.

127. Patton, A. R., Hill, E. G., and Foreman, E. M.
Amino Acid Impairment in Casein Heated with Glucose.
Science, 107, 623; 1948.

128. _____, and Pyke, W. E.
The Role of Amino Acids and Glucose in the Browning of Potato Chips.
Abst. Papers. 110th Meeting, A. C. S., 1946.

129. Pearce, J. A.
Fluorescence Development in Various Food Products.
Can. J. Res., 22F, 87; 1944.

129A. _____.
Fluorescence Measurements as a Quality Control Technique in Food Processing.
Food in Canada, Nov., 1945.

130. _____.
Dried Milk Powder. I. Methods of Assessing Quality and Some Effects of Heat Treatment.
Can. J. Res., 23F, 177; 1945.

131. _____.
Fluorescence Development in Egg Powder and in Glucose-Glycine Mixtures.
Ind. Eng. Chem., 41, 1514; 1949.

132. _____, and Bryce, W. A.
Fluorescence Spectra of Extracts of Dried Whole Egg Powder.
Food Technology, 1, 310; 1947.

133. _____, and Thistle, M. W.
Fluorescence as a Measure of Quality in Dried Whole Egg Powder.
Can. J. Res., 20D, 276; 1942.

134. _____, Woodcock, A. H., and Gibbons, W. E.
Dried Whole Egg Powder. X. Effect of Added Substances on Keeping Quality.
Can. J. Res., 22F, 34; 1944.

135. Peters, F. N., Carroll, R. W., Bunting, W. R., and Hensley, G. W.
The Influence of Environmental Conditions on the Behaviour and Composition of Cereal Proteins.
Quartermaster Food and Container Institute Report No. 1, 1947.

136. Pregl, F.
Quantitative Organic Microanalysis.
Blakistons, Philadelphia, 1924.

137. Pyne, G. T.
The Determination of Milk Proteins by Formaldehyde Titration.
Biochem. J., 26, 1006; 1932.

138. Radley, J. A., and Grant, J.
Fluorescence Analysis by Ultraviolet Light.
Chapman and Hall, London; 1933.

139. Ramsey, R. J., Tracey, P. H., and Ruehe, H. A.
The Use of Corn Sugar in the Manufacture of Sweetened Condensed Skimmilk.
J. Dairy Science, 16, 17; 1933.

140. Reisen, W. H., Clandinin, D. R., Elvehjem, C. A., and Cravens, W. W.
Liberation of Essential Amino Acids from Raw, Properly Heated and Overheated Soy Bean Oil Meal.
J. Biol. Chem., 167, 143; 1947.

141. Rice, R. G., Kertesz, Z. I., and Stotz, E. H.
Color Formation in Furfural Systems.
J. Am. Chem. Soc., 69, 1798; 1947.

142. Richert, P. H.
Darkening and Other Grape Products Problems.
Fruit Products J., 10, 1930.

143. Richmond, H. D., Elsdon, G. D., and Walker, G. H.
Dairy Chemistry, p. 10.
Griffin, London, 4th Ed., 1942.

144. Robison, W. L.
Swine Feeding Experiments.
Ohio Agr. Expt. Sta. Spec. Circ., 26; 1930.

145. Ross, A. F.
Deterioration of Processed Potatoes.
Advances in Food Research. Vol. I. Academic Press, 1948.

146. Ross, A. F., Hilborn, M. T., Jenness, L. C., and Bartlett, E. M.
Discoloration of Potatoes Can be Avoided.
Food Packer, 26, 38; 1945.

147. Samuely, F.
Uber die Eiweiss hervorgehenden Melanine.
Beitrage 2. Chem. Physiol. Path., 2, 355; 1902.
Chem. Zentr., 6, 2, 805; 1902.

148. Scallett, B. L., and Gardner, J. H.
Formation of 5-Hydroxymethylfurfural from D-Glucose in Aqueous Solution.
J. Am. Chem. Soc., 67, 1934; 1945.

149. Schmidt, C. L. A., Kirk, R. L., and Appleman, W. K.
The Apparent Dissociation Constant of Arginine and Lysine.
J. Biol. Chem., 88, 285; 1930.

150. Schneider, B. H.
Nitrogen-Balanced Studies with Various Fish Meals.
J. Agr. Res., 44, 723; 1932.

151. Seaver, J. L., and Kertesz, Z. I.
The "Browning (Maillard) Reaction" in Heated Solutions of Uronic Acids.
J. Am. Chem. Soc., 68, 2178; 1946.

152. Shigeki, M.
Change in Quality of Meat Protein.
J. Agr. Chem. Soc. (Japan), 20, 315; 1944.
Chem. Abstracts, 42, 3502; 1948.

153. Singh, B., Dean, G. R., and Cantor, S. M.
The Role of 5-(Hydroxymethyl)-furfural in the Discoloration of Sugar Solutions.
J. Am. Chem. Soc., 70, 517; 1948.

154. Splittgerber, A.
A Study of the Solids of Milk.
Z. Nahr. Genussm., 24, 493; 1912.
Chem. Abstracts, 7, 389; 1913.

155. Sprung, M. M.
A Summary of the Reactions of Aldehydes with Amines.
Chem. Rev., 26, 297; 1940.

156. Stadtman, E. R.
Nonenzymatic Browning in Fruit Products.
Advances in Food Research. Vol. I. Academic Press; 1948.

157. Stadtman, E. R., Barker, H. A., Mrak, E. M., and Mackinney, G.
Studies on the Storage of Dried Fruit.
Ind. Eng. Chem., 38, 99; 1945.

158. Stevens, J. M., and McGinnis, J.
The Effect of Autoclaving Lysine in the Presence of Carbohydrate on its Utilization by the Chick.
J. Biol. Chem., 171, 431; 1947.

159. Stewart, G. F., Best, L. R., and Lowe, B.
Some Factors Affecting the Storage Changes in Spray-Dried Egg Products.
Proc. Inst. Food Technologists, 77; 1943.

160. Stewart, R. A., and Carroll, R. W.
Abst. Papers. 109th Meeting, A. C. S., 1946.

161. _____, Hensley, G. A., and Peters, F. W.
The Nutritive Value of Protein. I. The Effect of Processing on Oat Protein.
J. Nutr., 26, 519; 1943.

162. Stewart, G. F., and Kline, R. W.
Dried Egg Albumen. I. Solubility and Color Denaturation.
Proc. Inst. Food Technologists, 48; 1941.

163. Stover, N. M., and Sandin, R. B.
Use of Boric Acid in Microkjeldahl Determination of Nitrogen.
Ind. Eng. Chem., Anal. Ed., 3, 240; 1931.

164. Supplee, G. C.
The Keeping Quality of Dry Milk.
Proc. World's Dairy Congress, 2, 1248; 1923.

165. Tarassuk, N. P., and Jack, E. L.
A Study of the Browning Reaction in Whole Milk Powder and Ice Cream Mix Powder.
J. Dairy Science, 31, 255; 1948.

166. Thistle, M. W., Pearce, J. A., and Gibbons, N. E.
Dried Whole Egg Powder. I. Methods of Assessing Quality.
Can. J. Res., 21D, 1; 1943.

167. _____, White, W. H., Reid, M., and Woodcock, A. H.
Dried Whole Egg Powder. XIV. Effects of Low Temperature, Low Moisture Content, Carbon Dioxide Pack and Copper Contamination on Keeping Quality.
Can. J. Res., 22F, 80; 1944.

168. Thompson, J. B., Kocher, R. B., and Fritzsche, H. W.
A Browning Reaction Involving Copper-Proteins.
Arch. Biochem., 18, 41; 1948.

169. Troy, H. C., and Sharp, P. F.
α- and β-Lactose in Some Milk Products.
J. Dairy Science, 13, 140; 1930.

170. Van Slyke, D. D.
The Analysis of Proteins by Determination of the Chemical Groups Characteristic of the Different Amino Acids.
J. Biol. Chem., 10, 15; 1911.

171. _____.
The Nature of the Free Amino Groups in Proteins.
J. Biol. Chem., 16, 539; 1914.

172. Wahab, A.
M.Sc. Thesis.
University of California, 1946.
Quoted from Stadtman, E. R.
Advances in Food Research, Vol. I. Academic Press, 1948.

173. Waisman, H. A., and Elvehjem, C. A.
The Effect of Autoclaving on the Nutritive Value of Edestin.
J. Nutr., 16, 103; 1938.

174. Weast, C. A., and Mackinney, G.
Nonenzymatic Darkening of Fruits and Fruit Products.
Ind. Eng. Chem., 33, 1408; 1941.

175. Webb, B. H.
Color Development in Lactose Solutions During Heating with Special Reference to the Color of Evaporated Milk.
J. Dairy Science, 18, 81; 1935.

176. Westfall, R. J.
Influence of Crude Trypsin Inhibitor on Utilization of Hydrolysed Protein.
Proc. Soc. Expt. Biol. and Med., 68, 498; 1948.

177. _____, and Hauge, S. M.
The Nutritive Quality and the Trypsin Inhibitor Content of Soybean Flour Heated at Various Temperatures.
J. Nutr., 35, 379; 1948.

178. White, W. H., and Thistle, M. W.
Dried Whole Egg Powder. II. Effect of Heat Treatment on Quality.
Can. J. Res., 21D, 194; 1943.

179. _____.
Dried Whole Egg Powder. IV. Effect of Moisture Content on Keeping Quality.
Can. J. Res., 21D, 211; 1943.

180. _____, and Reid, M.
Dried Whole Egg Powder. VI. Effect of Storage Temperature and Gas Packing on Keeping Quality.
Can. J. Res., 21D, 271; 1943.

181. Whittier, E. O., and Benton, A. G.
The Formation of Acid in Milk by Heating.
J. Dairy Sci., 10, 126; 1927.

182. Wilder, O. M., and Kraybill, H. R.
Effect of Cooking and Curing on the Lysine Content of Pork Luncheon Meat.
J. Nutr., 33, 235; 1947.

183. Wilgus, H. S., Norris, L. C., and Heuser, G. F.
Effect of Heat on Nutritive Value of Soy-Bean Oil Meal.
Ind. Eng. Chem., 28, 586; 1936.

184. _____.
Haddock Meal. Effect of Manufacturing Process upon the Nutritive Value.
Ind. Eng. Chem., 27, 419; 1935.

185. Wolfram, M. L., Cavalieri, L. F., and Cavalieri, D. K.
Chemical Interactions of Amino Compounds. II. Methylation Experiments.
J. Am. Chem. Soc., 69, 2411; 1947.

186. _____, Schuetz, R. D., and Cavalieri, L. F.
Chemical Interactions of Amino Compounds and Sugars. III. The Conversion of D-Glucose to (5-(Hydroxymethyl)-2-furaldehyde.
J. Am. Chem. Soc., 70, 514; 1948.

187. Woodcock, A. H., and Reid, M.
Dried Whole Egg Powder. IX. Effect of Drying Conditions on Quality.
Can. J. Res., 21D, 389; 1943.

188. Wright, N. C.
The Action of Rennet and of Heat on Milk.
Biochem. J., 18, 247; 1924.

189. _____.
The Effect of Heat on the Solubility of Milk Proteins.
J. Dairy Res., 4, 122; 1932.

190. Zerban, F. W.
Report on Polariscopic Methods.
J. Assoc. Official Agr. Chem., 11, 167; 1928.

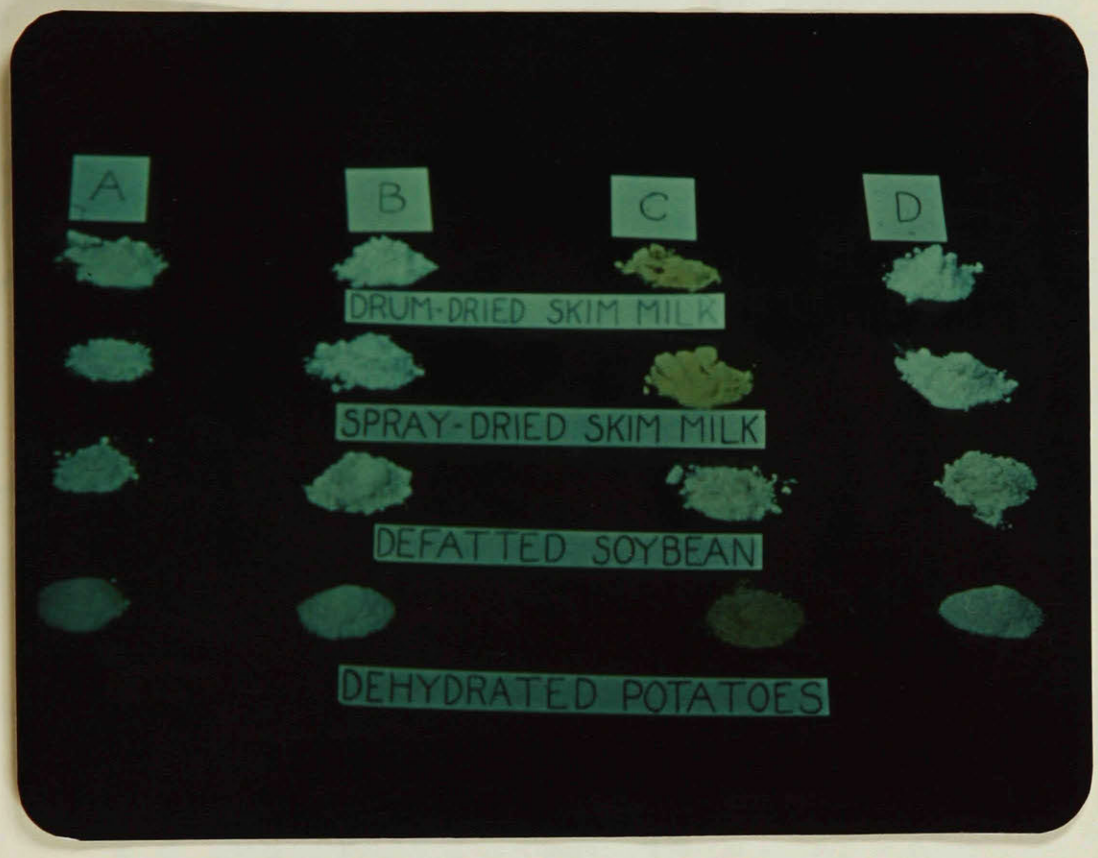
191. Zittle, C. A., and Eldred, N. R.
Determination of l-Lysine with a Specific Decarboxylase.
J. Biol. Chem., 156, 401; 1944.

Part II

Appendix

FIGURE 16.

Illustration of the Colour and Texture Changes of Foods after 18 Months Storage



	Storage Temperature	Storage Humidity
A	27°C	High
B	27°C	Low
C	37°C	High
D	37°C	Low

FIGURE 17.

Colour Changes of Stored Foods

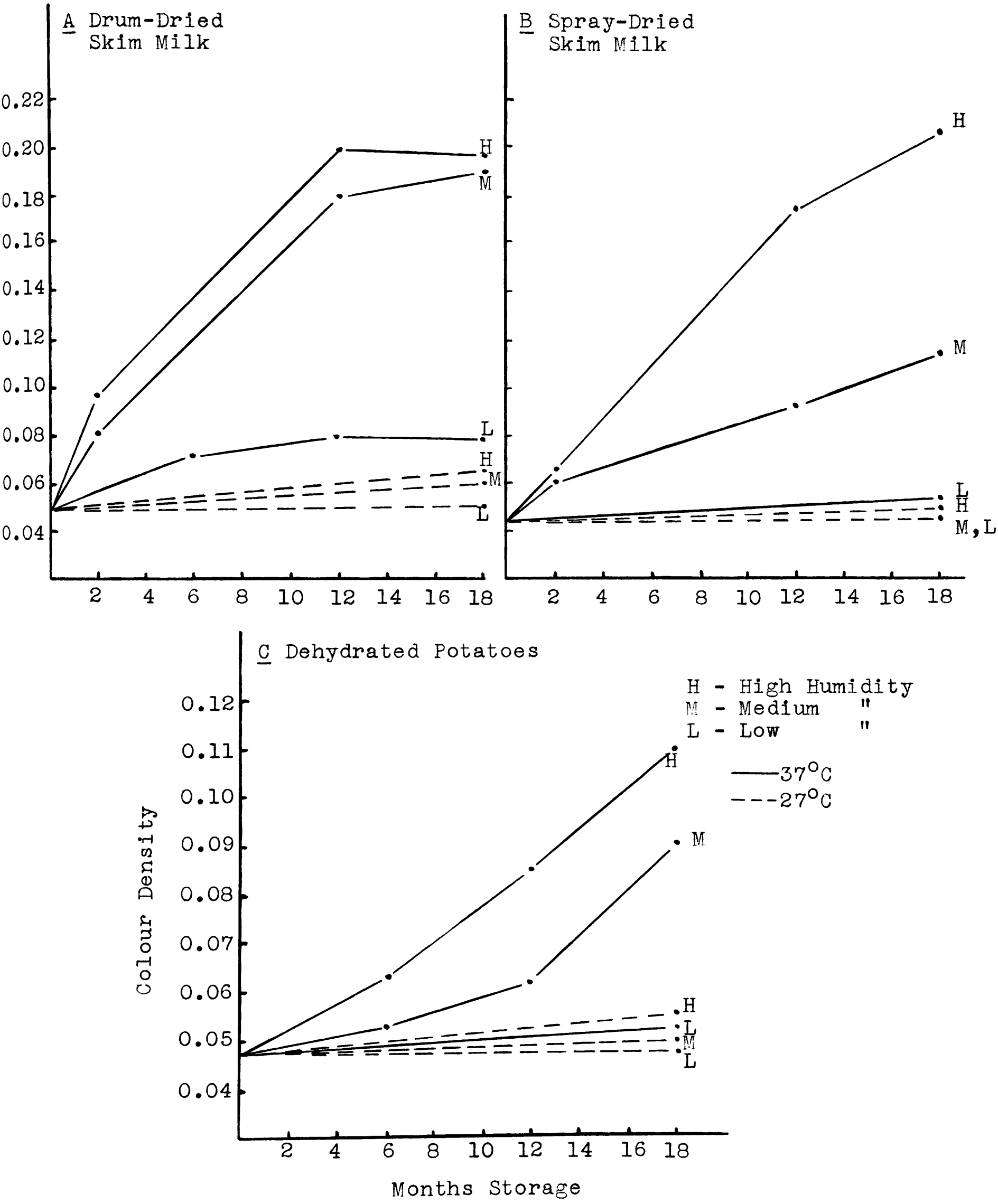


FIGURE 18.

Fluorescence of Stored Foods

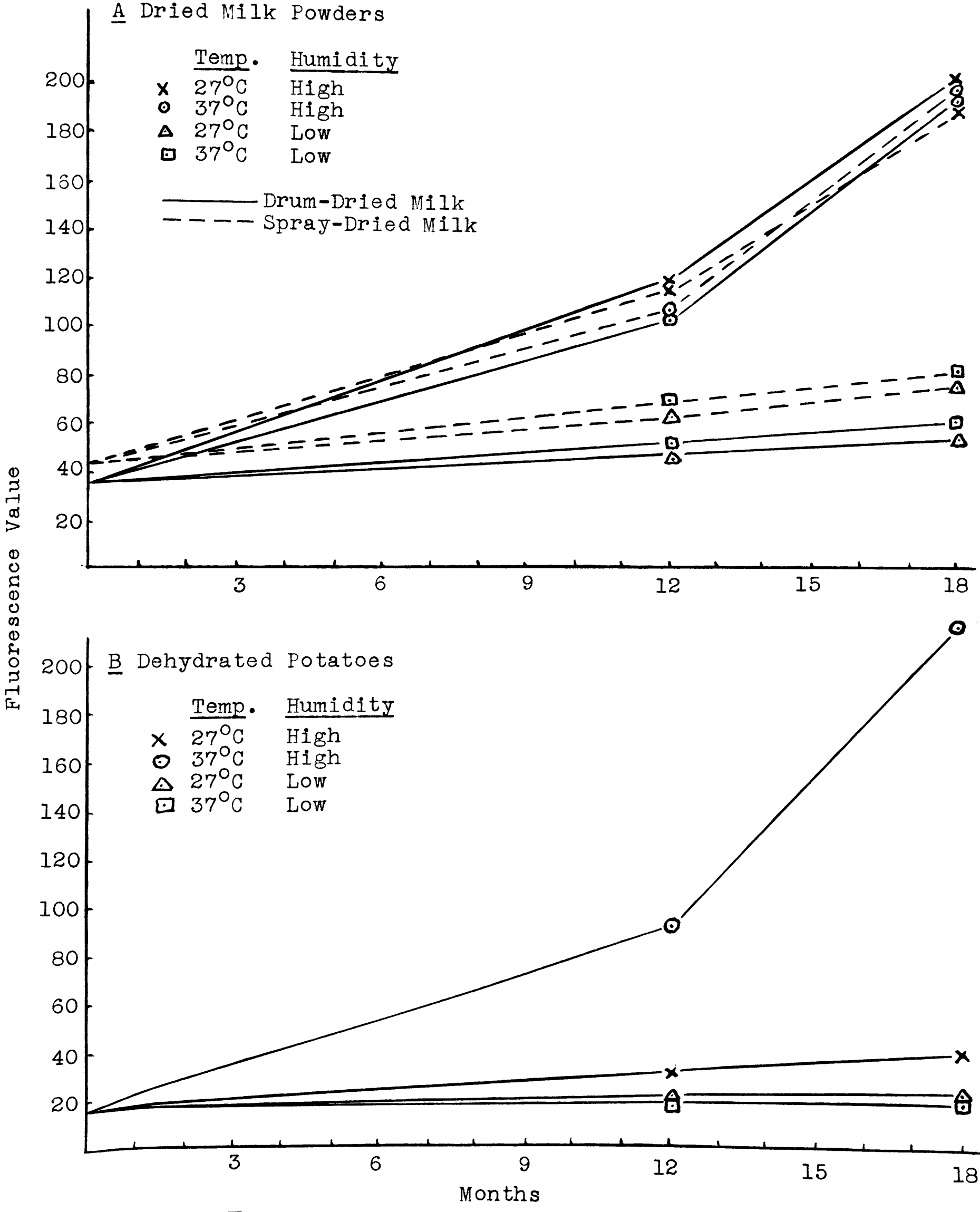


FIGURE 19.

Solubility of Nitrogen of Stored Foods

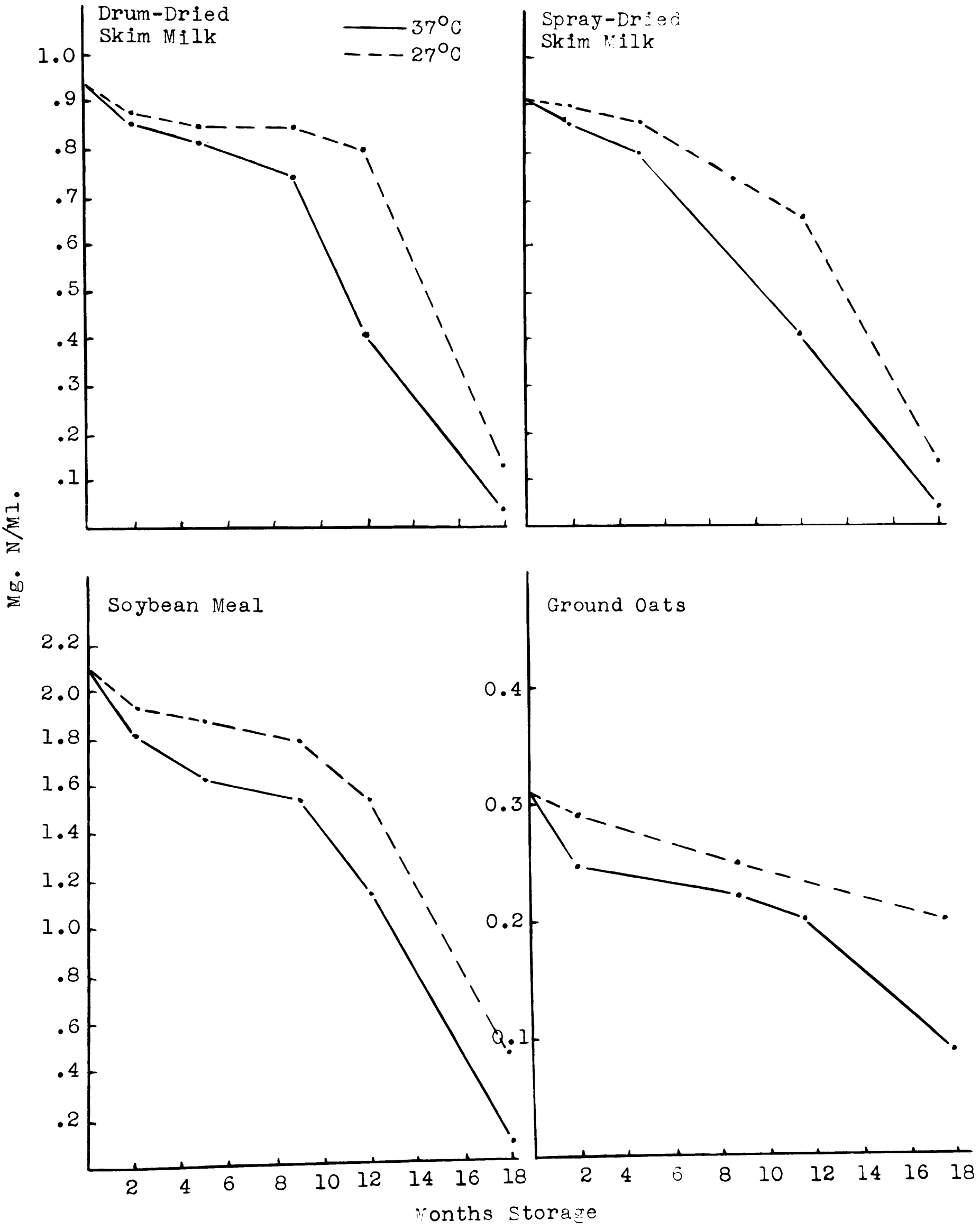


FIGURE 20.

Acid Hydrolysis of Stored Foods

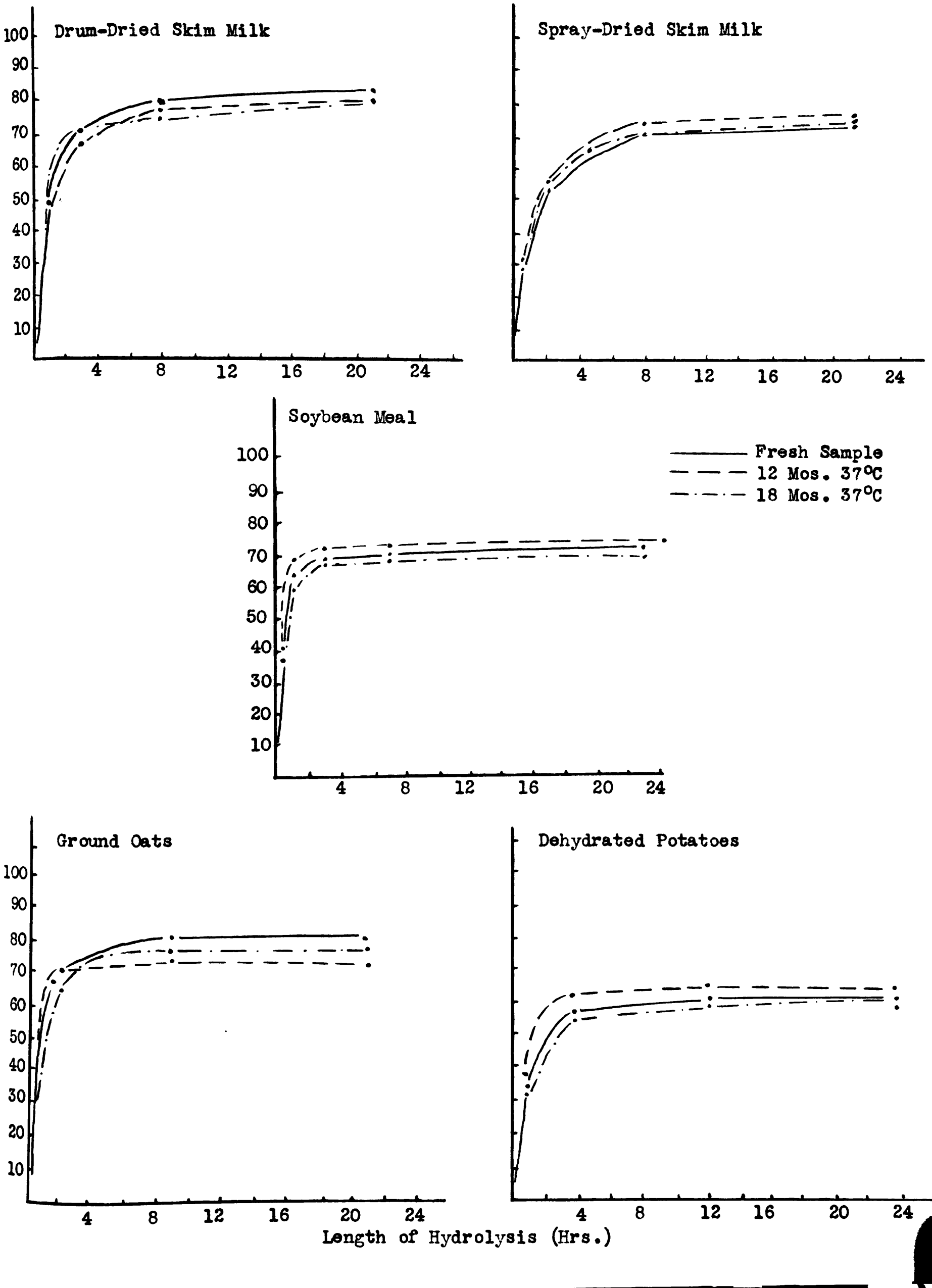


FIGURE 21.

Enzymatic Hydrolysis of Foods

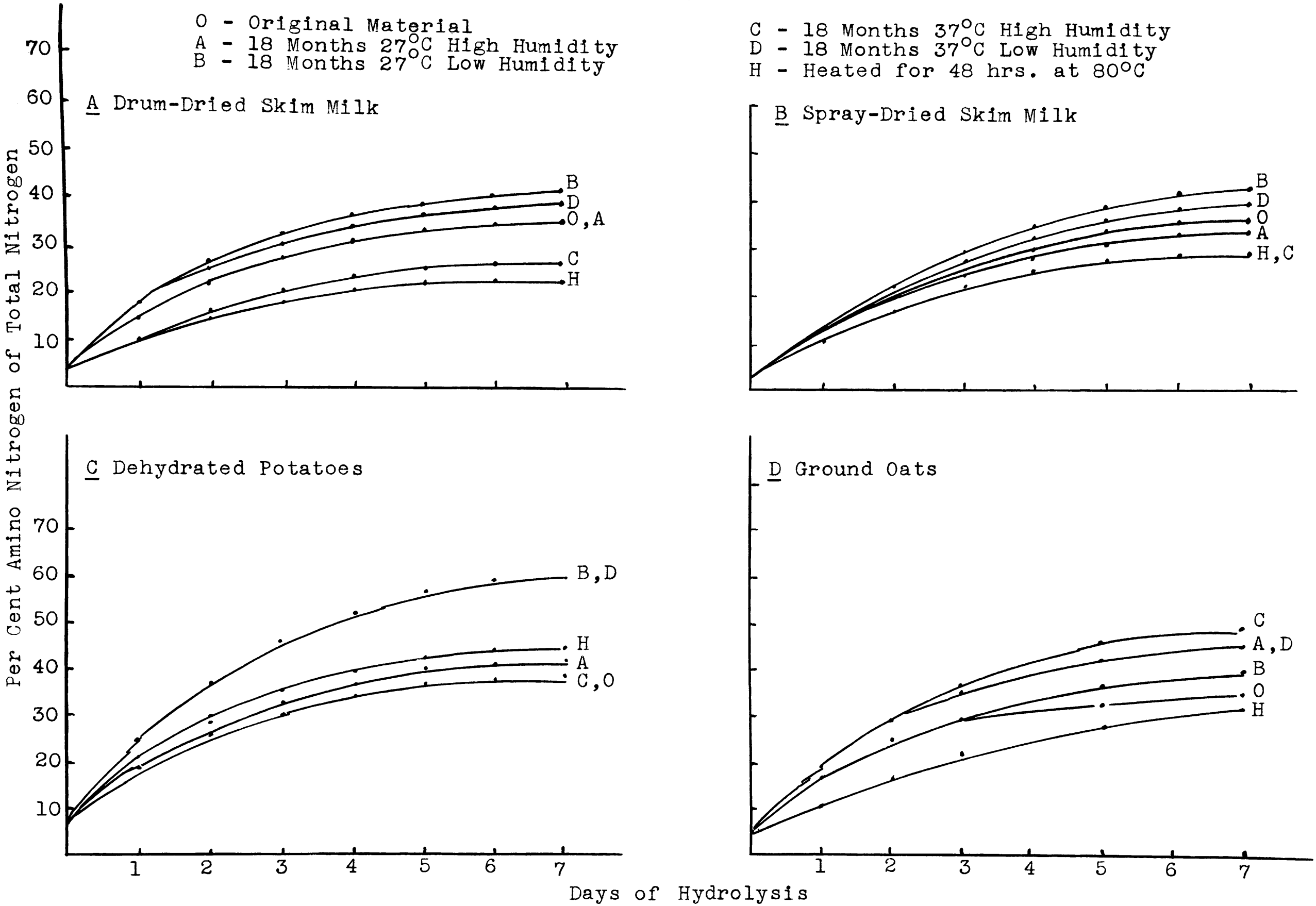


FIGURE 22A.

Titration Curves of Stored and Heated Drum-Dried Skim Milk Powder

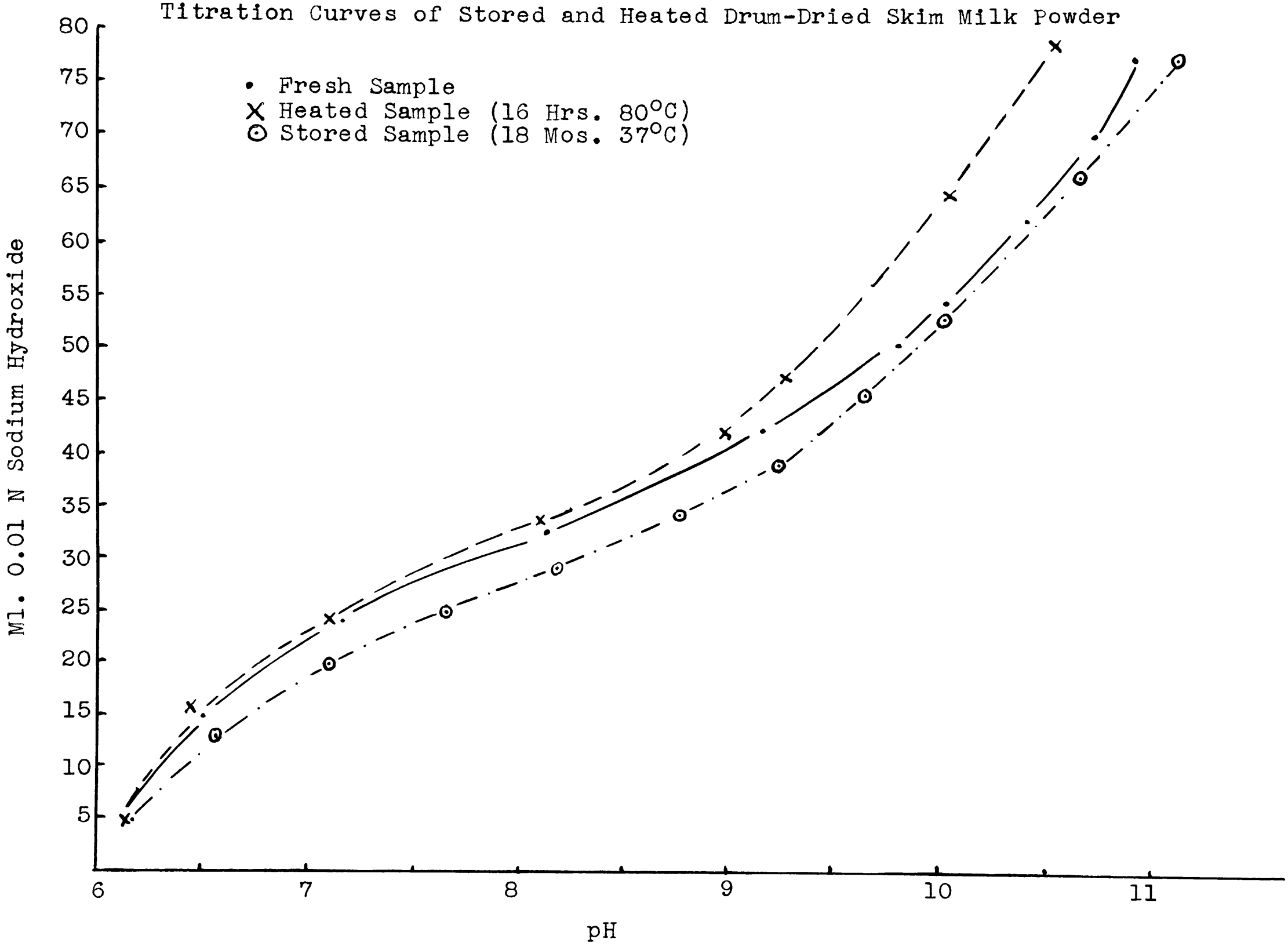


FIGURE 22B.

Titration Curves of Stored and Heated Spray-Dried Skim Milk Powder

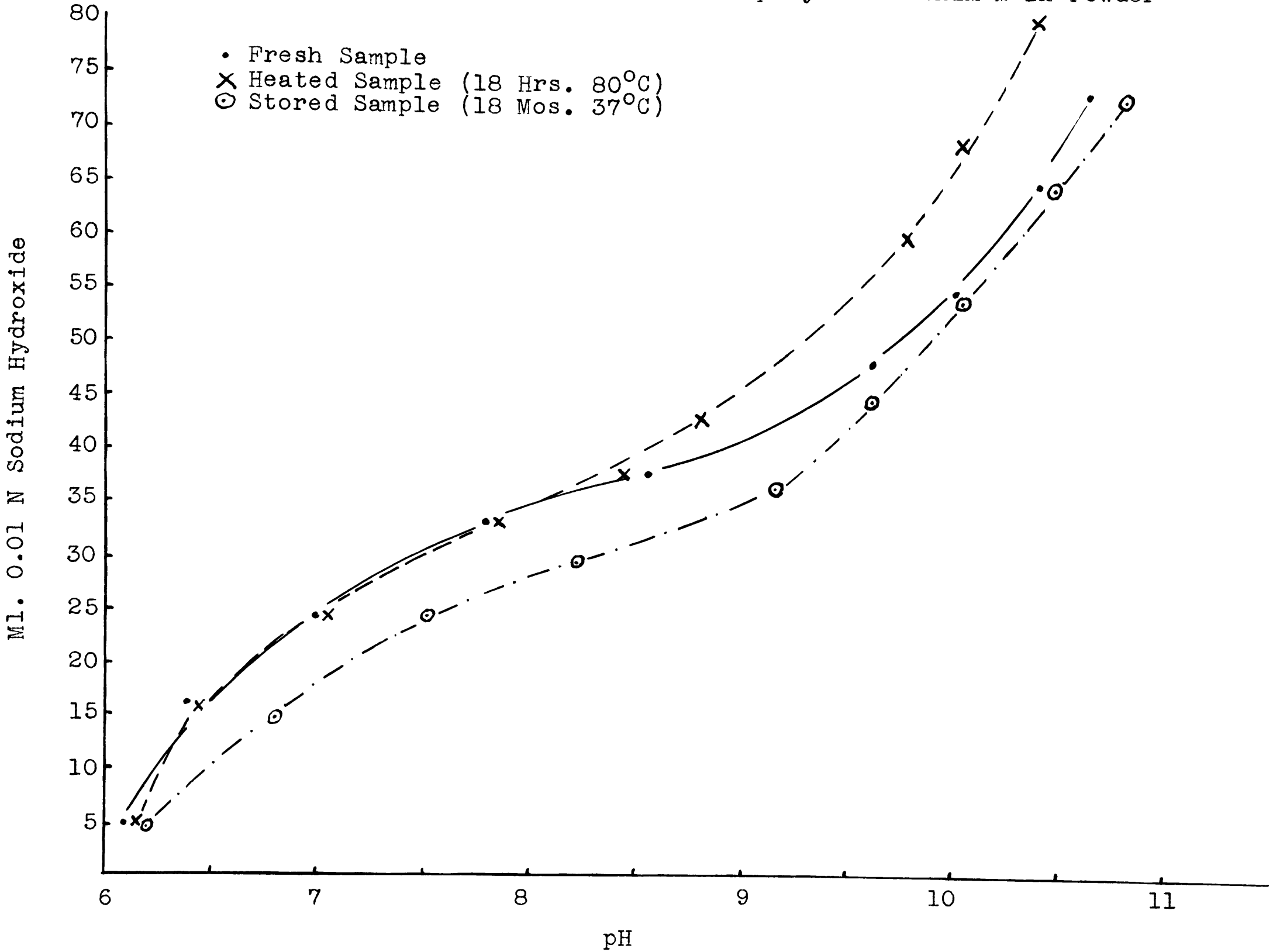


FIGURE 22C.

Comparison of the Titration Curves of Drum-Dried and Spray-Dried Skim Milk

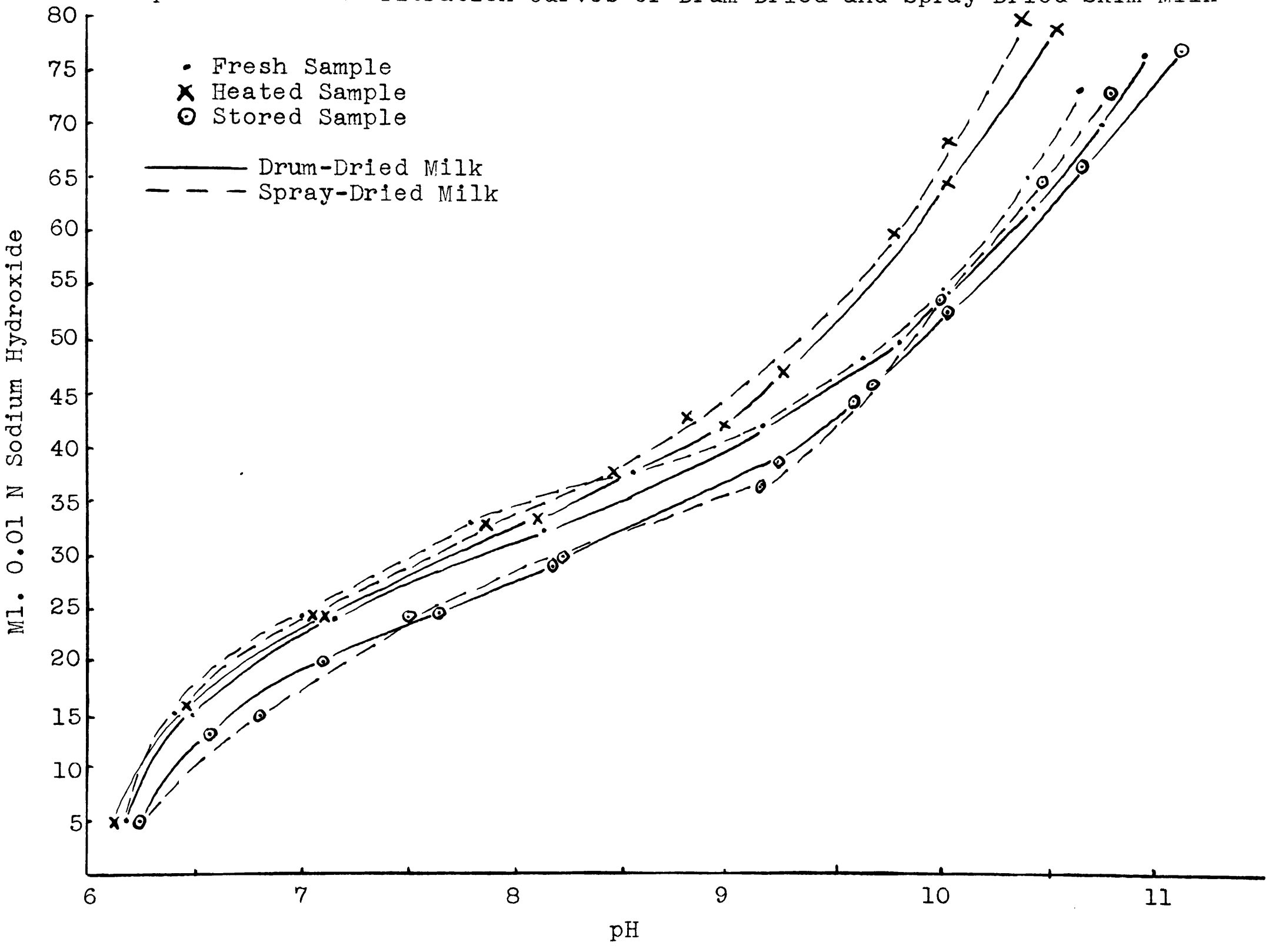


FIGURE 22D.

Titration Curves of Stored and Heated Dehydrated Potatoes

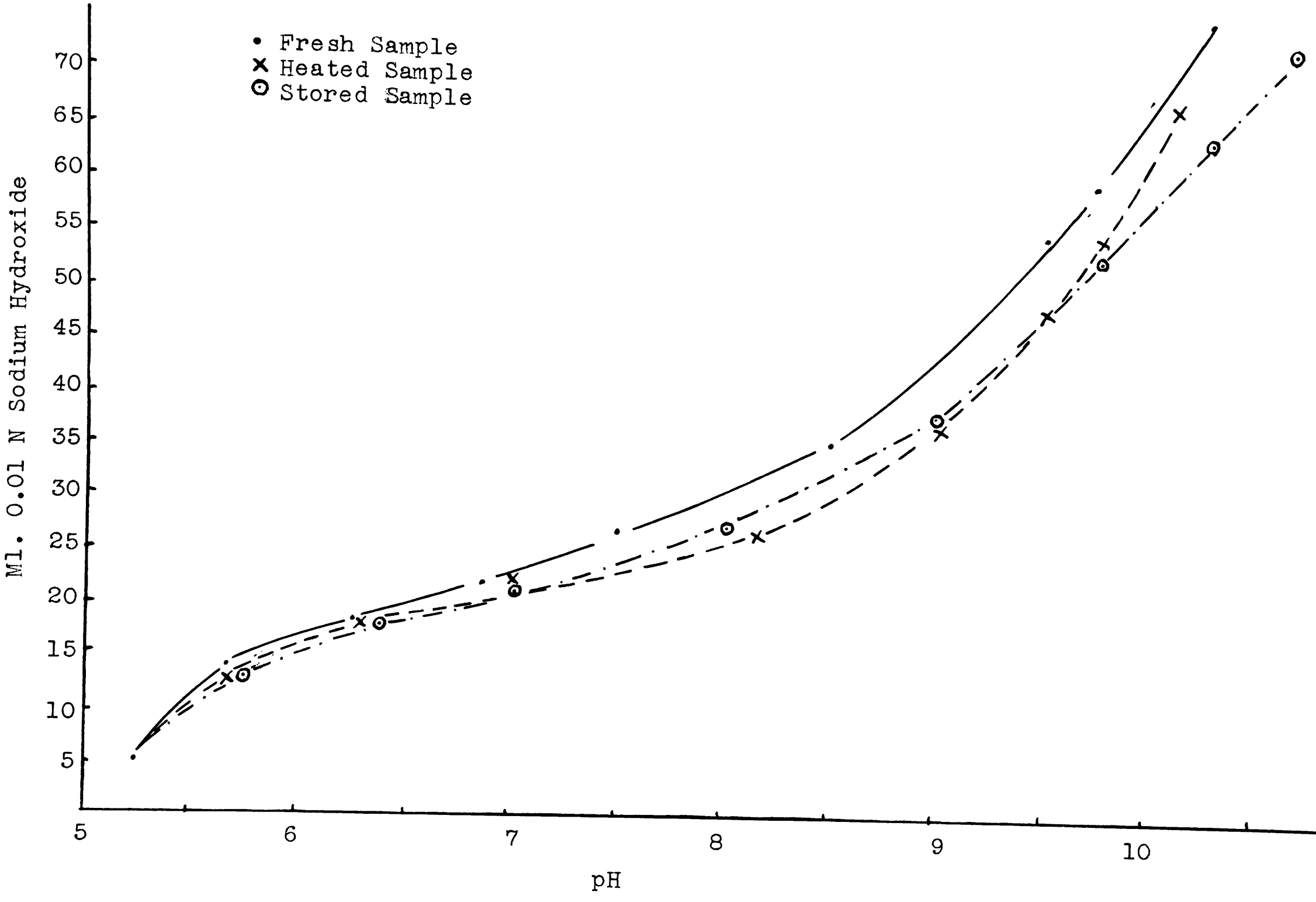
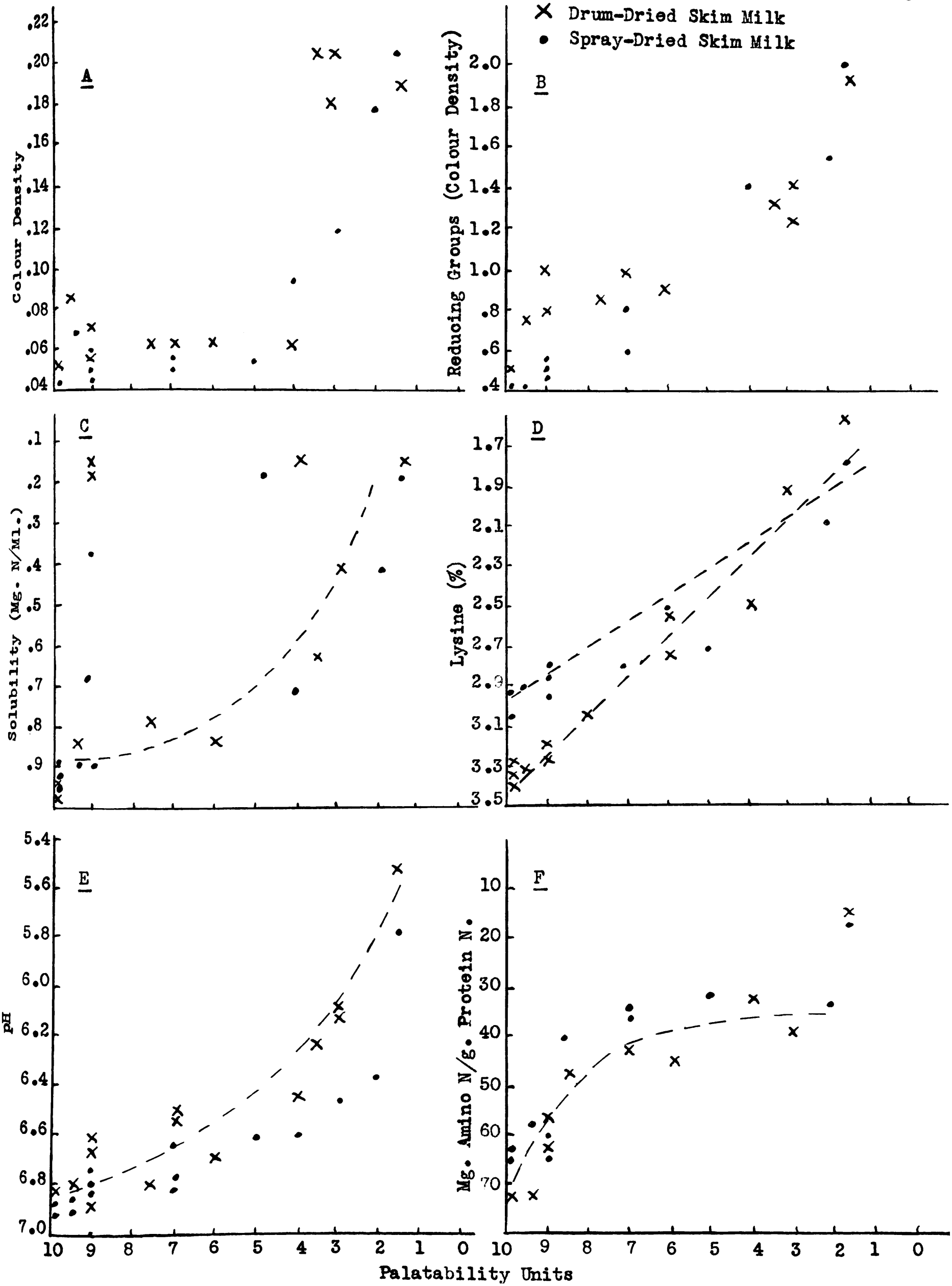
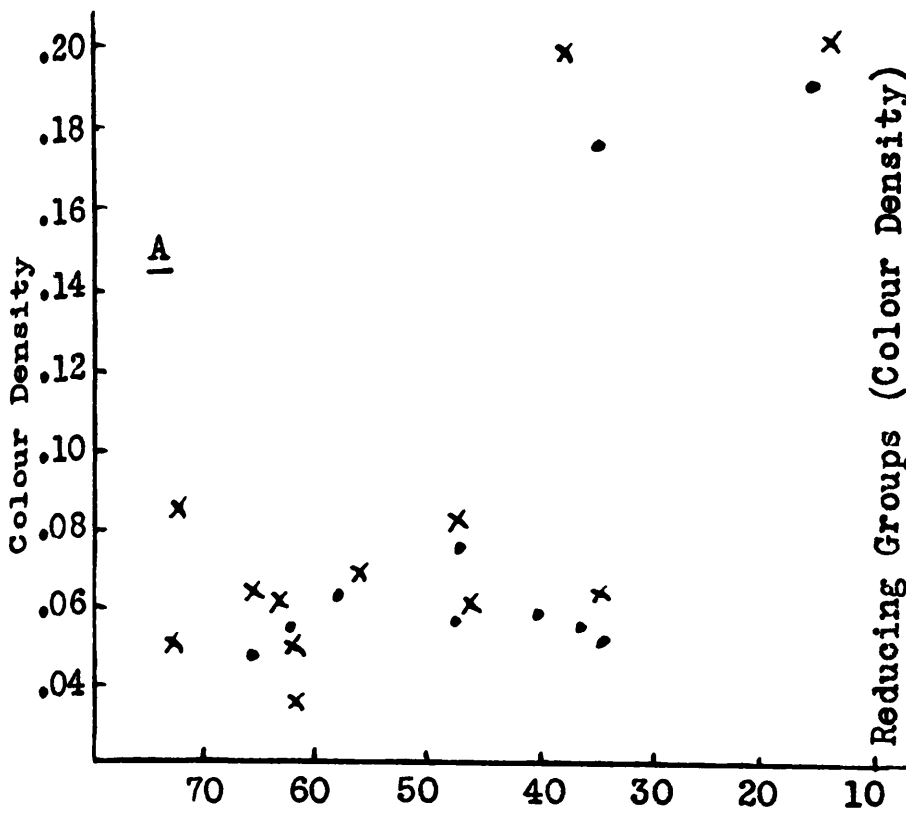


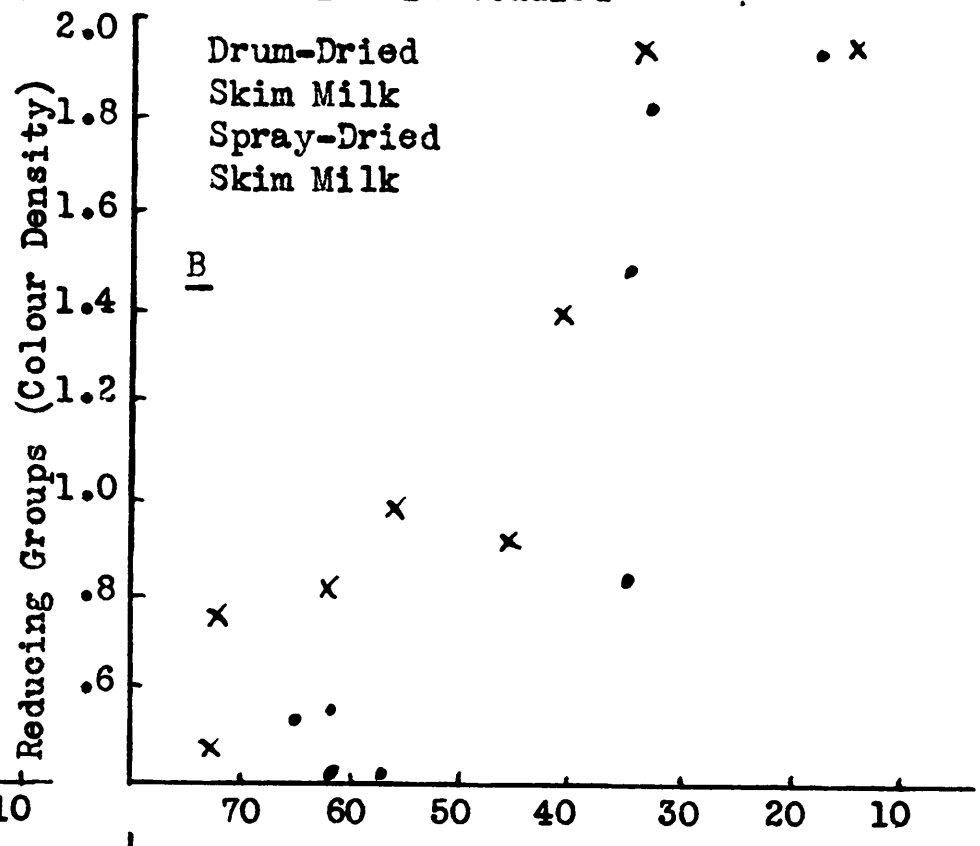
FIGURE 23.

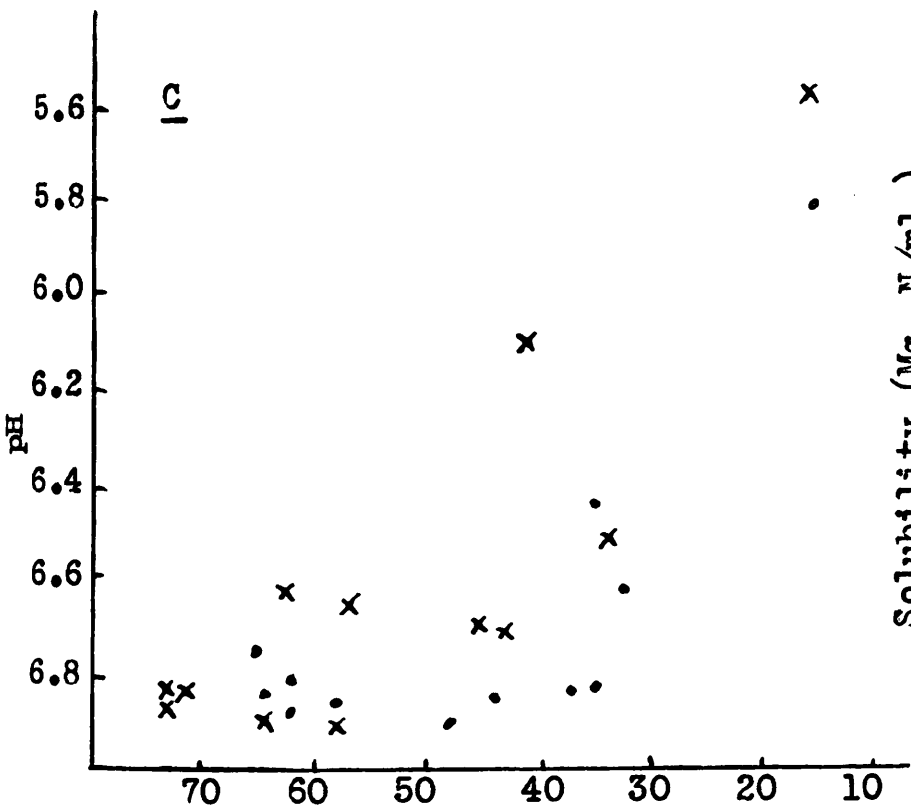
Relationships Between Palatability of Milk Powders and Other Measurements During Storage

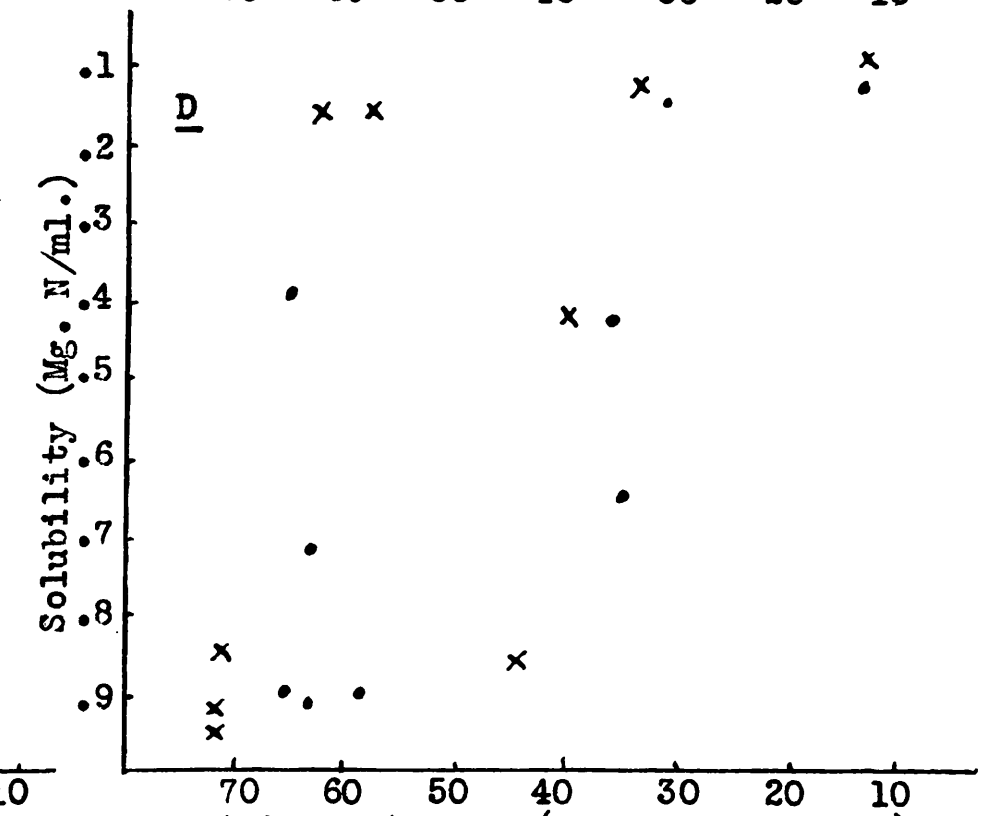


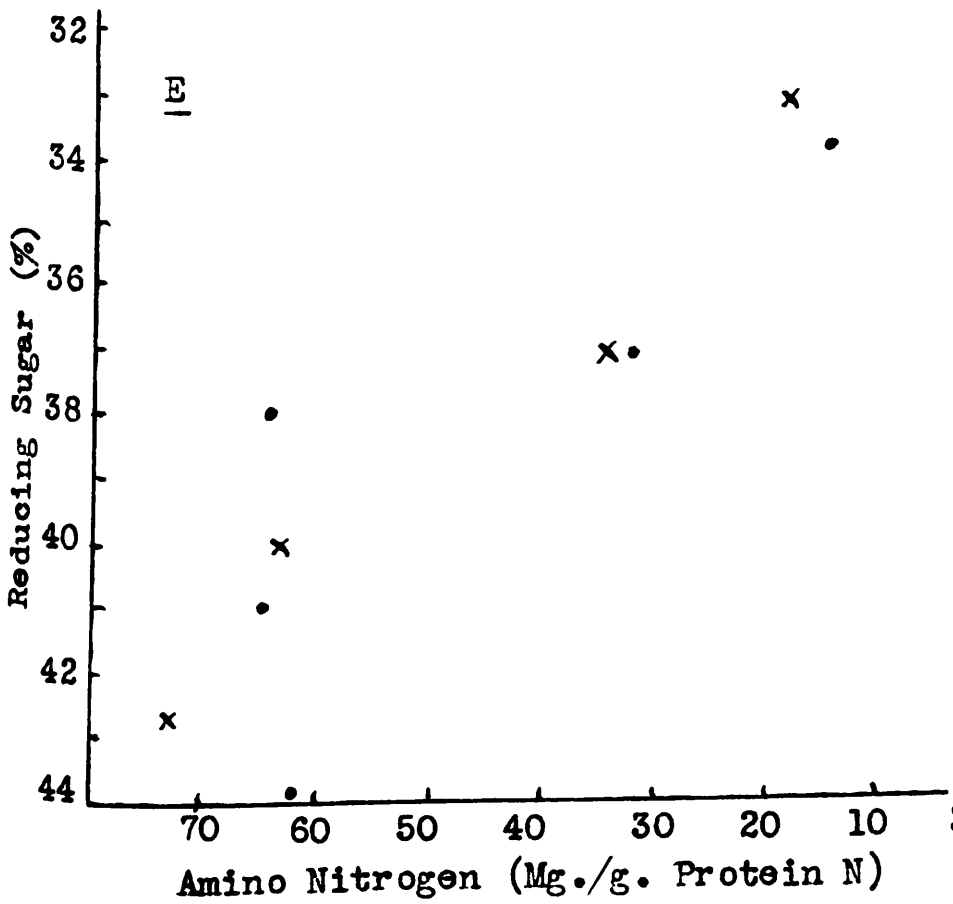
Interrelations Between Various of the Criteria Studied











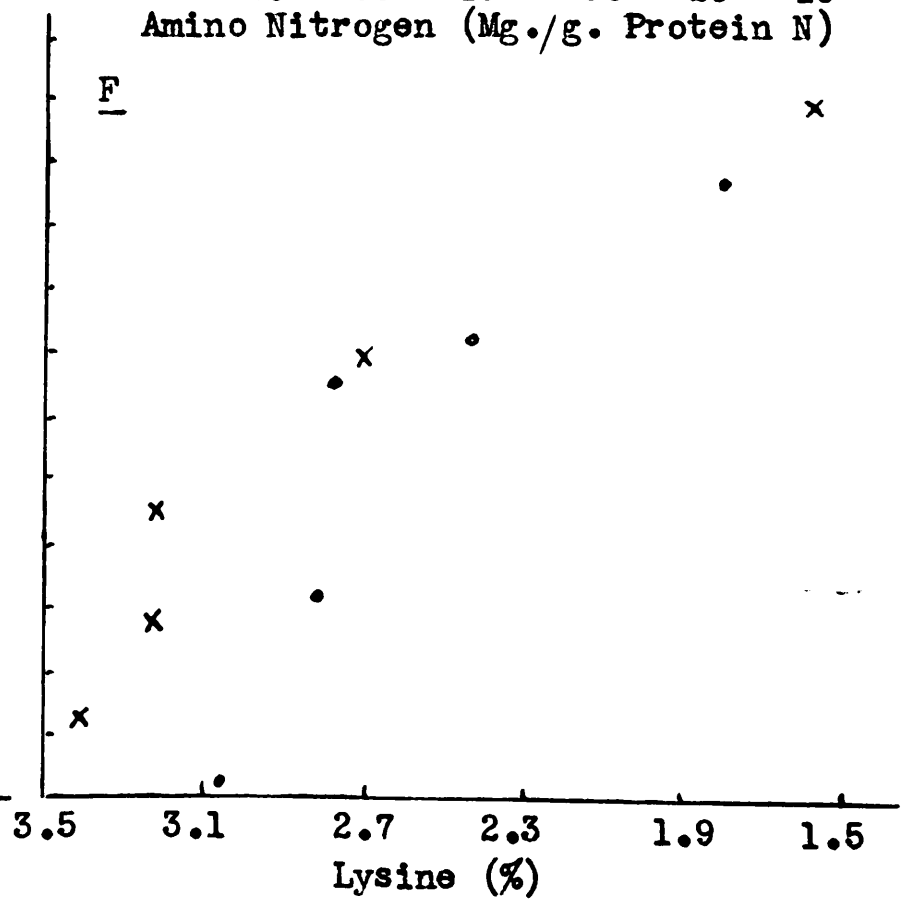


TABLE VIII

Proximate Composition of Food Products

Food Material	Moisture %	Protein %	Fat %	Ash
Drum-Dried Skim Milk Powder	6.79	36.52	0.88	6.72
Spray-Dried Skim Milk Powder	3.50	36.43	0.96	7.89
Defatted Soybean Meal	7.40	45.60	2.00	5.79
Meat Meal	5.77	50.69	5.96	17.24
Ground Oats	11.48	15.57	5.25	1.03
Dehydrated Potatoes	7.20	9.41	3.12	2.81

TABLE IX

Materials Used for Humidity Control at Different Temperatures

Temperature	Humidity Required	Materials Used
27°C.	80%	Saturated NH_4Cl
"	50%	Saturated $Ca(NO_3)\cdot 4H_2O$
"	0%	P_2O_5
37°C.	80%	Saturated KCl
"	50%	Saturated $CoCl_2\cdot 6H_2O$
"	0%	P_2O_5

TABLE X

Taste Score on Drum and Spray-Dried Skim Milk Powders

Scale: 10 - Excellent, fresh powder
1 - Bad, inedible powder

Drum-Dried Skim Milk Powder

Storage Temp.	Humidity	Flavour Score		
		6 Mos.	12 Mos.	18 Mos.
27°C	High	8	6	4
"	Medium	9	7.5	7
"	Low	10	10	9
37°C	High	6	3	1.5
"	Medium	7	3.5	3
"	Low	10	9.5	9

Spray-Dried Skim Milk Powder

Storage Temp.	Humidity	Flavour Score		
		6 Mos.	12 Mos.	18 Mos.
27°C	High	9	7	5
"	Medium	9.5	9	7
"	Low	10	10	9
37°C	High	6	2	1.5
"	Medium	7	4	3
"	Low	10	9.5	9

TABLE XI

Effect of Temperature and Humidity on the Rates of the Reaction(s) Causing Flavour Deterioration in Dried Milk Powders after 18 Month's Storage

Storage Temperature °C	Storage Humidity	Relative Rates of Reaction Causing Flavour Deterioration	
		Drum-Dried Skim Milk	Spray-Dried Skim Milk
37	High	8.5	8.5
37	Medium	7.0	7.0
37	Low	1.0	1.0
27	High	6.0	5.0
27	Medium	3.0	2.0
27	Low	1.0	1.0

TABLE XII

pH Change of Foodstuffs During Storage

	Original pH	12 Months						18 Months					
		27°C			37°C			27°C			37°C		
		H	M	L	H	M	L	H	M	L	H	M	L
Drum-Dried Skim Milk	6.82	6.68	6.81	6.83	6.11	6.25	6.83	6.47	6.52	6.65	5.53	6.09	6.65
Spray-Dried Skim Milk	6.87	6.81	6.88	6.84	6.44	6.60	6.91	6.60	6.71	6.75	5.81	6.48	6.80
Defatted Soybean Meal	7.12	6.84	6.84	6.84	6.80	6.92	7.00	6.67	6.74	6.79	6.44	6.66	6.78
Meat Meal	6.68	6.69	6.79	6.63	6.68	6.72	6.67	6.71	6.66	6.50	6.61	6.63	6.54
Ground Oats	6.34	6.07	6.11	6.17	5.44	5.88	5.93	5.94	5.98	6.03	5.31	5.64	5.73
Dehydrated Potatoes	6.21	5.95	5.98	6.02	5.80	5.93	6.03	5.79	5.84	5.89	5.48	5.72	5.90

H - High Humidity
M - Medium Humidity
L - Low Humidity

TABLE XIII

pH Decrease of Foods During Storage

Storage Conditions	Drum-Dried Skim Milk	Spray-Dried Skim Milk	Defatted Soybean Meal	Ground Oats	Dehydrated Potatoes
			12 Months		
37° H*	0.71	0.43	0.32	0.90	0.41
37° L**	0.00	0.00	0.12	0.41	0.18
27° H	0.14	0.06	0.28	0.27	0.26
27° L	0.00	0.03	0.28	0.17	0.19
			18 Months		
37° H	1.29	1.06	0.68	1.03	0.73
37° L	0.17	0.07	0.24	0.61	0.31
27° H	0.35	0.27	0.45	0.40	0.73
27° L	0.17	0.12	0.33	0.31	0.32

H* - High Humidity

L**- Low Humidity

TABLE XIV

Change of Reducing Substances of Foodstuffs During Storage

	Original	2 Months	4 Months	12 Months						18 Months					
				27°			37°			27°			37°		
				H	M	L	H	M	L	H	M	L	H	M	L
Drum-Dried Skim Milk	8.2*	9.4	17.4	12.8	22	16.2	13.2	8.3	22	20.6	12.8	28	16.8	14.6	28	19	17.4
Spray-Dried Skim Milk	2.2	2.4	3.6	4.4	5.2	14.6	7	4.8	23	22	5.4	27	11	8.2	29	12	8.8
Soybean Meal	8.4	6	5.9	3.8	2.6	4.6		5.4									
Meat Meal	13.6	16.2	10	11.4	5.6	7.6		7.4									
Ground Oats	1.2	1.5	1.1	1.0	0.8	1.2		1.9									
Dehydrated Potatoes	10.4	10	6	6.4	2.8	4.2		3.5									

* Moles x 10^{-5} Cysteine per gm. solids.
H - High Humidity
M - Medium Humidity
L - Low Humidity

TABLE XV

Reducing Sugar Change of Milk Powders in Storage

	Original	18 Months				% Lost			
		27°		37°		27°		37°	
		H	L	H	L	H	L	H	L
Drum-Dried Skim Milk	42.7*	37.1	39.6	32.8	41.3	13.1	7.3	23.2	3.3
Spray-Dried Skim Milk	44.6	37.2	40.8	34.0	37.6	16.5	8.5	23.8	15.7

* - Per cent of moisture-free powder.

H - High Humidity

L - Low Humidity

TABLE XVI

Changes of the Amino Nitrogen of Foodstuffs During Storage

	Original	2 Months		4 Months		6 Months		12 Months				18 Months			
		27°	37°	27°	37°	27°	37°	27°		37°		27°		37°	
		H	H	H	H	H	H	H	L	H	L	H	L	H	L
Drum-Dried Skim Milk	91.8 73*	64	47	58	43	52	43	46	73	41	72	88.1 34	91.8 62	68.5 16	89.3 57
Spray-Dried Skim Milk	88.1 64	48	40	44	37	41	37	35	62	36	58	85.6 33	88.1 65	66.1 15	88.1 62
Soybean Meal	50					51	53	61	61	75	70	70	72	70	71
Meat Meal	56					69	75	97		108	76	99	66	111	69
Ground Oats	82							94	80	98	85				94
Dehydrated Potatoes	172							166	171	136	170	163	173	140	173

* - Mg. Amino N/gm. protein N
H - High Humidity
L - Low Humidity

by Van Slyke
By Formol Titration

TABLE XVII

Loss of Lysine of Stored Milk Powders as Determined after Acid and Enzymatic Hydrolysis by Microbiological Assay

	Original	18 Months				% of Lysine Lost			
		27°		37°		27°		37°	
		H	L	H	L	H	L	H	L
Drum-Dried	2.15*	1.30	2.10	0.60	1.96	39.5	2.3	72.1	8.8
Skim Milk	3.43	2.51	3.21	1.54	3.24	26.8	6.4	55.1	5.5
Spray-Dried	2.18	1.35	2.15	0.68	2.15	38.0	1.4	68.8	1.4
Skim Milk	3.07	2.74	2.82	1.76	2.81	10.7	8.1	42.7	8.4

* - Per cent of Milk Powder (Moisture-free)

H - Enzymatic Hydrolysis

L - Acid Hydrolysis

McGILL UNIVERSITY LIBRARY
IxM ★ .1H21.1943
UNACC.