STUDIES ON THE CHEMICAL CHANGES IN THE PROTEIN AND CARBOHYDRATE FRACTIONS OF MILK POWDERS DURING STORAGE

A Thesis

by

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Submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

McGill University

September 1947

ACKNOWLEDGMENTS

The author is deeply indebted to Dr. R. A. Chapman for his continued interest and extensive advice during the course of this investigation.

The author is also grateful for the many suggestions and help of Dr. W.D. McFarlane and Dr. W. A. DeLong.

The numerous milk powders employed in this investigation were generously supplied by the following: Mr. W. C. Cole, Arden Farms Company, Los Angeles, California; Dr. S. T. Coulter, Division of Dairy Husbandry, Department of Agriculture, University of Minnesota; Mr. E. S. MacBride, Michigan Producers Dairy Company, Adrian, Michigan; Dr. A. R. M. McLean, Eastern Dairies Limited, Montreal; Dr. H. Pyenson, Department of Dairy Husbandry, College of Agriculture, University of Illinois; Dr. G. A. Richardson and Dr. N. P. Tarassuk, Division of Dairy Industry, College of Agriculture, University of California, and Dr. P. A. Wright, Bureau of Dairy Industry, United States Department of Agriculture, Washington, D. C.

A grant from Swift Canadian Company Limited, Toronto, is gratefully acknowledged.

TABLE OF CONTENTS

GENERAL INTRODUCTION

The drying of foods was widely employed by ancient people as a means of food preservation. The original techniques were extremely crude but during the succeeding centuries the methods were gradually modified and improved. As is often the case with progress, the growth of the dried food industry in recent times has been motivated by war-time needs. These requirements have accelerated the development of improved dehydrating processes, which have resulted in products of relatively high quality.

Unfortunately, the quality of dried foods deteriorates rapidly on storage. This instability has been especially apparent under the drastic condtions encountered by armies in the field. Consequently, the need for information in regard to the factors leading to instability, and their control, has been recognized, and a large scale program of research on food deterioration is being conducted by the Quartermaster Food and Container Institute for the Armed Forces of the United States. The results of this program and of private investigations, to date, have been very informative and useful, but many aspects of the problem still require further study.

Changes occurring in the vitamin content and fat fraction of foods have been investigated by many workers. However, relatively few studies have been made on the protein or sugar fractions. It is known that chemical reac-

tions occurring in these fractions lead to discoloration and the production of off-flavours and odours. The present investigation is concerned with the chemical changes occurring in milk powders during storage, especially those associated with the protein fraction. Particular attention has been paid to the development of reducing substances and to their relationship with browning.

HISTORICAL INTRODUCTION

There is relatively little information in the literature about the chemical changes occurring in milk powders during storage. Consequently, before the experimental work of the present investigation was undertaken, the literature concerning deterioration in the processing and storage of other food products was examined. The knowledge obtained from this examination was very useful in planning the research that followed. Therefore, a comprehensive discussion of the changes occurring in foods other than milk powders is being included in this review. These changes not only give rise to discoloration and the production of off-flavours and odours, but decreases in nutritive value may occur as well.

Jones and Gersdorff have shown that during storage there is a decrease in the solubility and digestibility of soybean meal (1) and wheat flour (2). These investigators have also found the nutritive value of corn to be considerably lower after a storage period of twelve months (3). Similar results have been reported for corn by Mitchell (4), and for skim milk powder by Henry and co-workers (5, 6, 7) and Sourkes (8).

A number of workers have also reported a detrimental effect of heat on the biological value of proteins. Stewart and associates (9) have demonstrated a decrease in the growth-promoting properties of oat protein subjected to heat during processing. Morgan and King (10), Morgan (11) and

Mitchell and Block (12) have observed similar results with cereal proteins. Casein has been shown to be nutritively damaged by heat by McCollum and Davis (13) and Morgan (11), and Fixsen and Jackson (14) and Fairbanks and Mitchell (15) have demonstrated a similar effect with skim milk powder. Other proteins showing a decrease in nutritive value because of heating are those of fish meal (16, 17, 18), beef muscle (19) and dried liver (20). On the other hand, Yarusova (21) and Scheunert and co-workers (22, 23), and more recently, Wilder and Kraybill (24) have reported that heat has no detrimental effect on the biological value of various proteins.

Henry and co-workers (7) have shown that the addition of lysine to milk powder resulted in the restoration of most of the nutritive value lost during storage. Greaves and Morgan (25) have also found that the biological value of heated casein could be restored by the addition of lysine. Destruction of lysine by heat has been observed by Hodson and Krueger (26) during the processing of milk, and by Greaves and associates (27) in casein. Similar results have been reported by Waisman and Elvehjem (28) for autoclaved edistin, and by Voegtlin and Thompson (29) for autoclaved whole milk powder.

Block and co-workers (30) have been unable to detect any difference in the lysine content of acid hydrolysates of heated and unheated casein, but Eldred and Rodney (31)

have found a significant loss when enzymatic hydrolysis was employed. Seegers and Mattill (20) have reported that the nutritive value of heated protein can be partially restored by acid hydrolysis. It has been suggested by Morgan (11) and Seegers and Mattill (20) that heating may render certain amino acids less available for digestive absorption, owing to a relative decrease in the ease of liberation of these amino acids by proteolytic enzymes.

Recently, Riesen and co-workers (32) have studied the liberation of essential amino acids from soybean oil meal before and after autoclaving for four hours. Following autoclaving, a marked decrease in the liberation of lysine, arginine and tryptophane by acid or alkaline hydrolysis was evident, while enzymatic hydrolysis resulted in a decreased liberation of all the essential amino acids. Hodson and Krueger (33), using alkaline and acid hydrolysis,have reported a loss of lysine, arginine, tryptophane, histidine and methionine in stored skim milk powders. Block and associates (34) have shown that baking and toasting of proteins cause a decrease in biological value, but have no effect on the value of their non-enzymatic hydrolysates. These workers have suggested that the decrease in the nutritive value is due to the formation of a bond between a free ϵ-amino group of lysine and a free carboxyl group of a dicarboxylic acid. This bond can be readily hydrolyzed by acid or alkaline hydrolysis but not

by digestive enzymes, and thus the lysine involved is unavailable for nutritional purposes. There is no evidence to support this hypothesis, and a theory suggested by Henry and co-workers (7), that the ϵ -amino group of lysine condenses with the aldehyde group of a reducing sugar seems more probable.

The condensation of amino acids and reducing sugars is often referred to as the Maillard reaction, because the first extensive reports on the reaction were made by Maillard (35, 36). The products of this reaction are dark-coloured melanoidins and, consequently, it is also known as the melanoidin or "browning" reaction. The work of the early investigators has been adequately reviewed by Ambler (37). These studies revealed that heating solutions of amino acids and sugars resulted in the destruction of a portion of the reactants and the production of carbon dioxide, aldehydes and melanoidins.

Subsequently, the kinetics and equilibria of the reaction have been extensively studied by physical-chemical techniques. The principal investigators have been Borsook and Wasteneys (38), Neuberg and Kobel (39, 40), Von Euler and associates (41, 42, 43) and Frankel and Katchalsky (44, 45, 46). They have agreed in general, that a combination of amino acids and sugars occurs in solutions. However, a considerable number of reports are mutually contradictory. These discrepancies can be resolved, though only

in part, by a careful consideration of the specific conditions of the reaction. It is certain that both the nature and extent of reaction vary widely with the principal variables, i.e., concentration, temperature, hydrogen ion concentration, specific reactants and time. Unfortunately, many papers give insufficient details of the conditions for an adequate evaluation of results.

Many reports have been made on the isolation of products from reactions between amines and sugars. The general type of reactions involved has been reviewed by Sprung (47). Although numerous qualitative and quantitative studies have been conducted to show that amino acids and sugars interact similarly to other amines and aldehydes, in only a few instances (43, 48, 49, 50) have pure products been isolated and characterized. These products have been obtained by artificial means, not duplicated in natural systems and hence their use in establishing the course of the Maillard reaction in biological systems is limited.

The most serious forms of food deterioration occurring during storage are discoloration and the production of off-flavours and odours. Although dark-coloured substances may arise from oxidation of natural pigments, the tannins, the degradation of chlorophylls and various enzymatic reactions, only the non-enzymatic browning associated with the Maillard reaction and the caramelization of sugars will be considered in this review.

Much of our knowledge of the chemistry of food discoloration has been obtained from studies on the browning of sugar and malt products during processing. Two general hypotheses have been developed: (1) that browning is essentially a caramelization of sugars; (2) that browning is essentially a Maillard-type reaction between reducing sugars and amino acids or proteins. The literature concerning the identity, separation or possible relationship between these two reactions is replete with contradictions.

Merhle (51) and Orth (52) have pointed out that the principal physical factors causing darkening of sugar juices during processing are high temperature and prolonged heating. Janák (53) has reported that at a given hydrogen ion concentration, the colour formed is proportional to the product of the temperature and time of heating. Within practical limits, the higher the alkalinity, the greater is the increase in colour (54, 55).

Christiani (56), Pshenichnuii and Shumkov (54), Smoleński (57) and Macků and Langer (58) have observed a relationship between reducing sugars and formation of colouring matter in beet and cane products. Other studies on the heating of sucrose solutions have shown that humic and caramel products are formed. These products are a mixture of derivatives of dehydrated sucrose, varying in their degree of dehydration and polymerization (59, 60, 61).

A number of investigators have attempted to identify

the intermediate products of sugar decomposition and polymerization. Seaver and Kertesz (62) have shown that glucuronic acid solutions brown more rapidly than ordinary sugar solutions. Montgomery and Wiggins (63) have autoclaved sucrose solutions and have identified a number of the products formed. These include 5-hydroxymethyl 2-furfural, levulinic acid, formic acid and acetol. The latter may arise from a preliminary formation of methyl glyoxal. Enders and associates have studied the breakdown of sugars in the presence of amino acids, and methyl glyoxal has been suggested as an intermediate in the Maillard reaction (64). The caramelization of aldehydes under milder conditions has been attributed to the catalytic action of amino acids (65). In the later stages of the reaction, amines may react with the breakdown products to form humin (melanoidins). The substances formed from sugars, by amino-group catalysis, have the same characteristics as those obtained from heat or alkaline treatment in the absence of amino compounds. Cavalieri and Wolfrom (66) have presented evidence which indicates that the browning of sugar in alkaline solution is due to the catalytic action of the hydroxyl ion.

The possibility of the Maillard reaction being involved in the darkening of sugar or sugar products was suggested by the early work of Stoltzenberg (67) and Stanek (68), who isolated from beet molasses, dark-coloured substances con-

taining 6.6 and 7.2 per cent nitrogen, respectively. Stanek (69) was able to synthesize compounds, with similar properties to those he had isolated, from invert sugar and certain amino acids. Invert sugar alone, treated in the same manner as sugar-amino acid solutions, gave products that were lighter and had different solubility properties than the darker nitrogenous compounds. Oudemans (70) has confirmed the work of Stanek. Friedrich (71) has claimed that the nitrogenous colouring matter from beet molasses is formed by a condensation involving caramel rather than sugar, but Stanek (72) has disagreed, pointing out that amino acid-reducing sugar compounds are formed at much lower temperatures than those at which caramel is prepared. Ripp (73) has produced caramel substances by heating levulose solutions in the absence and presence of amino acids. In the latter case, the depth of colour formation was greater but not in proportion to the interacting nitrogen. Browne (74) has found, in the colouring matter of cane molasses, a substance which agrees closely with the composition of a melanoidin, prepared by Sattler and Zerban (75) from fructose and aspartic acid.

A close correlation between the nitrogen content of sugar-cane juices and colour developed during processing has been reported by Ayyar and Bhushanam (76). Lothrop and Gertler (77) have found a similar correlation between the amount of amino nitrogen in honey and the tendency for

the honey to caramelize or darken on heating. Schuette and Baldwin (78) have noted that dark-coloured honeys generally contain more amino nitrogen than lighter ones.

Fowler and Kopfler (79) studying the darkening of raw sugar in storage, have reported that the final colour is darker when the nitrogen content of the sugar is higher. Kopfler (80) has attributed the increase in colour of cane molasses during storage to a glucose-amino acid interaction. Ambler and Byall (81) have shown that even refined sugar may contain up to ten parts per million of amino acids and 186 parts per million of total nitrogen. Thus, the yellowing of sugar, stored for long periods of time may be caused, in part, by the formation of amino acid condensation products, as well as caramelization. Recently Fosnot and Haman (82) have attributed the darkening of malt syrups during storage to the Maillard reaction.

In general, the occurrence of the melanoidin reaction in foods is to be avoided, but in malt and malt products certain features of the reaction may give rise to desirable qualities. Pleasant "malty" aromas and flavours are produced by a controlled melanoidin-type reaction. However, if allowed to proceed too far, charring or bitterness may result. The technique of controlling sugar-amino acid interactions in kilning is employed to produce different types of malts. If the reaction is minimized, a lighter malt is formed, but if temperature and moisture

conditions are favourable then darker malts are produced.

Even before Maillard's fundamental publications (35, 36), Ling (83) had pointed out the importance of the sugar-amino acid condensation in the kilning of malt. He showed that dark-coloured substances, similar to those produced in malting, could be formed by heating a mixture of glucose and asparagine. Later, Lintner (84) and Ruckdeschel (85) confirmed and extended Ling's work. Ruckdeschel (85) has reported that on heating a concentrated solution of dextrose and an amino acid, the resulting decrease in free amino acid is proportional to the increase in colour intensity. The colour and aroma development in these synthetic solutions occurred in a manner analogous to their development during kiln-drying. Many subsequent workers have confirmed the importance of the melanoidin reaction in kilning.

Schneider and Harries(86) have observed a marked decrease in formol nitrogen without a corresponding increase in the colour of the finished malt. This has led them to discredit the role that the melanoidin reaction may play in colour and aroma development. On the other hand, Bloch (87), in a recent review, has reported that no decrease of formol nitrogen occurs during kilning, although dark-coloured substances may be formed. However, he has supported the melanoidin theory, suggesting that a quantity of amino acids, approximately equal to the amount

reacting, must be liberated by enzymatic activity.

In addition to its effects on the malt, the melanoidin reaction plays a similar role in the subsequent stages of the brewing process: mashing, boiling and fermentation. Laufer (88) has recently given an excellent review on the factors influencing the colour of beer and ale. He has pointed out that in addition to the melanoidin reaction, caramelization of sugars during the boiling process may contribute to the deepening of colour. Gray and co-workers (89) have also attributed the colour of beer to both of these reactions.

Most fruit products readily undergo changes in colour during preparation, processing or storage. Joslyn (90) has recently reviewed the topic. These changes are caused, in part, by non-enzymatic reactions, but the chemical evidence to support the melanoidin theory is actually very meagre and not altogether consistent.

Joslyn and co-workers (91, 92), Loeffler (93) and Beattie and associates (94, 95) have linked the darkening of citrus juices with losses in ascorbic acid. Joslyn and Marsh (92) have suggested that the first step in the browning of orange juice involves oxidation and loss of ascorbic acid, followed by a Maillard type of reaction with the primary products formed. These workers have isolated from orange juice a pigment strikingly high in nitrogen content.

Hall and Nedvidek (96) and Wilson (97) have associated the Maillard reaction with discoloration in fruit juices. Although the former have reported a decrease in total amino nitrogen during the darkening of orange juice concentrates, no such decrease could be detected by Joslyn and Marsh (92) and Nelson and co-workers (98). Loeffler (93) could find no change in the amino nitrogen content of bottled orange juice during storage. However, Joslyn and Marsh (92) did find a marked increase in browning when amino acids and other amines were added to orange juice, and Richert (99) has reported similar results with studies on sugar syrup and grape juice concentrate. Beattie and associates (94) could detect no change in the total sugar or reducing sugar content of fruit juices during storage.

A few investigators have reported a decrease in the amino nitrogen content of dehydrated fruits during storage. Katz (100) has shown that the water and 50 per cent acetone-extractable amino nitrogen of dried apricots decreased 20 and 43 per cent, respectively. Although Bedford (101, 102) has stated that amino acids and reducing sugars are probably not involved in the darkening of dried apricots, in a later study (103), a progressive decrease of amino nitrogen (14 per cent in eight weeks) and amide nitrogen (17 per cent in eight weeks) was noted. Weast and Mackinney (104) have observed a marked decrease

in the glutamic and aspartic acids content of dried apricots on darkening during storage. They have isolated a dark compound containing 3.26 per cent nitrogen. This compound is indistinguishable from a synthetic substance prepared from an aspartic acid-sugar mixture. Consequently, these authors have attributed a major role to the Maillard reaction in the darkening of dried apricots.

There is relatively little definite information available on the mechanism of the non-enzymatic "browning" reaction in vegetable products. Ruschmann (105) has reported brown discoloration and inferior silage from potatoes of high sugar content. The colour was intensified by the presence of ammonium salts or amino compounds. This discoloration was attributed to the formation of humus-like substances by a reaction between partly-caramelized sugars and nitrogen compounds. A study on discoloration of dehydrated potatoes during storage has been made by Burton (106, 107). Brown substances were isolated which gave a positive test for caramel. Although storage resulted in a reduced sugar content, there was no correlation between browning and decrease in sugar. However, there was a correlation between browning and loss in amino nitorgen. The author has concluded that in the early stages of browning, caramelization of glucose is more important than the Maillard reaction, but that the latter is more prominent in the later develop-

ment of more intense browning. Lewis and Doty (108) have recently isolated a nitrogen-containing pigment, which they have claimed is responsible for the discoloration of cooked potatoes.

There is considerable disagreement in the literature in regard to the mechanism of the browning of heated, autoclaved or stored milk products. Kass and Palmer (109) have adequately reviewed the subject. They have discussed the work of Kometiani, who found no significant changes in amino nitrogen during the heating of milk, and has attributed browning to a caramelization of lactose. Previously, Leeds (110) and Wright (111) had suggested a similar theory. On the other hand, Ramsey and co-workers (112) have stated that, "Caramelization plays no role in discoloration of dairy products". These investigators heated solutions of various amino acids and sugars. The colours developed, particularly in the mixtures containing glycine and lysine, were similar to those obtained by heating the sugars with milk protein.

Wright (111) and Kass and Palmer (109) have disagreed completely with the theory of Ramsey and co-workers. The results of their experiments have led them to conclude that the browning of autoclaved milk is mainly caused by caramelization, with casein acting as a caramelizing agent. The dark colour of casein precipitated from autoclaved milk is not due to a chemical reaction but to a physical adsorp-

tion of the lacto-caramel. Kass and Palmer (109) have shown that the browning action of amino acids is similar to the catalytic effect of various salts and buffers on sugar solutions. Webb (113) has suggested that caramelization and sugar-amino acid interaction may both be of importance in colour development in autoclaved milk.

Although much concern has been shown over the deterioration of stored milk powder, actually very little research has been carried out on the chemical reactions involved. Henry and co-workers (7) have reported a 64 per cent loss of amino nitrogen in skim milk powders of 7.3 per cent moisture content, after storage for sixty days at 37°C. This loss was accompanied by an eightfold increase in the amount of reducing sugar combined with protein. Lea (114) has recently attributed a major role in milk powder deterioration to the reaction of lactose with free amino groups of the protein, and to the degradation products of this complex.

Of the numerous foods being dehydrated in the last decade or so, egg powders have been the most extensively investigated for causes of deterioration during storage. Stewart and co-workers (115) have demonstrated that certain changes, developed during storage, in the colour and solubility of dried eggs are related to the presence of free sugar. Stewart and Kline (116) and Hawthorne and Brooks (117) were able to retard deterioration by removal of sugar.

A loss of amino nitrogen and reducing sugar during storage has been reported by Bate-Smith and Hawthorne (118). These workers have attributed some of the changes occurring in stored dried eggs to a Maillard-type reaction. The addition of amino acids to liquid eggs prior to drying helped to retain the solubility of the powdered product during storage, but resulted in increased discoloration. Kline and Fox (119) have reported a similar effect. Probably the sugar present reacts with the added amino acid, forming a dark-coloured compound. In the absence of the added amino acid, the egg protein is involved in the reaction, and decreased solubility of the protein results. Olcott and Dutton (120) have supplied strong evidence in favour of the Maillard reaction. They have shown that at least four egg proteins react with glucose in synthetic mixtures, under conditions simulating those found in dried eggs undergoing deterioration. In these experiments, 35 to 45 per cent of the amino nitrogen disappeared after storage. The decrease was accompanied by an increase in fluorescence and discoloration.

However, not all detrimental changes in stored egg powders are caused by the protein-sugar interaction (115, 121, 122). Fevold and co-workers (121) have claimed that the development of off-flavours and odours is caused mainly by reactions occurring in the phospholipide fraction of egg yolk. Edwards and Dutton (123, 124, 125) have reported a

reaction, in egg powders, between cephalin and an aldehyde.

Although relatively little research has been conducted on the mechanism of reactions concerned with deterioration of dried foods during storage, extensive investigations have been made on the physical conditions responsible for these reactions. In general, high temperature, high moisture content, prolonged storage and air-packing have induced deterioration, although exceptions have been reported. Other factors, such as the source and quality, processing treatment, hydrogen ion concentration, the addition of inhibitors and packaging may also effect the quality of the product, but will not be considered in this review.

Many workers have observed the detrimental effect of high moisture content and high temperature on dried fruits (126, 127), dried vegetables (128, 129, 130), milk powders (131, 132), egg powders (133, 134, 135) and dried meats (136). In each case only a few representative references are given. Although minor discrepancies have arisen, as to the effect of temperature on the degree or rate of discoloration, in general, rapid discoloration has resulted from high temperature. The subject has recently been reviewed by McConnell and co-workers (137).

In some instances, reports of moisture effects on stored dehydrated foods have been contrary to the general rule discussed in the foregoing paragraph. Thus, Stadtman and co-workers (138) have shown that under anaerobic con-

ditions the storage life of dried apricots was progressively increased when the moisture content was raised from 10 to 25 per cent. Maximum rate of darkening occurred between 5 and 10 per cent. Lowering the moisture content below 5 per cent retarded deterioration. The beneficial effect of high moisture content became progressively smaller as the oxygen in the pack was increased. Previously, Nichols and Reed (139) had reported that drying to 10 per cent moisture resulted in decreased colour stability in pears. By similar treatment, apples were improved, and apricots were unchanged. Bryce and Pearce (140) have observed a 3 per cent moisture content to be preferable to 2 or 5 per cent in milk powders.

Many conflicting reports have been made concerning the effect of oxygen and other gases on the darkening of dehydrated foods. This lack of agreement is undoubtedly due to differences of experimental conditions under which the observations have been made. Beneficial effects for gas or vacuum-packing have been reported in the majority of cases (132, 135, 138, 140, 141, 142). However, there are a number of instances where food deterioration was found to be independent of atmosphere (107, 126, 129).

Pearce and Thistle (143) have detected the development of fluorescence in egg powders during storage, and have used fluorescence as a means of assaying quality. This technique has proved successful with egg powders and

may be useful for dehydrated pork, dried bananas, dried parsnips, ration biscuits, butter (144) and liquid and frozen eggs (145). Fluorescence was found to be unsatisfactory for measuring quality in dried milk or soya flour (144).

Pearce (146) has suggested that the decomposition of egg protein is a factor contributing to fluorescence. Olcott and Dutton (120) have shown that the fluorescent substances are protein-sugar reaction products. In addition, Edwards and Dutton (123) have reported that cephalin and aldehydes react to form a brown pigment that fluoresces. The latter is extracted from egg powders with ether and is different from the substances referred to by Pearce, who extracted with salt solutions. Boggs and co-workers (122) have found that salt-extraction fluorescence correlated well with palatability scores of egg powders containing 4 to 5 per cent moisture, but poor correlation was obtained in samples below 2.7 per cent. With the samples of lower moisture content, the fluorescent properties of the lipide fraction extracted with ether were a better measurement of quality than salt-water fluorescence.

Chapman and McFarlane (147) have observed an increase in reducing groups during the storage of whole milk powders. Henry and co-workers (7) have reported similar results with skim milk powders. These reducing groups are believed to be associated with the protein fraction (147).

EXPERIMENTAL

It appeared that the ferricyanide method, proposed by Chapman and McFarlane (147) for the estimation of the reducing capacity of milk powders,might prove useful in studying chemical changes occurring during storage. The present investigation is concerned with the application of this method and the production, identification and role of the reducing substances involved.

1. THE USE OF THE FERRICYANIDE METHOD IN STUDIES ON MILK POWDERS.

(a) Modification of the Ferricyanide Method.

Chapman and McFarlane (147) have referred to the compounds in milk powders, which reduce potassium ferricyanide at pH 5, as reducing groups. In the present study, for reasons that will become more apparent in later discussions, these compounds are called reducing substances. Throughout the investigation, these reducing substances are determined by the method of Chapman and McFarlane, with the following modifications.

In the original method, the blue-coloured ferric ferrocyanide (Prussian blue) was developed by adding ferric chloride to the acid solution containing the ferrocyanide ion. Since the intensity of the colour changed with time, an empirical time factor was used; all colorimeter readings being made ten minutes after the addition of ferric chloride. In the present study, it was found that the

change of colour on standing was compensated by a similar change in the blank. Therefore, the ferric chloride was added first to the blank and then to the other solutions at half-minute intervals. The colorimeter measurements were made in the same order with the same time interval. In this manner, the time of standing was the same for each solution, and satisfactory intensity measurements could be taken from five to ten minutes after the addition of ferric chloride. The calibration curve prepared by using either the original or the modified method did not follow Beer's Law. Recently, Lea (114) has stated that if the colour intensity was measured one minute after the addition of ferric chloride, a calibration curve could be prepared in which there was no deviation from Beer's Law. This modification, however, has not been employed in the present investigation.

In preparing the calibration curve, Chapman and McFarlane (147) subjected varying concentrations of glutathione to the entire procedure of the method. The results were expressed as moles x 10^5 reducing groups per g. of milk powder. In the present study, the calibration curve was prepared using solutions of potassium ferrocyanide. A solution, containing 25 ml. of 1 per cent potassium ferricyanide, 25 ml. of 10 per cent trichloracetic acid and 25 ml. of potassium acid phthalate-sodium hydroxide

buffer of pH 5, was prepared. To 5 ml. aliquots of this solution, 5 ml. of potassium ferrocyanide solution of known concentration was added, and the colour was developed by the addition of ferric chloride. The amounts of potassium ferricyanide required to form the quantities of potassium ferrocyanide added, were calculated. The results are expressed as mg. of potassium ferricyanide reduced per g. of milk powder. This unit has proven more satisfactory than the cumbersome "moles x 10^5". A calibration curve, prepared as described above, is shown in Fig. 1. Coulter (148) has used the ferricyanide method for investigating milk powders of relatively high reducing capacity. He has found that a blue precipitate appeared on the filter paper during the filtration step, and that this difficulty could be avoided by carrying out the reaction at pH 6.6 rather than pH 5. While this modification may be useful for milk powders of high reducing capacity, it possessed no advantage for powders used in the present investigation. In fact, it was found that greater differences could be detected between fresh and stale milk powders when the reaction was carried out at pH 5.

(b) Application of the Ferricyanide Method.

It seemed possible that if a large number of milk powders, processed and stored under different conditions,

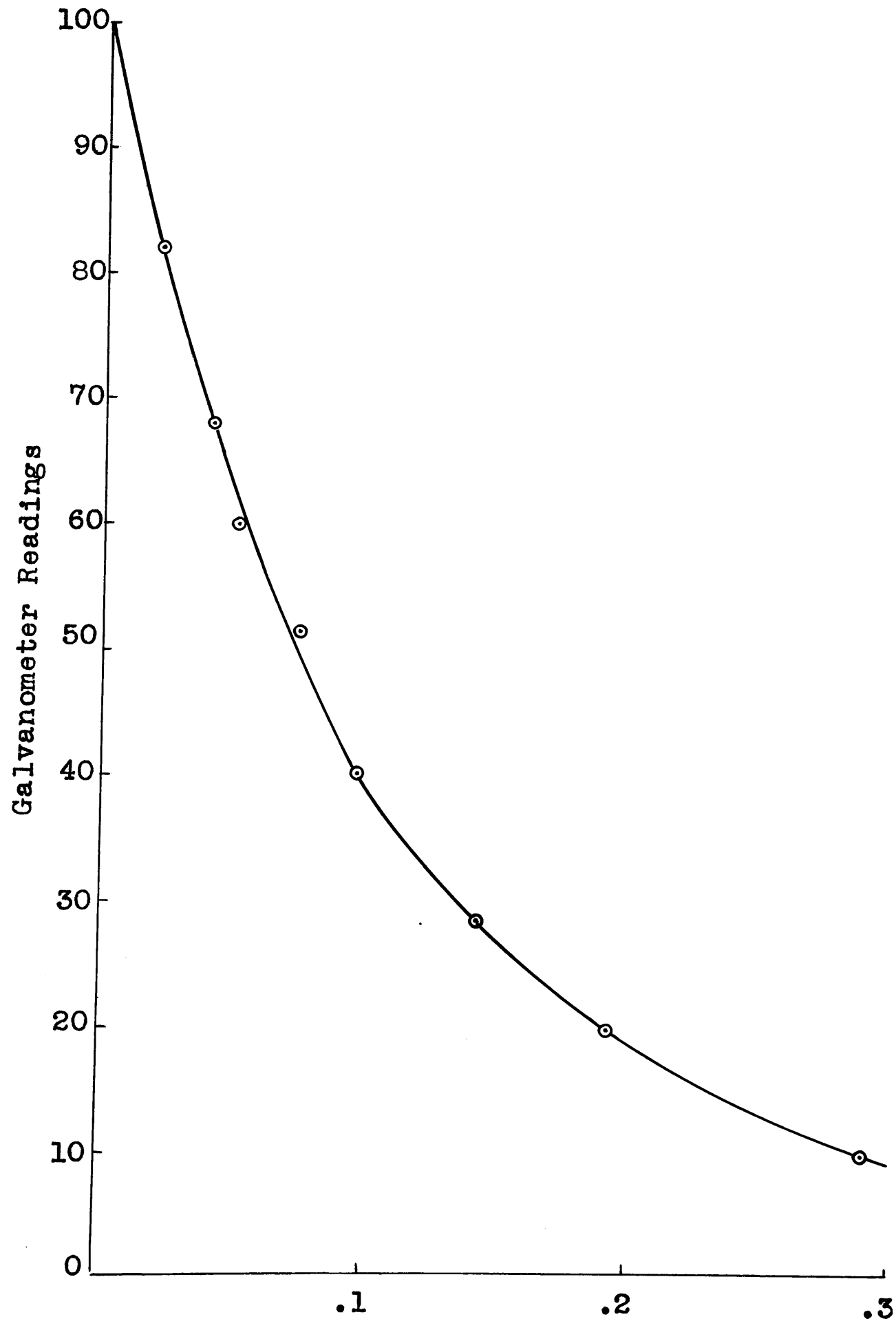

Fig. 1. Calibration Curve using Potassium Ferrocyanide.

were examined, the results might give some useful information concerning the production and nature of the reducing substances present. In addition, this examination might indicate the feasibility of using the ferricyanide method for assaying quality in milk powders.

There are two ways in which such an investigation could be conducted. Milk powders could be prepared and stored by the investigators themselves, or different samples could be obtained from various outside sources. The former is more advantageous, because the conditions of processing and storage can be carefully controlled. Unfortunately, the facilities for preparing milk powders were not available for the present study. Accordingly, a number of samples have been obtained from various sources in the United States. The reducing capacity of these samples has been determined by the ferricyanide method, and the results are presented in Tables I to IV. All samples were packed in sealed tins, except for the drum-dried powders in Set F, Table III, which were packed in cardboard containers with slip-on covers. On opening the tins, no differences in colour could be detected in the spray-dried samples.

In order to facilitate the interpretation of the results, the samples have been grouped in sets. In Tables I and II, the time and temperature of storage and the moisture content of individual samples, within a set, are approximate-

ly the same. Consequently, the only variable is that which is under study in each of the tables. Since the results in Tables I and II indicate that differences in processing treatment or atmosphere in the pack do not effect the production of reducing substances, these factors are not considered to be variables in Tables III and IV; i.e., the individual samples, within a set in Tables III and IV, may have been processed differently or packed under different atmospheres.

Before attempting to interpret the data presented in Tables I to IV, the reproducibility of results with the ferricyanide method will be discussed. In this investigation it was possible to reproduce results on duplicate samples within 2 per cent transmission of light by the galvanometer. At transmissions of approximately 55 per cent, this difference involved a variation in reducing capacity of 5 per cent. At higher galvanometer readings the error would be greater. Thus, Samples 30 and 30AB in Set A, Table II, with a difference of approximately 8 per cent in their reducing capacity, gave light transmissions of 62 and 64 per cent, respectively. The limitations of the method should be kept in mind in the interpretation of the results.

Except for slight variations, within the limits of experimental error, the results in Table I show that the

TABLE I

The Effect of Time and Temperature of Preheat Treatment and of Temperature of Drier on the Development of Reducing Substances in Spray-Dried Whole Milk Powders

Set	Sample	Preheat Treatment		Air Temp. in Drier		Reducing Capacity[1]
		Temp. °F.	Time Min.	Inlet °F.	Exhaust °F.	
A	145S	155	30	300	145	6.93
	146S	165	30	300	145	7.47
	147S	175	30	300	145	7.06
B	146H	165	30	300	145	4.76
	147H	175	30	300	145	4.41
C	WM31	173	15	-	-	4.03
	WM32	175	10	-	-	3.94
	WM36	160	20	-	-	4.02
D	WM34	160	15	-	-	4.24
	WM44	190	10	-	-	4.56
E	WM56	180	30	-	-	4.18
	WM63	165	30	-	-	4.08
F	30	165-170	-	320-340	175	3.42
	40	190-200	-	320-340	175	3.58

[1]Reducing capacity in mg. of potassium ferricyanide reduced per g. of milk powder (dry weight).

TABLE II

The Effect of Atmosphere in the Pack on the Development of Reducing Substances in Spray-Dried Milk Powders.

Set	Sample	Atmosphere in Pack	Reducing Capacity[1]
Whole Milk Powders:			
A	30	Air	3.90
	30AB	Nitrogen (2)[2]	3.58
B	40	Air	3.58
	40ABH	Helium (2)	3.42
	40ABN	Nitrogen (2)	3.58
Skim Milk Powders:			
C	A11	Nitrogen	8.87
	A14	Air	8.87
D	A31	Nitrogen	6.70
	A34	Air	6.47
E	B11	Nitrogen	8.13
	B14	Air	8.10
F	B41	Nitrogen	6.40
	B44	Air	6.13
G	20	Air	6.03
	20A	Nitrogen	5.77
	20AB	Nitrogen (2)	5.56

[1]Reducing capacity in mg. of potassium ferricyanide reduced per g. of milk powder (dry weight).

[2]Double gassed--2nd gassing 1 or 2 days after 1st.

TABLE III

The Effect of Time and Temperature of Storage on the Development of Reducing Substances in Milk Powders.

Set	Sample	Storage: Time Weeks	Storage: Temp. F°	Storage: Additional Storage At Room Temp. Weeks.	Reducing Capacity[1]
Spray-Dried Whole Milk Powders:					
A	1	114	10	3	9.95
	48	8	10	3	3.73
	146H	77	10	3	4.76
	147H	77	10	3	4.41
	146S	77	100	3	7.47
	147S	77	100	3	7.06
B	WM12	30	95	26	5.93
	WM44	6	86	24	4.56
C	255	35	-15	29	4.24
	262	35	72	29	4.75
	264	35	100	29	5.62
D	17ABR	-	-	86	3.14
	17ABT	65	90-100	21	4.48
Spray-Dried Skim Milk Powders:					
E	20	-	-	85	6.03
	20T	64	90-100	21	8.09
Drum-Dried Skim Milk Powders:					
F	D1	-	-	26	16.75
	D2	-	-	8	13.48

[1]Reducing Capacity in mg. of potassium ferricyanide reduced per g. of milk powder (dry weight).

TABLE IV

The Effect of Moisture Content on the Development of Reducing Substances in Spray-Dried Milk Powder.

Set	Sample	Moisture Content[1] %	Reducing Capacity[2]
Whole Milk Powders:			
A	WM12	1.46	5.93
	WM19	3.69	10.13
B	WM31	2.60	4.03
	WM32	2.61	3.94
	WM34	1.98	4.24
	WM36	2.43	4.02
	WM44	1.65	4.56
Skim Milk Powders:			
C	A11	2.49	8.87
	A31	4.56	6.70
	A14	2.49	8.87
	A34	4.85	6.47
D	B11	2.76	8.13
	B41	4.67	6.40
	B14	2.38	8.10
	B44	4.54	6.13
E	1AR	5.91	6.48
	2AR	3.66	4.57

[1]Loss of weight after heating at 100°C. in vacuum oven for 6 hours. (149).

[2]Reducing Capacity in mg. of potassium ferricyanide reduced per g. of milk powder (dry weight).

differences in preheat treatment and temperature of the drier have no apparent effect on the production of reducing substances in milk powders. Similarly, an examination of Table II indicates no correlation between reducing substances and the atmosphere in the pack.

As would be expected, the reducing capacity of milk powder rises rapidly with increases in time and temperature of storage. This fact is amply demonstrated by the results shown in Table III. It is interesting to note that the drum-dried samples (Set F) have a much higher reducing capacity than the spray-dried samples.

It is evident, from the results presented in Table IV, that no definite statement can be made concerning the effect of the moisture content of milk powders on the development of reducing substances. Set A shows a marked increase in reducing substances with increased moisture content, and similar results are shown in Set E. On the other hand, Sets C and D reveal an opposite trend. A number of contradictory reports, concerning the effect of moisture content on stability of stored dehydrated foods, have been reviewed in the Historical Introduction. Bryce and Pearce (140), using palatability studies, have reported that a moisture content of 3 per cent is preferable to 2 or 5 per cent for retaining quality in skim milk powders. It is

apparent from the foregoing that further investigation is necessary before any definite conclusions can be reached.

Pearce (150) has attempted to assess the quality of milk powders using objective tests. After trying a large number of different methods, he concluded that palatability studies, involving errors up to 35 per cent, were the most satisfactory. In the present investigation, it was not possible to correlate the appearance of reducing substances in milk powders with a loss in palatability, since facilities for conducting satisfactory controlled taste panels were not available. However, it is interesting to note that prolonged storage and high temperatures, which are known to accelerate the deterioration of food products, also result in a definite increase in reducing substances.

2. THE IDENTIFICATION OF THE REDUCING SUBSTANCES IN MILK POWDERS.

(a) Oxidation of Tyrosine and Other Amino Acids.

Chapman and McFarlane (147) have concluded that the proteins of milk powder were denatured during storage, and reducing groups thereby became accessible to the ferricyanide reagent. However, cysteine was the only amino acid found to react to any appreciable extent, under the conditions of the test. The reaction between potassium ferricyanide and cysteine occurred stoichiometrically, one mole of the oxidant reacting with one mole of the reductant.

It was noted, however, that the cysteine content of milk protein could account for only a small fraction of the reducing capacity shown by milk powders. Anson (151) has reported that tyrosine and tryptophane could be oxidized by potassium ferricyanide at pH 6.8 and 9.6. The extent of the oxidation was greater at the lower hydrogen ion concentration, and at this level both of these amino acids were stronger reducing agents than cysteine.

It appeared of value to investigate the reducing properties of tyrosine and tryptophane in greater detail. It was found that in alkaline solution (0.067 N sodium hydroxide), tyrosine was completely oxidized after heating for twenty minutes in a boiling water bath. Longer periods of heating did not result in further reduction of the reagent, while with shorter periods and lower temperatures, less ferrocyanide was produced. The high reducing capacity of tyrosine, under the conditions employed, is illustrated in Fig. 2. The reducing properties of tryptophane were not investigated as extensively, but at pH 10 this amino acid was found to have only 50 per cent of the reducing capacity of tyrosine. Several other amino acids, including alanine, arginine, aspartic acid, cystine, glutamic acid, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine and valine, were all found to possess reducing properties in alkaline solution,

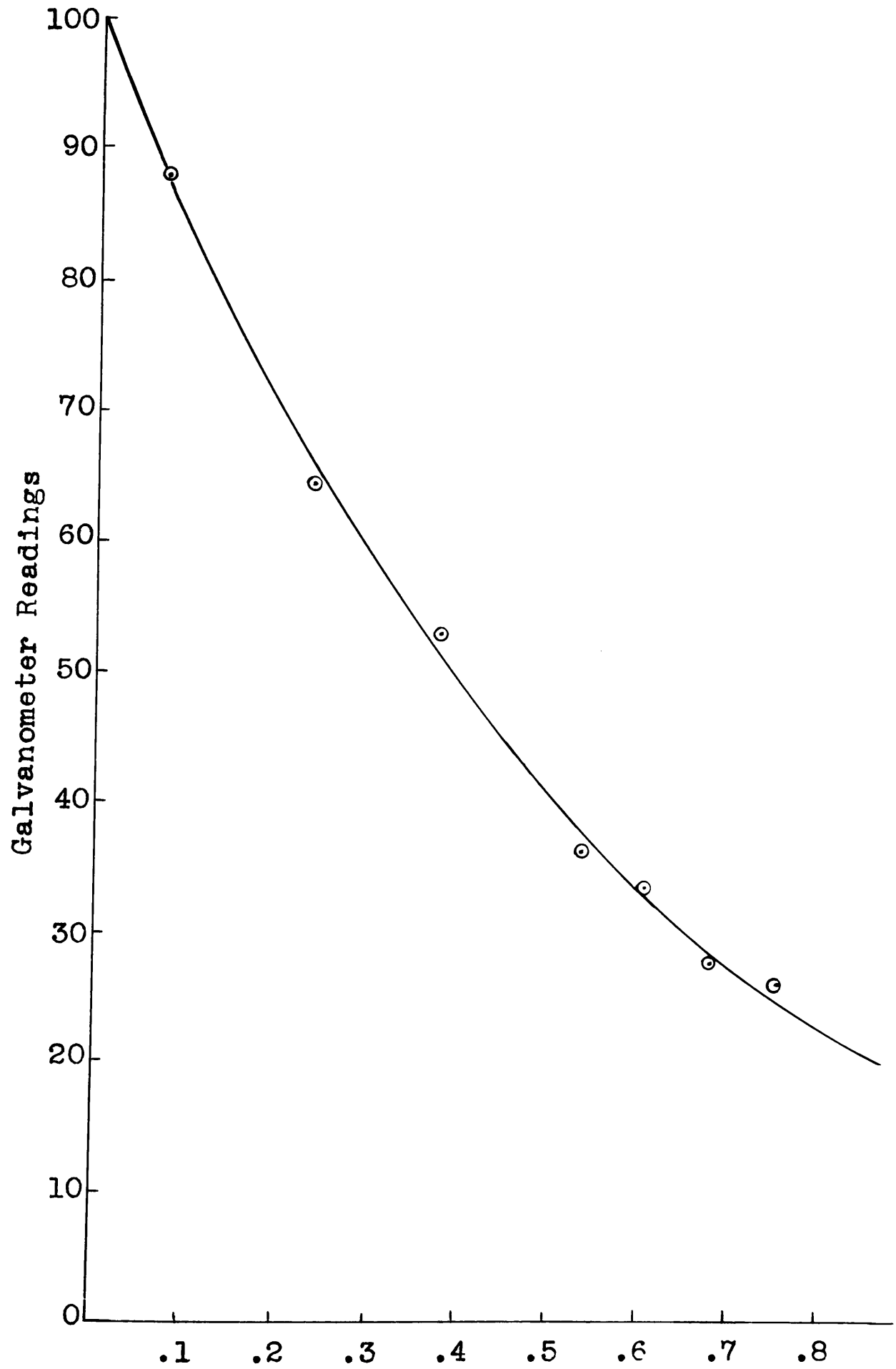

Fig. 2. Oxidation of Tyrosine by Potassium Ferricyanide.

but were much weaker reducing agents than tyrosine or tryptophane.

It appeared possible that certain substances in milk powder might catalyze the reaction between tyrosine or tryptophane at pH 5. Also, these amino acids might act differently as units in a peptide structure as compared to the free state. Bowman (152) has shown that the reaction between iodine, silver or potassium permanganate and tyrosine is accelerated by the time, temperature or alkalinity of the reaction medium. By using a phosphate buffer as a catalyst, the reduction of tyrosine at pH 6.8 occurred in two minutes, while in the absence of the catalyst the reaction did not reach completion after a week. Therefore, an attempt was made, in the present study, to accelerate the reaction between potassium ferricyanide and tyrosine, using phosphate buffers. The effect, however, at pH 5 was negligible, although at pH 7, there was a slight catalytic action. Tyrosine was also added to milk powder and the reducing substances determined by the ferricyanide method, in order to detect the presence of compounds that might exhibit a catalytic effect, but no increase in reducing capacity was observed. Later experiments have indicated that the reducing substances in milk powder are of an entirely different nature.

(b) Reducing Substances in Synthetic Mixtures.

Borsook and Wasteneys (38) have shown that solutions of glycine and glucose were capable of reducing methylene blue, when incubated together, but exhibited no such effect when heated separately. The reducing property of the solution was not destroyed by hydrolysis, in a boiling water bath for one hour, with approximately 0.025 N hydrochloric acid. Therefore, it seemed possible that heated amino acid-lactose mixtures might reduce potassium ferricyanide. A considerable amount of information, regarding the role of the Maillard reaction and caramelization in the browning of food products, has been obtained from experiments with solutions of amino acids and sugars, but there are no reports of experiments employing these substances in the solid state. Since this investigation was concerned with milk powders, it appeared advisable to conduct studies on dry mixtures.

Glycine and lysine monohydrochloride have been used as representative amino acids in the following experiments. The former was chosen because it is readily obtainable in adequate quantities, and the latter has been employed because it contains an ϵ -amino group. It has been assumed that a large percentage, if not all (153), of the free amino groups in casein are ϵ -amino groups of lysine. The concentrations of amino acids and lactose employed have

varied from equal parts of each to one part by weight of the amino acid to thirteen parts of lactose. In cases where only qualitative results are described, it was considered unnecessary to specify the concentrations used. Three methods were employed for the preparation of the synthetic mixtures of the amino acids and lactose.

(1) The components were ground together in a mortar.

(2) The components were ground together in a mortar and placed in a closed vessel over a saturated solution of sodium nitrate.

(3) The components were mixed, dissolved in distilled water and lyophilized.

In all cases, control samples of both the amino acid and lactose were treated in a similar manner to the synthetic mixtures.

It was found that, if a glycine-lactose mixture, prepared by the first method, was heated at 100°C. for two days, no browning or production of reducing substances occurred. If the temperature was raised to 130°C.,intense browning developed and the mixture possessed strong reducing powers. After a week at 130°C., the glycine control still remained colourless, while the lactose control showed only slight signs of browning. The latter was kept at 130°C. for over a month. After this treatment, a definite increase in browning was evident, and the lactose strongly reduced

the ferricyanide reagent. However, there was still less discoloration and a lower reducing capacity than in the glycine-lactose mixture which had been heated for only two days. At 130°C.,the lysine monohydrochloride-lactose mixture browned only slightly in two days.

The mixtures prepared by the second method were heated for seven hours. Intense browning occurred and the reducing capacity increased in both the glycine-lactose and lysine monohydrochloride-lactose mixtures. The lyophilized glycine-lactose sample browned to a greater extent at 115°C. than the sample prepared by the first method and heated at 130°C. Marked browning of the lyophilized lysine monohydrochloride-lactose mixture also occurred at 130°C. The control samples of the mixtures prepared by the second and third method remained colourless.

A mixture of casein and lactose was also prepared by the first method. Intense browning and strong reducing capacity developed in two days at 130°C. In this case, however, the casein control also browned and developed reducing substances, but to a lesser extent than the mixture. This effect was attributed to appreciable amounts of lactose, which the casein was known to contain. Lea (114) has been successful in retarding browning and the development of reducing substances in accelerated storage tests on milk powders, by removal of the milk sugar by dialysis.

It is apparent,from the results of the experiments just described,that some correlation must exist between the development of reducing substances and browning in the synthetic mixtures. Unfortunately, at the time that these experiments were being carried out, the identification of the possible source of development of reducing substances found in milk powders was the main interest, and all reducing substances were determined qualitatively only. Due to contamination by molds, these mixtures were discarded, and hence it is not possible to present any quantitative data concerning the relationship of browning and the development of reducing substances.

In order to determine whether amino acids react with lactose or only catalyze its breakdown in heated synthetic mixtures, amino nitrogen determinations by the Van Slyke manometric technique (154) were made on the samples, before and after the heat treatments. It was thought that the presence of decomposition products might slow down the reaction between nitrous acid and the amino group. However, it was found that the free amino nitrogen of heated glycine-lactose mixtures reacted completely in five minutes, and no nitrogen was liberated on further treatment. Dunn and Schmidt (155) have shown that the deamination of the ϵ-amino group by nitrous acid at 23°C. was complete after

thirteen and a half minutes of shaking. In the present study, the samples containing lysine were shaken for twenty minutes to ensure a complete reaction. Subsequent shaking did not result in the release of additional nitrogen. The synthetic mixtures used in this experiment were prepared in two concentrations: (1) one part by weight of the amino acid to thirteen parts of the sugar, and (2) equal parts of the amino acid and sugar. The former was an approximation of the ratio of lactose to free amino groups in fresh milk powders. The losses of the amino nitrogen, caused by heating the synthetic mixtures, are presented in Table V. Marked browning occurred in all samples, unless otherwise indicated.

TABLE V

The Effect of Various Heat Treatments on the Loss of Amino Nitrogen in Synthetic Mixtures of Amino Acids and Lactose.

Preparation[1]	Treatment		% Loss of Amino Nitrogen			
	Temp. °C.	Time	glycine-lactose ratio by weight		lysine HCl-lactose ratio by weight	
			1:13	1:1	1:13	1:1
(1)	130	2 days	20.3	0	0[2]	-
(2)	100	7 hours	39.2	0	28.8	0
(3)	115	2 days	18.8	0	-	-
(3)	130	2 days	-	-	19.7	-

[1]Methods of preparation are given on p. 38.

[2]Browned only slightly. All other samples browned considerably.

It is seen from Table V that, in the mixtures containing amino acid and lactose in the ratio of one part to thirteen, a greater loss of amino nitrogen occurred when moist heat, rather than dry heat, was applied. Similarly, lyophilized mixtures showed a greater loss than the mixtures prepared by the first method. This was probably due to the proximity of the sugar and amino acid molecules that must exist in a lyophilized mixture. In all cases, treatment with moist heat produced the darkest-coloured mixtures, the lyophilized samples were intermediate, and the mixtures prepared by the first method were the least discoloured. No attempt has been made to correlate the degree of browning with loss of amino nitrogen.

Since all the samples in Table V were discoloured, with one exception, it is apparent that browning may occur without a loss of amino nitrogen. This fact suggests that amino acids under certain conditions may act as catalysts in the browning of lactose. However, since the amino acid-lactose mixtures, in the ratio of one part to thirteen, exhibited a loss of amino nitrogen on browning, it was anticipated that milk powders might behave similarly. Such a loss has been demonstrated in accelerated storage tests by Henry and co-workers (7) and in the present investigation on stored and heated milk powders, and these results will be discussed in greater detail in Section 3. Thus, it is apparent that in addition to their catalytic

action in the browning of lactose or milk powder, amino acids or proteins may also take part in a Maillard-type reaction.

An attempt was made to determine the relative extent of reaction of the α - and ϵ -amino groups of lysine in the browning of synthetic mixtures. It is known that α -amino groups react almost completely with nitrous acid in three minutes, but that ϵ -amino groups react more slowly (155). Therefore, it was hoped, by determining the amino nitrogen on the unheated lysine monohydrochloride-lactose mixture, after three and twenty minutes of shaking, to calculate the percentage of ϵ -amino groups not reacting in the first three minutes of treatment. Assuming that the same percentage of the total free amino groups in the heated mixture would also remain unreactive, after three minutes of shaking, it would be possible to estimate the number of ϵ -amino groups that had condensed with the lactose. This experiment was only partially successful, because it was not possible to obtain reliable reproducible results with the three minute shaking treatment. This was attributed to the uncontrollable change in the rate of shaking of the Van Slyke apparatus. Although the results obtained did not accurately indicate the amounts of each of the amino groups involved in the reaction, it was obvious that both the α - and ϵ -amino

groups were capable of condensing with lactose.

From the preceding experiments, it is evident that reducing substances present in milk powders could be totally accounted for by the degradation products of lactose and by complexes, formed by a condensation of an amino group of milk protein with lactose or the decomposition products of lactose. However, this theory does not exclude the possibility that other factors may contribute to reducing properties as well. It also seems evident that discoloration and reducing substances result from the same reaction. No complete explanation can be offered for the more reactive properties of glycine, as compared to lysine monohydrochloride, exhibited in these studies. The presence of the hydrogen chloride molecule in the latter would result in a higher hydrogen ion concentration in the lysine mixtures. However, there may be additional factors which also contribute to the difference in reactivity.

(c) Fractionation of the Reducing Substances in Milk Powders.

The preceding experiments have indicated that the reducing substances in milk powders may be associated with both the protein and carbohydrate fractions. To test this theory, powders have been fractionated and the reducing substances in the fractions obtained have been estimated.

Three methods of fractionation were tried: (1) salting-out of the protein with saturated solutions of ammonium sulphate; (2) precipitation of the protein with 10 per cent trichloracetic acid; (3) removal of the sugar by dialysis. The latter method was found to give the most complete separation and was, therefore, employed in this investigation.

Lea (114) has also used dialysis for separating the sugar and protein fractions of milk powders. This investigator has determined the reducing substances in both fractions and has reported that their sum is approximately equal to the total reducing substances in the unfractionated powder. In the present study, determinations were made on the non-dialyzable or protein fraction only, in order to permit a frequent change of the dialyzing medium.

The following procedure was employed. Twenty g. of milk powder was reconstituted im distilled water and made up to 200 ml. One-half of this volume was retained as a control, and the remaining half was placed in a tubular cellophane dialyzing sac, and the sac was suspended in distilled water at 2°C. Approximately every six hours the solution containing the dialyzable fraction was removed and replaced with fresh distilled water. These solutions were collected,and at the end of the experiment were concentrated to a few ml. and tested with

Millon's reagent. A negative test was obtained, indicating that no appreciable amount of protein fragments was passing through the membrane. The dialysis was continued for four days. At this point, the solution containing the dialyzable fraction was not added to the combined dialysates but was concentrated to a few ml. and tested with Benedict's reagent. Again, a negative test was obtained, indicating that all the sugar had been removed from the reconstituted milk. The estimation of reducing substances in the dialyzed samples and the controls were made in the usual manner employing the ferricyanide reagent. The results are presented in Table VI.

TABLE VI

The Fractionation of Reducing Substances in Spray-Dried Whole Milk Powders.

Sample	Total Reducing Capacity[1]	Reducing Capacity in non-dialyzable fraction % of total capacity
A	8.72	57
B	6.60	49
C	4.88	66
D	4.28	67
E	4.24	45
F	3.92	40
G	3.60	33
H	3.60	27

[1]Reducing capacity in mg. of potassium ferricyanide er g. of milk powder.

Lea (114) has reported that during storage of milk powders, not only was there an increase in total reducing substances, but that there was also an increase in the percentage of these substances in the protein fraction. The data in Table VI do not substantiate Lea's observations. For example, Sample A possesses a reducing capacity of 8.72 units, with 57 per cent of this capacity being associated with the milk protein. On the other hand, Sample C, possessing a reducing capacity of only 4.88 units, has 66 per cent associated with the protein fraction. A careful comparison of the experimental conditions employed by Lea with those employed in the present investigation, has suggested an explanation for the discrepancies in the results. Lea has used a milk powder of high moisture content (7.6 per cent), and the total reducing substances possessed by this powder, after storage, were approximately twenty-five times greater than the amount found in the average milk powder used in the present study. The powder, employed by Lea, darkened considerably during storage, while the powders listed in Table VI showed no appreciable differences in colour.

It should be pointed out that none of the powders employed in this experiment showed any difference in their amino nitrogen content, indicating that the proportion of

amino groups involved in the production of reducing substances could not be detected by the Van Slyke manometric technique. The ferricyanide method, however, is much more sensitive and thus the relatively large changes in reducing capacity may involve only a relatively few amino groups. It is evident, from the results given in Table VI, that reducing substances may be associated with both the carbohydrate and protein fractions of milk powders.

3. CHANGES IN THE PROTEIN FRACTION OF MILK POWDERS DURING STORAGE AND HEATING.

(a) Studies on Milk Powders.

This study has been concerned with the changes in amino nitrogen content and in the dissociation properties of milk powders during storage and heating. Amino nitrogen has been determined by the Van Slyke manometric technique and by the formol titration method.

Dunn and Schmidt (155) and Van Slyke and Birchard (153) have reported that at 21°C. and 24°C., respectively, the reaction between casein and nitrous acid reached completion in thirty minutes. However, recently, Lieben and Loo (156) have shown that the liberation of nitrogen may continue even after this period of treatment. In the present study, the thirty minute shaking treatment has been adopted. Although, in view of Lieben and Loo's observations, this

procedure may have given low results, it should be pointed out that the present investigation was mainly concerned with a comparison of different milk powders, and it has been assumed that any error involved would be the same for all powders, and hence, the results would be comparable. Milk powders, reconstituted in distilled water, tended to foam over into the gas burette of the Van Slyke apparatus when treated with glacial acetic acid and sodium nitrite, even in the presence of an anti-foam reagent. It was found that excessive foaming could be prevented by placing the reconstituted milk in a boiling water bath and agitating it vigorously with a mechanical stirrer for fifteen minutes. This mild heating treatment did not result in any noticeable browning of the reconstituted milk. It was also found, by titrating the reconstituted milk with standard alkali before and after heating, that there were no measureable changes in the formol titration values or in the dissociation properties of the milk protein. Ten g. of milk powder was reconstituted, as described, in about 40 ml. of distilled water, and, after cooling, the volume was made up to 50 ml. Amino nitrogen was determined on 10 ml. aliquots.

Levy (157) has reported that maximum accuracy in the formol titration of amino acids was obtained when the following conditions were employed. The formaldehyde was

neutralized to pH 7 before using, and no correction was made for a blank. The concentration of formaldehyde was between 6 and 9 per cent at the end of the titration. For a mixture of amino acids, a titration range between pH 7 and 9.1 was recommended. Sahyun (158) has preferred to carry out the formol titration without first neutralizing the formaldehyde, and accordingly, has applied a correction for a blank. This worker has suggested that protein hydrolysates should be titrated between pH 6 and 9. In the present investigation, both of these methods were tried, and both gave results which were relatively high as compared to the values obtained by the Van Slyke manometric technique. Determinations carried out by Sahyun's method, in particular, resulted in very high values. This fact might be expected, since groups, other than amino groups, would probably be titrated between pH 6 and 7. In the present investigation, the conditions suggested by Levy have been adopted.

Measurements of pH were made with a Beckman pH meter (Model G), equipped with a standard glass electrode assembly. The pH meter was standardized with a potassium dihydrogen phosphate-disodium hydrogen phosphate buffer of pH 6.86 and a borax buffer of pH 9.18. These buffers were prepared from salts obtained from the U.S. Department of Commerce, National Bureau of Standards, Washington, D.C.

The following procedure was employed. Ten g. of milk powder was reconstituted in 200 ml. of distilled water, and the pH was adjusted to 7. Sixty ml. of neutralized 40 per cent formaldehyde was added, and the solution was titrated, at 25°C., to pH 9.1 with 0.1 N sodium hydroxide. The solution was vigorously agitated with a mechanical stirrer while pH readings were being made. The rate of addition of the standard solution and the time of titration (seven minutes) were the same for all determinations.

It was found that the milk powders, described in Section 1, did not show any appreciable differences in their amino nitrogen content, even though large differences in reducing capacity could be detected by the ferricyanide method. Therefore, for the present experiment, samples of fresh, heated and very stale milk powders were employed. The heated sample was prepared by heating the fresh sample, at 100°C., for two days. It was noted that the brown discoloration that resulted was greater when the sample was heated in vacuo, than when heated at the same temperature in a non-evacuated oven. No explanation can be offered for this phenomenon. The loss of volatile substances during the heating of the powder was determined, and an amount of the heated sample, equivalent to 10 g. of the fresh sample, was employed for the amino nitrogen and the titration curve experiments. Only two stale

samples of milk powder were available for this investigation. Both of these samples were prepared in 1943, and have since been stored at room temperature in containers with slip-on covers. The samples were obtained from different sources, and Sample A was darker in colour than Sample B.

The results of the amino nitrogen determinations are presented in Table VII. It is seen that the amino nitrogen of the fresh sample, as determined by the manometric technique, is about 20 per cent lower than the value obtained by the formol titration. This difference may be the result of an incomplete reaction between nitrous acid and the milk protein, but more likely, is due to the fact that substances or groups, other than amino groups were titrated in the formol titration. It is also seen, from an examination of Table VII, that the Van Slyke manometric technique gave much lower values for the stale and heated milk powders than for the fresh sample. However, corresponding differences were not obtained when the formol titration method was employed. In an attempt to explain this discrepancy, the titration curves of the samples were determined.

The titration of the milk powders was carried out at 25°C., between pH 6 and 12. The pH readings were made directly, employing the same pH meter used for the formol

TABLE VII

The Free Amino Nitrogen Content of Fresh, Heated and Stored Spray-Dried Whole Milk Powders.

Sample	Mg. of Amino N_2 per g. of Milk Powder[1]	
	Van Slyke	Formol
Fresh	2.93	3.64
Heated	1.73	3.71
Stale A	1.79	3.64
Stale B	1.65	3.53

[1]dry weight

titrations. In addition to the buffers, previously described, an acid potassium phthalate buffer of pH 4.01 and a borate-sodium hydroxide buffer of pH 12.13 (159) were also employed. The former was also prepared from a salt obtained from the U. S. Department of Commerce, National Bureau of Standards, Washington, D. C. It was found that if the glass electrode was standardized, for example, with the phosphate buffer of pH 6.86, a reading of pH 8.83 was obtained with the borax buffer of pH 9.18. It was, therefore, necessary to apply a correction factor to all readings between pH 6.86 and 8.83. This factor was variable, increasing as the pH was increased. It was assumed that this increase in the correction factor would

bear a linear relationship to the increase in pH. Although this assumption may not be correct, any error involved would be approximately the same for all milk powders examined, and hence, the final results should be comparable. Similar corrections were applied to the pH readings between 4.01 and 6.86, and 9.18 and 12.13. Since only comparable results were desired, it was not considered necessary to standardize the glass electrode against a standard cell. For all readings above pH 9, a Beckman "Type E" glass electrode (1190-E)[1] was employed.

In order to minimize the error caused by changes in ionic strength during titration, sufficient sodium chloride was added to the reconstituted milk to make the solution 1 N. The solution was titrated with 0.1 N sodium hydroxide, to which had been added sufficient sodium chloride to make the solution 1 N with respect to the sodium ion. The alkali was standardized against acid potassium phthalate, after the sodium chloride had been added. Thus, while the standard solution was only 0.1 N, its ionic strength was approximately the same as that of the reconstituted milk, and, consequently, no change in the ionic strength of the solution being titrated occurred throughout the titration. It was assumed that the contribution of the milk powder

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[1] The manufacturer claimed that this electrode would give relatively accurate pH reading in the presence of large amounts of sodium ion. Later experiments (p. 71) proved this claim to be invalid.

to the ionic strength of the reconstituted solution need not be considered. Since only 10 g. was reconstituted in a liter of distilled water, the effect on the ionic strength of diluting this volume with a 100 ml. of the standard solution would be negligible.

The titration of milk powder was carried out in the following manner. Ten g. of the powder was reconstituted in 500 ml. of distilled water, and 58.5 g. of sodium chloride was added, and the volume was made up to 1 liter. Samples being compared were always titrated at the same time. The electrode was then standardized, and the solutions, in turn, were adjusted to pH 6 using 1 N hydrochloric acid. The electrode was then restandardized, and if any change in the assymmetry potential of the electrode had occurred, the Zero Adjustor on the meter was adjusted to give the correct reading of the buffer used, and the pH of the solutions was taken again. This entire procedure was repeated until satisfactory readings were obtained. An aliquot of standard alkali was then added to each of the solutions, and the pH was taken. This procedure was continued until the titration was complete. A change in the assymmetry potential of the standard electrode seldom occurred, although these changes were common when the "Type E" electrode was employed. In order to prevent the accumulation of a layer on the surface of the electrodes,

the solution was vigorously agitated with a mechanical stirrer, while pH readings were being made.

A blank solution, consisting of 1 liter of 1 N sodium chloride was titrated in a similar manner and at the same time as the reconstituted milk. The volume of standard solution required to bring the blank to a particular pH, was substracted from the volume required to bring the milk powder solution to the same pH. The volumes thus obtained were then plotted against the pH values. The accuracy obtained by this method became progressively smaller as the alkalinity of the solution increased. This fact was due to the increase in the slope of the blank titration curve, as the pH increased. Thus, it was found that above pH 11.3 it was not possible to obtain reliable results. However, this has not proven to be a serious hindrance, since a satisfactory study could be made on the portion of the curves below pH 11.3.

The titration curves of the fresh and heated samples are presented in Fig. 3. A number of other samples of fresh and heated milk powders have been titrated, and in all cases similar results were obtained. An examination of these curves indicates that more groups are titrated in the heated sample, between pH 6 and 9.25, than are titrated in the fresh sample. However, between pH 9.25 and

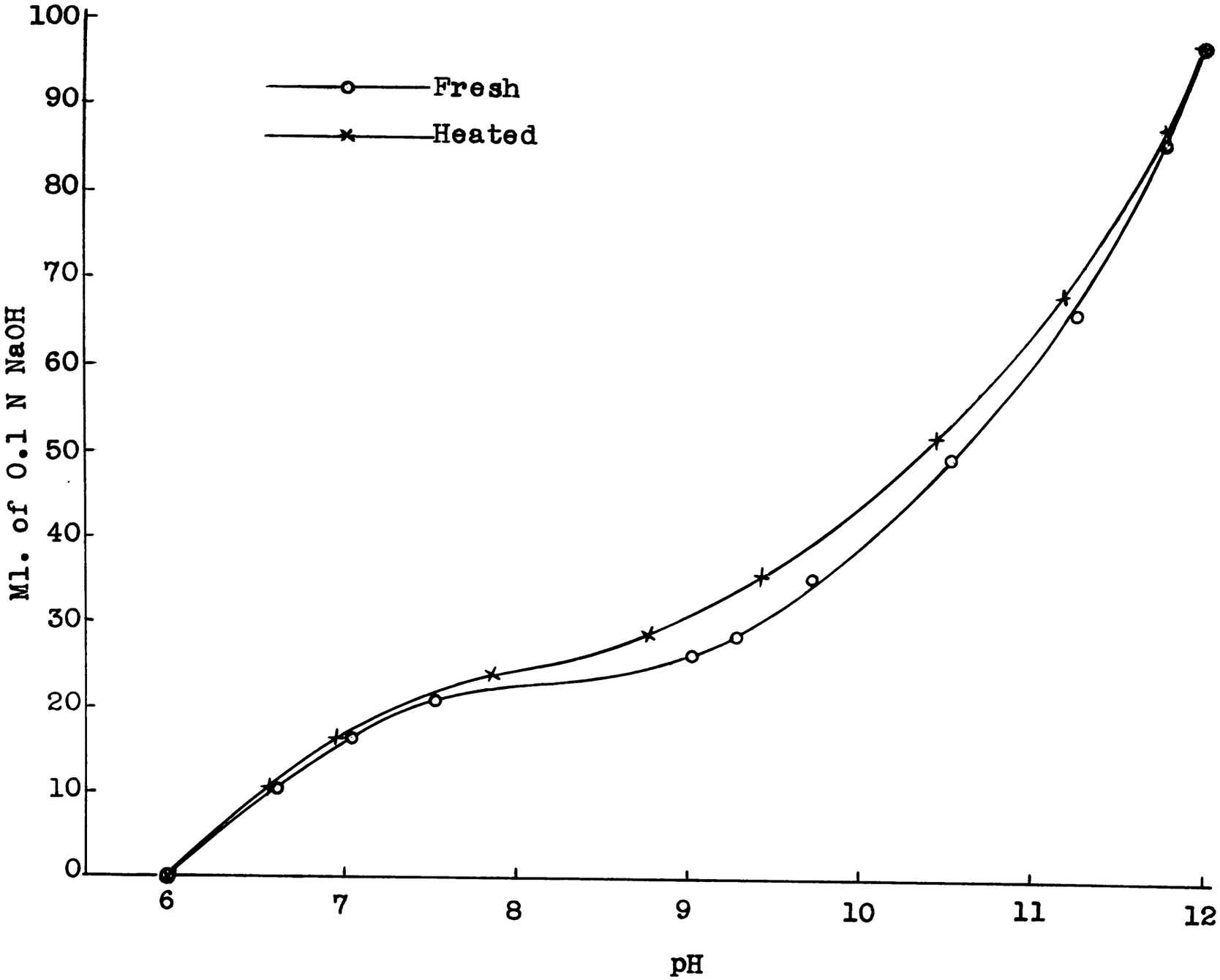

Fig. 3. Titration Curves of Fresh and Heated Milk Powders.

11.95, the reverse is apparent. The fact that the curves converge at pH 11.94, and are only slightly separated at pH 7, is in accordance with the results obtained using the formol titration method. Since the pK' value of the ϵ-amino group of lysine is 10.53 (160), it is evident that the heated sample contains fewer such groups than the fresh sample. This fact was in agreement with the results obtained using the Van Slyke manometric technique, and it added support to the theory that discoloration and decrease of nutritive value of heated milk powder were due, in part, to a reaction between the ϵ-amino group of lysine and lactose. It has been assumed that the majority, if not all (153), of the free amino groups of milk protein are ϵ-amino groups of lysine.

Thus, the titration curves, shown in Fig. 3, satisfactorily explain the discrepancy of the results presented in Table VII. However, the foregoing discussion does not account for the additional titratable groups revealed in the heated sample below pH 9.25. It is known that the denaturation of proteins, during heating, exposes the phenolic group of tyrosine and the sulphydryl group of cysteine. Cannan (161) has reported that the former group does not dissociate below pH 11 and that the sulphydryl group would probably behave in a similar manner. Thus,

these groups should not affect the curve between pH 6 and 9.25. However, the possibility of interference due to sulphydryl groups was checked by blocking these groups with iodine.

Edsall (162) has stated that iodine reacted with several groups on the protein molecule, but Anson (163), working with egg albumin, has shown that at pH 3.2, the reaction with iodine was restricted to the sulphydryl groups. The following experiment was, therefore, carried out. A 2 per cent solution of iodine in 1 N potassium iodide was prepared. This solution was slowly added to reconstituted milk powder, at pH 3.2, containing a few ml. of starch solution, until a slight excess was evident. The excess iodine was reduced with sodium thiosulphate. The reconstituted milk was then adjusted to pH 6, and was titrated in the usual manner to pH 10. The curve, thus obtained, was identical with the titration curve of the milk powder, which had not been treated with iodine. Therefore, assuming that the reaction between iodine and milk protein would occur in a manner analogous to that obtained by Anson, with iodine and egg albumin, it was considered that sulphydryl groups were not responsible for the increased base-combining capacity of the heated milk powder in the pH range 6 to 9.25.

The difference in the dissociation characteristics of fresh and heated milk powders may be explained by assuming that a condensation of a free amino group of the protein with lactose or a decomposition product of lactose occurs to form a product which contains an imino group. The mechanism of such a condensation may be similar to the generally accepted mechanism of the reaction between formaldehyde and amino acids. This reaction, proposed by Balson and Lawson (164) and Levy and Silberman (165), is represented by the following equation.

$$R.NH_2 + CH_2O \rightleftharpoons R.NH.CH_2OH + CH_2O \rightleftharpoons R.N(CH_2OH)_2 \quad (1)$$

Compound A Compound B

The imino group in the condensation product corresponding to Compound A would be expected to ionize at a lower pH than the amino group, and would account for the greater base-combining capacity of the heated milk protein in the pH range 6 to 9.25. It is possible that in the solid state, or that in the presence of only small amounts of water, the reaction would be prevented from proceeding beyond the first step. It is also probable that the molecules of sugar or of the degradation products of sugar would be larger than the relatively small formaldehyde molecule, and hence, might be prevented by steric hindrance from forming a product similar to Compound B.

The theory, suggested in the preceding paragraph,

would explain the fact that similar values were obtained for the amino nitrogen content of heated and unheated milk powders by the formol titration method. Every amino group reacting would form an imino group. While this reaction would result in a decrease in free amino nitrogen by the Van Slyke method, it would not effect the formol titration values, for as Levy and Silberman (165) have shown, both amino and imino groups are titrated in the presence of formaldehyde.

It should be pointed out that reconstituted milk powder is not a true solution, but rather a complicated system, with most of the protein existing in the colloidal state. The heating of milk powders definitely has an effect on the physical properties of such a system. For example, it is known that heating results in decreased solubility. It was, therefore, conceivable that the results obtained in this investigation might be explained by changes in the physical, as well as the chemical properties, of milk powder. However, decreased solubility of milk powder, caused by heating, would be expected to result in a decrease in the base-combining capacity, in the neutral and slightly alkaline ranges of the titration curve, and in an increase in the more alkaline range, because of the greater solubility of the milk powders in

alkaline solution. Since an examination of Fig. 3 indicates that the base-combining capacity is greater in the lower pH range and less in the more alkaline region, after heating, it is evident that solubility factors are not responsible for the results obtained. Howat and Wright (166) have shown that the loss of solubility, caused by heating spray-dried milk powders in the dry state, could be restored by heating the reconstituted milk at 100°C. for thirty minutes. This treatment has been tried, in the present investigation, in order to offset any effect of decreased solubility, but has not resulted in any change in the titration curve.

The titration curves of the stale milk powders are presented in Fig. 4. For comparative purposes, the curve of the fresh powder is also given. It is evident that the curve of stale Sample A is similar to the curve of the heated powder given in Fig. 3. Thus, it appears that the reactions responsible for the changes in the titration curves caused by heating, may also be operative during long periods of storage. These reactions would also explain the differences, in the amino nitrogen values of Sample A (Table VII), obtained by the Van Slyke and formol titration methods. However, the titration curve of Sample B is almost identical with that of the fresh powder, despite the fact that a lower value for Van Slyke amino

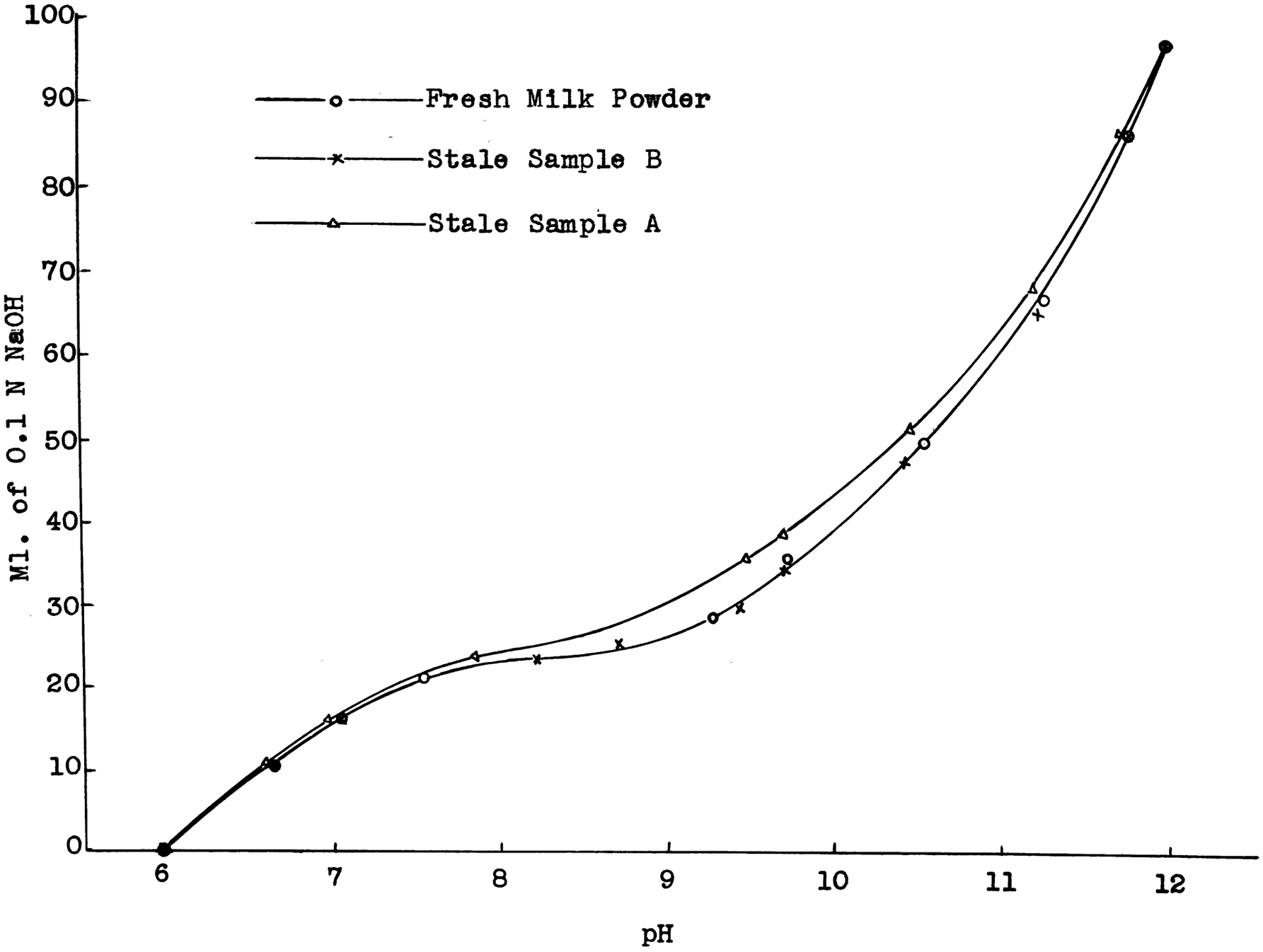

Fig. 4. Titration Curves of Fresh and Stale Milk Powders.

nitrogen (Table VII) was obtained on the stale sample. The amino nitrogen and the titration curves of the stale powders have been determined in triplicate, and the same results, within the limits of experimental error, have been obtained each time. It has already been pointed out that, although these powders were manufactured at approximately the same time and were stored under similar conditions, they were obtained from different sources, and Sample A was definitely more discoloured than Sample B. It is, therefore, surprising that Sample B has a slightly lower free amino nitrogen content, by the manometric technique, than Sample B. It seems possible that some factor may be interfering with the reaction between nitrous acid and the protein of Sample B, and that the discrepancy in the results of the two stale samples is due to an error in the Van Slyke amino nitrogen determinations, rather than the titration curves. However, no evidence can be presented to support this statement. Unfortunately, only two samples of stale milk powder were available for this study.

(b) Studies on Synthetic Mixtures of Amino Acids and Lactose.

It has been suggested that the changes, caused by heating, in the free amino nitrogen content and in the titration curves of milk powders are due to a reaction between an amino group of the milk protein and lactose.

Since milk is a complex substance, it seemed advisable to check this theory, using simple amino acid-lactose systems. Therefore, the following experiments were conducted, employing lysine monohydrochloride and glycine as representative amino acids. One part by weight of the amino acid and thirteen parts of lactose were dissolved in distilled water and the solutions lyophilized. Fourteen g. of each of the dried glycine-lactose and the lysine monohydrochloride-lactose mixtures was heated for two days at 115°C. and 130°C. respectively. The browned mixtures were then dissolved in distilled water and the volume made up to 200 ml. Ten ml. aliquots were taken for the Van Slyke amino nitrogen determinations, and 40 ml. aliquots were used for the formol titration and titration curves experiments. Unheated lyophilized mixtures were used as controls. The amino nitrogen values and the titration curves were determined using the same procedures employed in the investigations on milk powder, except that the shaking treatment in the Van Slyke determination was conducted for five minutes on the glycine-lactose mixtures and for twenty minutes on the lysine monohydrochloride-lactose mixtures. The results of the amino nitrogen losses, measured by the Van Slyke manometric technique and the formol titration method, are presented in Table VIII.

TABLE VIII

The Effect of Heating on the Loss of Amino Nitrogen in Synthetic Mixtures of Amino Acids and Lactose.

Mixture	% Loss of Amino N_2 in Heated Mixtures	
	Van Slyke	Formol
Glycine-Lactose	18.8	6.6
Lysine HCl-Lactose	19.7	9.7

An examination of Table VIII reveals that there is a variation in the amino nitrogen losses as determined by the two different methods. This variance is similar to that found with heated milk powders (Table VII). In the milk powder experiment, it was noted that no loss of amino nitrogen was detected by the formol titration method. Table VIII, however, shows losses of 6.6 and 9.7 per cent, for the respective mixtures. These results, in Tables VII and VIII, are not necessarily contradictory, since the systems and conditions of treatment were not the same in each experiment. The heated synthetic mixtures were much darker in colour than the heated milk powder. Although heating decreased the solubility of the milk powder, the insoluble components could be readily and evenly dispersed by shaking. On the other hand, the amino acid-lactose mixtures contained insoluble dark-coloured

precipitates, which would not go into solution or suspension with vigorous shaking at room temperature. It is possible that these insoluble materials might have been formed by a condensation between the amino acid and lactose or the decomposition products of lactose, the reaction proceeding beyond the first step shown in Equation 1 and resulting in the formation of a product which would have no dissociation properties, and hence, would not be titrated by the formol titration.

Further evidence, in support of the foregoing theory, is found on examination of the titration curves in Fig. 5 and 6, since, in each case, the total of the titratable groups present in the unheated mixtures is greater than in the heated mixtures. In addition to this fact, the differences in the curves of the heated and unheated synthetic mixtures are similar to those exhibited by the heated and unheated milk powders, shown in Fig. 3. This evidence supports the theory, that the interaction of lactose and protein is responsible for the changes, caused by heating, in the amino nitrogen values and titration curves of milk powders, and that this interaction probably results in the formation of a compound containing an imino group.

It is possible, but unlikely, that the additional titratable substances, in evidence in the lower portion of the curves of the heated mixtures, are completely non-nitrogenous in nature, i.e., that sugar decomposition

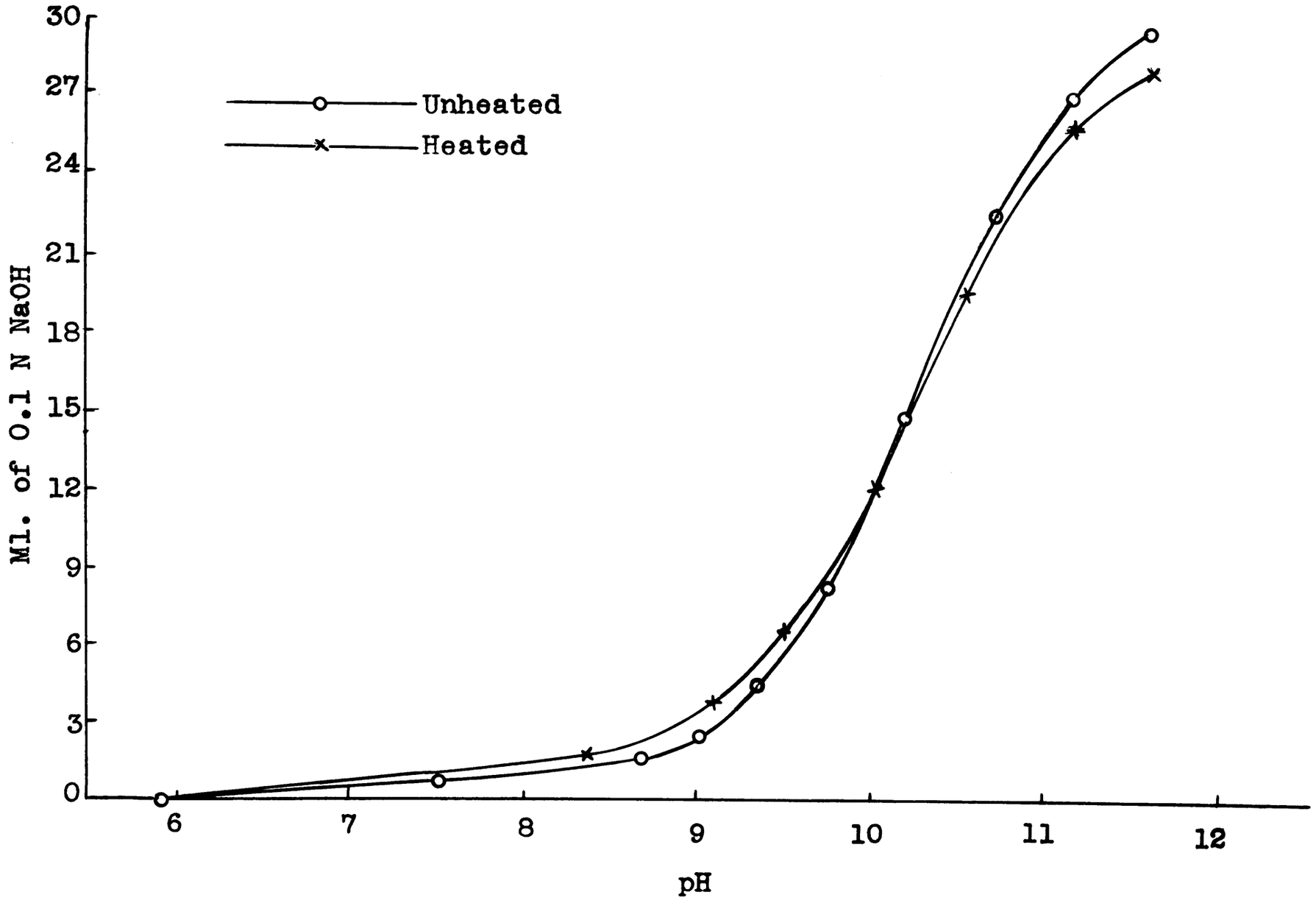

Fig. 5. Titration Curves of Glycine-Lactose Mixtures.

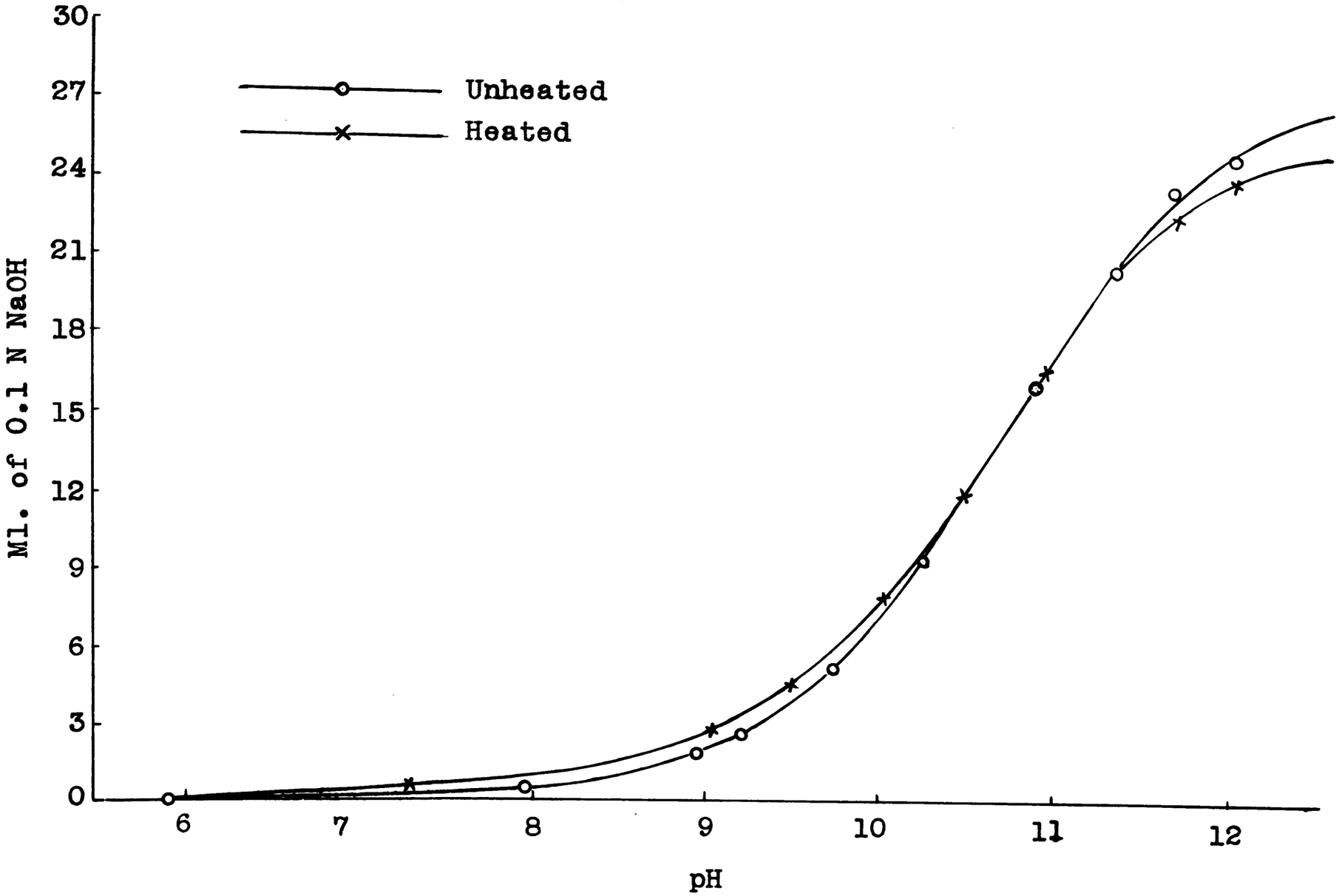

Fig. 6. Titration Curves of Lysine HCl - Lactose Mixtures.

products would contain groups capable of ionizing. It has been demonstrated (Table V) that amino acid-lactose mixtures, prepared with equal parts by weight of each component, showed no loss in amino nitrogen content as measured by the Van Slyke manometric technique. In the present experiment, an investigation of the titration curves of some of these mixtures also revealed no difference between the heated and unheated samples.

A close examination of the titration curves, presented in Fig. 5 and 6, reveals certain discrepancies between the curves of the unheated mixtures and the expected theoretical curves. For example, in Fig. 5, since 200 mg. of glycine was employed, the amino acid should have been completely titrated after approximately 27 ml. of standard alkali had been added. This volume is approximately 3 ml. less than the actual volume required. A similar trend is evident in Fig. 6. There is no apparent explanation for this deviation, although it may have been due to the presence of lactose. It has already been pointed out that the degree of reliability of pH determinations decreased as the alkalinity of the solution increased. It is also seen, in Fig. 5 and 6, that the points on the curves of the unheated samples represent pH values which are higher than would be theoretically expected. Batchelder

and Schmidt (167, 168, 169) have shown that, on the alkaline side of the isoelectric point, the effect of adding sodium chloride in concentrations up to 1 N, to amino acid solutions, was to decrease the pH of the solutions. This fact is contradictory to the results obtained in the present study. Although the lower portion of the curve of the heated mixture containing lysine, shown in Fig. 6, is on the acid side of the isoelectric point, and the shift towards the alkaline side, therefore, would be expected, the extent of the shift is greater than can be accounted for by the salt effect.

It seemed from the results obtained, that the accuracy of "Type E" electrodes, employed for all pH readings above 9, may have been affected by the presence of sodium ion, in spite of the manufacturer's claim that the error would be negligible. This fact was verified in the following manner. A 1 N sodium chloride solution and a blank, consisting of the same volume of distilled water, were adjusted to pH 6, and sufficient standard alkali solution was added to the blank to reach pH 10.5. The same volume of standard alkali was then added to the sodium chloride solution, and a pH value of 10.92 was obtained. Sufficient sodium chloride was then added to the blank to make the solution 1 N, and a shift in the pH from 10.5 to 10.92 was observed. This procedure was applied to solutions at

other pH levels, and similar results were obtained. At pH 8.7, the standard glass electrode was also affected, in the same manner as the "Type E" electrode, by the presence of the salt. It was therefore concluded that the electrodes gave inaccurate pH readings in the presence of the sodium ion, and that this factor was responsible for the displacement of the titration curves. Unfortunately, the error due to the electrodes was not discovered until the experimental work in the investigation was completed. However, it was not considered necessary to attempt to apply correction factors to the results, as the error involved would be approximately the same in all cases, and, consequently, the values obtained would be suitable for comparative purposes.

4. THE EFFECT OF REDUCING SUBSTANCES ON THE STABILITY OF FAT IN SYNTHETIC MIXTURES.

Chapman and McFarlane (170) have reported that the relative stability of the fat in milk powders, stored at elevated temperatures, was related to their content of reducing substances. Powders with a higher reducing capacity, as measured by the ferricyanide method, were found to be more stable than those with lower reducing capacities. In the present investigation, an attempt was made to obtain more direct evidence in support of these observations, by studying the effect of reducing substances in synthetic

mixtures on the stability of milk fat.

Synthetic mixtures of amino acids and lactose were prepared in the following manner. Three g. of lactose and 0.23 g. of glycine were mixed in 1.5 ml. of distilled water and heated in a closed vessel over a saturated solution of sodium nitrate for four hours at 100°C. This treatment had been previously found to produce large amounts of reducing substances. Three g. of lactose and 0.275 g. of lysine monohydrochloride were treated in the same manner. One sample of the lysine mixture and two samples of the glycine mixture were prepared. After heating, the samples were cooled and dissolved in approximately 100 ml. of distilled water. To these solutions, 15 ml. of 15 per cent cream was added. In addition to the cream, 6.5 g. of fresh skim milk powder was also added to one of the glycine-containing solutions. The solutions were then passed through an emulsifier, and the resulting emulsions were lyophilized. Control samples, in which the amino acid-lactose mixtures were not heated, were also prepared. It should be pointed out that, except in the case of the sample to which skim milk powder had been added, sufficient amounts of the various components of the mixtures were employed to give the approximate proportions of amino nitrogen, lactose and fat present in whole milk powders.

The lyophilized samples were stored at 53°C., and were examined periodically for their peroxide content, by the method of Chapman and McFarlane (171). The results are presented in Fig. 7. It can be seen that, in all cases, the samples containing the heated amino acid-lactose mixtures, i.e., those containing a large quantity of reducing substances, are more stable than the corresponding controls. Thus, it is evident that these reducing substances have a stabilizing effect on milk fat.

Fig. 7 shows that a different degree of stability exists between the three mixtures employed. It should be pointed out that no comparison of these results can be made, because certain experimental conditions, such as the hydrogen ion concentration and time of standing of the solutions, before and after emulsification, were not the same for the three mixtures.

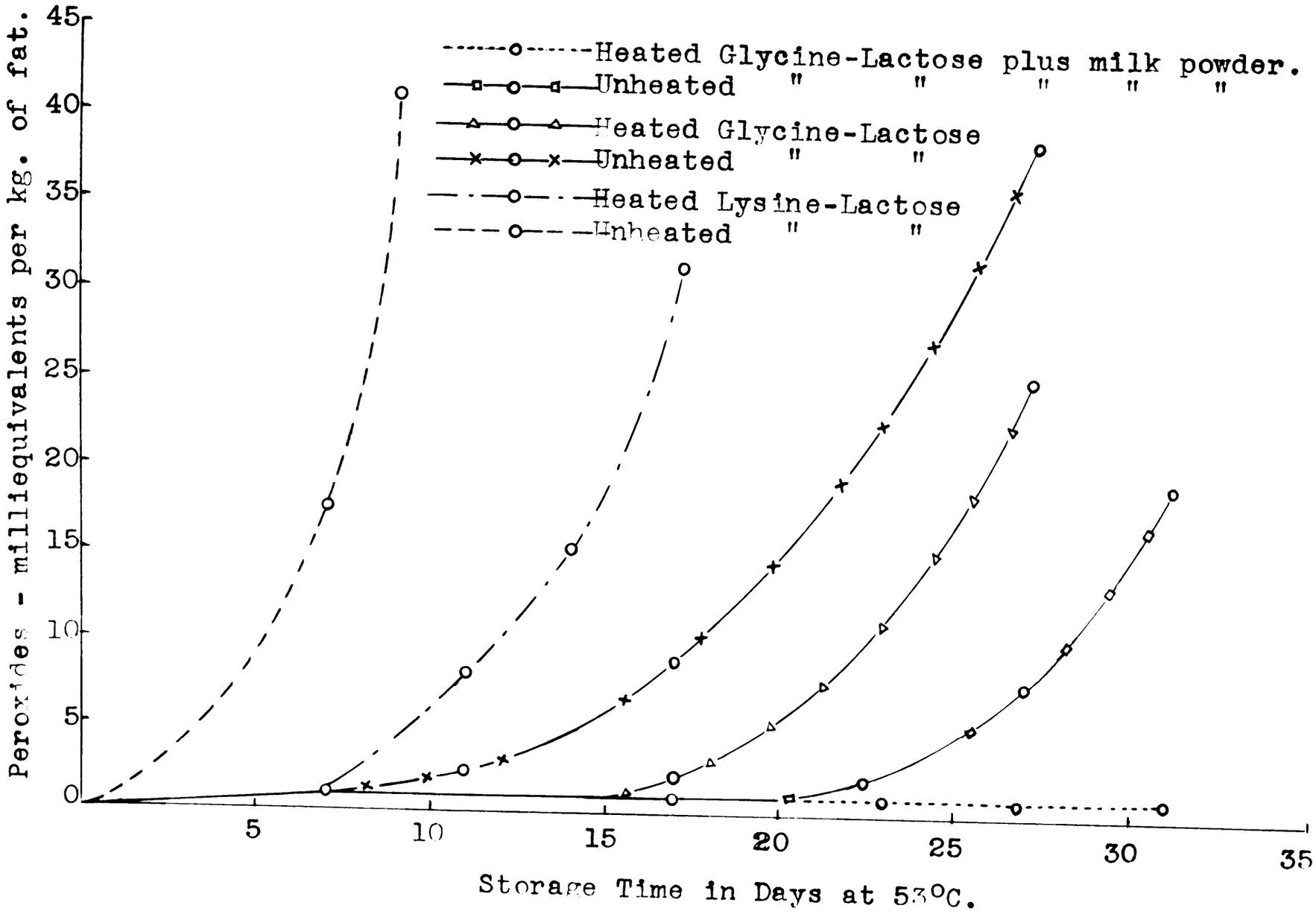

Fig. 7. Development of Peroxides in Accelerated Storage Tests on Synthetic Mixtures.

SUMMARY AND CONCLUSIONS

A number of modifications have been suggested for the Chapman and McFarlane method for the determination of reducing substances in milk powder. The modified method has been applied to a number of milk powder samples, processed and stored under different conditions. It has been shown that the production of reducing substances was independent of processing temperatures and of the type of atmosphere in the pack. A relationship has been found between the development of reducing substances and increases in the temperature and time of storage, but no direct correlation between reducing substances and moisture content was noted. These results show that the development of reducing substances is accelerated by those condtions of storage which are usually considered to increase the rate of deterioration of milk powders. Thus, it is suggested that the ferricyanide method of Chapman and McFarlane may prove useful for assessing the quality of milk powders, although further studies, involving palatability tests, are necessary, before more definite conclusions can be made.

The reduction of potassium ferricyanide by tyrosine and other amino acids has been studied, and it has been found that amino acids, and tyrosine in particular, were strong reducing agents in alkaline solution. However, no

reaction between potassium ferricyanide and tyrosine or other amino acids, with the exception of cysteine, has been noted at pH 5. An attempt to catalyze the reaction between tyrosine and the ferricyanide reagent at this pH level has been made, employing phosphate buffers as catalysts, but negative results were obtained. The addition of tyrosine to milk powders did not result in an increased reducing capacity of the mixture, as measured by the ferricyanide method. These results suggest, but do not definitely prove, that the groups on the protein molecule, which are known to be exposed during the denaturation of a protein, are not responsible for the reducing capacity of milk powders.

The development of reducing substances in dry synthetic mixtures of amino acids and lactose has been studied. Mixtures of lactose and glycine or lysine have been heated under various conditions, and intense browning and production of reducing substances were noted, while no such changes were detected in the control samples, consisting of either the amino acid or lactose. In all the heated mixtures, a qualitative correlation between browning and the development of reducing substances was observed. These reducing substances were developed in sufficient quantities to account for the total reducing capacity exhibited by milk powders.

The effect of heating on the amino nitrogen content of synthetic mixtures of amino acids and lactose has been studied, employing the Van Slyke manometric technique. No loss was detected in the mixtures containing equal parts by weight of the amino acid and lactose, although marked browning and the production of reducing substances occurred. However, in the mixtures, consisting of one part by weight of the amino acid to thirteen parts of lactose, a marked loss of amino nitrogen was observed. It was also shown that prolonged heating (one month) at 130°C. resulted in discoloration and the development of reducing substances in lactose, even in the absence of amino acids. Employing dialysis as a means of fractionating milk powders, it was shown that the reducing substances in milk powders are associated with both the protein and sugar fractions. It, therefore, seems justifiable to conclude that the development of reducing substances and browning in milk powders are caused by the same reactions, and that these reactions involve the degradation of lactose, which is catalyzed by the milk protein, and the condensation of lactose or its decomposition products with the amino groups of the protein.

The heating of milk powders has resulted in a loss of amino nitrogen as measured by the Van Slyke manometric technique, but no loss was observed when the formol titration method was employed. The investigation of the

titration curves of the heated and unheated powders has shown that, after heating, there is an increase in the number of dissociating groups between pH 6 and 9.25 and a corresponding decrease between pH 9.25 and 11.94. Similar results have been obtained with lyophilized amino acid-lactose mixtures. This phenomenon has been explained by assuming that a reaction involving an amino group, possibly of lysine, takes place, with the formation of an imino group, which dissociates at a lower pH. This reaction would not result in lower amino nitrogen values by the formol titration method but would give lower results by the Van Slyke manometric technique. A mechanism for such a reaction has been suggested.

The amino nitrogen content, by the Van Slyke and formol titration methods, and the titration curves of two stale milk powders have also been investigated. The amino nitrogen values of both of these samples were similar to those obtained with the heated powders, and the titration curve of the stale Sample A was almost identical with that of the heated powder. However, the titration curve of stale Sample B showed no significant deviation from the curve of the fresh milk powder. In view of this discrepancy, it would not be advisable to draw any definite conclusions regarding the mechanism of the reactions

occurring in the protein fraction of milk powder during storage.

Employing synthetic lyophilized systems, it has been shown that the reducing substances in heated mixtures of amino acids and lactose have a stabilizing effect on the milk fat.

It appears that the reactions occurring in the protein and carbohydrate fractions are responsible for many changes that milk powders undergo during storage and heating. These reactions result in browning and the production of reducing substances, and as other workers have suggested, may also give rise to fluorescent materials and the development of off-flavours and odours. The free amino groups of milk proteins play an important role in these reactions. These groups act as catalysts in the breakdown of the sugar and are also involved in sugar-protein condensations. Since a large proportion of the free amino groups in milk protein are ϵ-amino groups of lysine, it appears that lysine is the key amino acid involved in these chemical changes. These facts are in agreement with the results of numerous nutritional experiments, which have indicated that a proportion of the lysine of proteins is rendered unavailable to the animal body by heating or prolonged storage.

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