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Howard Keltner Zimmerman
Iowa State College

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PROTEOLYTIC ENZYMES OF RIPENING CHEDDAR CHEESE

by

Howard Keltner Zimmerman

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In Charge of Major Work

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Head of Major Department

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INTRODUCTION

Research during the past half century has shown that the ripening of cheddar cheese is a relatively complex process. Many investigations have been carried out relative to the physical, chemical and bacteriological nature of cheddar cheese. Notable achievements, such as the development and use of lactic starter cultures, the pasteurization of the milk used for cheese making, and, more recently, improvements in packaging design, have led to a gradual but continued improvement in our knowledge of the subject. However, many problems related to cheese remain unsolved, and chief among these are the problems associated with the ripening or curing processes.

Notwithstanding the fact that a considerable segment of the cheese-consuming public has become accustomed to the more mild flavor of pasteurized milk cheddar cheese and process cheese, a rather large demand still exists for the full, sharp, characteristic flavor normally found in raw milk cheddar cheese of good quality. Cheddar cheese made from pasteurized milk develops flavor more slowly than raw milk cheese, and it has been stated that, if properly made, it never develops the sharp flavor characteristic of raw milk cheese.

The development of a full, sharp-flavored raw milk cheddar cheese ordinarily requires a ripening period of at least six months, and some cheese is held for somewhat longer periods

before being offered to the consumer. Proper ripening obviously is an important economic problem when one considers the storage, refrigeration and handling costs involved.

So far, no satisfactory method of controlling the flavor of cheddar cheese has been devised. It quite generally is held that the development of flavor in cheddar cheese is associated with concurrent proteolytic and lipolytic changes. No simple explanation seems to account for all of the changes involved. However, the hydrolytic breakdown of cheese protein has been shown to be rather intimately associated with the ripening of cheddar cheese. This protein breakdown changes the tough, green and rubbery curd of young cheese to the smooth, waxy curd of well-ripened cheese of good quality. These changes occurring in the body and texture of the cheese appear to be associated to some degree with the development of the characteristic flavor of cheddar cheese.

Much of the work that has gone before has indicated that the proteolytic changes in ripening cheddar cheese arise as a result of the action of enzymes. Investigations into the enzymatic degradation of cheese protein during the ripening process, whether by the enzymes of rennet or by the enzymes of the bacteria contained in the cheese, would be expected to yield valuable information in regard to the ripening problem of cheddar cheese. Therefore, this investigation was initiated to study some of the proteolytic enzymes which were considered to be important in the ripening process of cheddar cheese.

Emphasis has been placed on the enzymes of commercial rennet in an attempt to obtain additional information on the effect of these enzymes on pasteurized milk cheddar cheese. It also was decided to study the proteolytic enzyme system of Streptococcus lactis in an attempt to determine how this particular organism might affect the changes observed in ripening cheddar cheese.

HISTORICAL

The Influence of Bacteria on the Ripening Process of Cheddar Cheese with Special Reference to *Streptococcus lactis*

In 1903, Harding (33) investigated the lactic acid bacteria in relation to the ripening process of cheddar cheese. He noted that the lactic acid bacteria were always present in factory milk, that they inhibited the growth of other microorganisms through production of acid, and finally made up more than 90 per cent of the total number of microorganisms present in the cheese. The acid formed by these bacteria was of further benefit in that it hastened the curdling action of the rennet and was necessary for the action of the pepsin contained in the rennet extract.

Harding and Prucha (34) carried out one of the first extensive investigations of the bacterial flora of cheddar cheese. They found that the *Bacterium lactis acidi* (*Streptococcus lactis*) group was the only one always present, and it almost always made up over 99 per cent of the total number of organisms found in the cheese. These workers further stated that there was no evident connection between the number of bacteria present in the cheese and the rate at which it ripened.

Hastings, Evans and Hart (35), in 1914, presented a very extensive report on the factors concerned in the ripening of cheddar cheese. They reported that B. lactis acidi (S. lactis) was predominant in cheese. The acid formed by these bacteria favored the shrinking of the curd with expulsion of the whey, activated the pepsin contained in the rennet extract and permitted "matting" of the curd. These investigators further stated that the bacteria contained in the milk were largely retained in the curd formed during the manufacture of the cheese.

Hucker and Marquardt (38) found that Streptococcus paracitrovorus (Leuconostoc dextranicum), when added to milk to be used for cheese making, had a desirable effect on the flavor of the resulting cheese. Streptococcus citrovorus (Leuconostoc citrovorum) apparently had no effect, while S. lactis gave as favorable results as did commercial starters.

Spitzer, Parfitt and Epple (61) studied proteolysis by a number of different organisms and reported that S. lactis was one of the least proteolytic organisms investigated. These workers noted that the acidity of the medium was one of the factors which most influenced proteolytic changes.

In another study of the proteolytic action of certain lactic acid bacteria, Petersen, Pruess and Fred (51) investigated proteolysis by 22 strains of lactic acid bacteria. These workers used formation of non-protein nitrogen, amino

nitrogen and ammonia as criteria of proteolysis. Non-protein nitrogen was the most abundant form of nitrogen produced by these organisms growing in several different kinds of media. In general, only small quantities of ammonia were produced, and in some cases none at all. The lactic acid bacteria, in general, were not very proteolytic; very small increases in the respective protein breakdown products were obtained, and these increases in most cases required many days incubation.

The casein-splitting properties of starters have been studied by Barthel and Sadler (14). The amount of amino nitrogen produced by mixed lactic starter cultures was much greater than that produced by pure cultures of the streptococcus group. However, the amount of soluble nitrogen produced by both groups in milk at room temperature frequently was the same.

Hucker, in 1929, (37) noted that certain strains of streptococci had the ability to increase the amino nitrogen content of milk after prolonged incubation. Working with chemically pure casein as the only source of nitrogen, these strains of S. lactis did not produce visible growth if washed cells were used as an inoculum. If large amounts of unwashed cells were used as an inoculum, these streptococci usually produced visible growth.

Anderegg and Hammer (2) reported that some strains of S. lactis produced an increase in the soluble nitrogen of a skim milk culture medium. Addition of peptone decreased the

proteolysis, while addition of calcium carbonate increased it. Organisms causing proteolysis generally coagulated the milk quicker, but proteolysis was not due to the acidity developed.

Hammer and Patil (28) found that certain cultures of S. lactis were able to proteolyze milk, while others were not. This proteolysis was evident in 1.5 days without addition of calcium carbonate, but was more evident if calcium carbonate was added to the milk. Neither air supply nor addition of sterile lactic acid to the milk influenced the proteolysis. Addition of 0.1 per cent alanine to the milk slightly retarded proteolysis by S. lactis.

Janke and Holzer (40) reported that untreated cultures of S. lactis hydrolyzed casein more readily than did chloroform treated cultures. They concluded that non-liquefying organisms such as S. lactis have an endo-cellular proteinase which was able to attack the casein only after autolysis of the cell. They thought that this would make possible the ripening of cheese by degenerating cells of S. lactis.

Kelly (43), in determining the influence of certain lactic acid streptococci on the chemical changes in cheddar cheese during ripening, noted that, with the exception of the protein fraction soluble in water, little change in protein degradation occurred in the first 10 to 13 days. The increase in protein digestion at the end of 10 days was thought to be related to rapid destruction of the bacteria, since it was during this period that the lactose was destroyed and the bacteria (strepto-

cocci) made their last increase in numbers.

In a comparison of the influence of different starters on the quality of cheddar cheese, Hansen, Bendixen and Theophilus (30) found that flavor of cheese made with a pure culture of S. lactis was almost as good as that of cheese made with two commercial lactic starters, while the body and texture of the cheese made with the pure culture were superior to that made using the commercial starters.

The influence of the starter culture on the ripening process also was studied by Allen and Knowles (1), in 1934. In cheese made from milk having a very low bacterial count, a vigorous starter contributed materially to the cheese ripening process and was much more effective in this respect than a slow starter. The effect was not due to greater acid production, since the pH's of all cheeses studied were approximately the same.

Sherwood and Whitehead (59) found that the proteolytic powers of the various pure cultures of lactic streptococci employed by them as starters had but little influence on the rate of proteolysis in the resultant cheddar cheese. No connection was found between the numbers of streptococci present in the milk at the time of renneting and the rate of cheese ripening. These workers suggested that the rennet enzyme was the most important agent in cheese ripening, and that the most significant function of the streptococci was the formation of acid, which affected the rate of proteolysis

by the rennet enzyme.

In an extensive study of raw milk cheddar cheese, Freeman and Dahle (26) reported that the rate of proteolysis in cheddar cheese during ripening was directly related to the numbers of bacteria initially found in the cheese. However, these workers stated that the development of flavor in the same cheese was not related to the numbers of bacteria initially present. By raising the ripening temperature from 45° F. to 63° F., the rate of proteolysis in cheddar cheese could be increased 40 to 100 per cent. Maximum flavor score was reached more quickly at 63° F.

Protein metabolism and acid production by the lactic acid bacteria in milk were studied by Braz and Allen (17), in 1939. They noted that proteolysis was inhibited by high acidity. Neutralizing the acid by the addition of chalk eliminated the inhibitory effect of the acid and resulted in greater protein degradation. These workers stated that provision of additional quantities of amino acids in the form of yeast extract usually resulted in a tendency to stimulate the anabolic process, so that either smaller quantities of amino acids or negative values were obtained after growth of the cultures.

Extracts of Streptococcus cremoris and S. lactis were shown by Hansen (31) to stimulate the development of Lactobacillus casei and Betacoccus cremoris (Leuconostoc citrovorum). These extracts were obtained both by autolyzing

the cells under toluene and by disintegration of the cells by means of sonic oscillation. The extracts were sterilized by autoclaving before being tested. The extracts were found to be much more stimulatory than were the control suspensions of killed cells.

Peterson, Johnson and Price (52) showed the presence of proteolytic activity in a water extract of cheddar cheese. The optimum pH for casein hydrolysis by the cheddar cheese proteinase system appeared to be 5.0, while a secondary optimum occurred at pH 7.0 to 8.0. The proteolytic activity at pH 5.0 was increased in the presence of reducing agents, particularly cysteine hydrochloride. These workers reported, concurrently, on the proteinase content of raw milk cheddar cheese and pasteurized milk cheddar cheese during making and ripening (53). The active proteinase in ripening cheddar cheese was considered to be of bacterial origin, and only a small part of the total proteinase activity of cheddar cheese was contributed by the milk and rennet extract. Pasteurized milk cheddar cheese differed from raw milk cheddar cheese in that it contained a lower content of cysteine-activated proteinase.

Collins and Nelson (19) investigated the effect of S. lactis, E. coli and A. aerogenes growing singly and in combination on the soluble nitrogen of milk. S. lactis caused a rapid increase in the soluble nitrogen of skim milk during the first day or two of growth. This was followed

by a small, gradual increase which continued for at least 15 days. E. coli and A. aerogenes grown alone caused a deficit at first and then an increase in soluble nitrogen. Mixed cultures of S. lactis and coliform organisms caused increases in soluble nitrogen which were intermediate between the results of the component organisms.

In a review of the ripening process of hard cheese, Orla-Jensen (49) gives one of the most popular theories of this phenomenon. He suggested that ripening was caused by the proteolytic activity of the rennin combined with the proteolytic endo-enzymes of certain lactic acid bacteria. He states that: "Living bacteria have no direct influence because the liberation of their digestive enzymes in the surrounding medium takes place only after death and subsequent autolysis of the bacterial cells."

The Influence of Rennet and Other Enzymes of Non-Bacterial Origin on the Ripening of Cheddar Cheese

Babcock, Russell, Vivian, and Hastings (8), as early as 1899, recognized the importance of enzymes in the ripening process of cheddar cheese. These workers studied the enzyme galactase and stated that it more closely resembled the group of bacterial enzymes, rather than those enzymes of animal origin, insofar as the types of proteolytic decomposition products were concerned. These workers concluded that galactase played the principal role in the hydrolytic

degradation of cheese protein.

Babcock, Russell and Vivian (7) found that increasing the amount of rennet extract used increased the amount of soluble nitrogenous products, which measured the progress of cheese ripening. They also stated that the digestive action of rennet was due to action of pepsin contained therein and that the products of peptic digestion in milk and cheese were confined to the higher decomposition products, such as albumoses and peptones.

Van Slyke, Harding, and Hart (65), in a comparison of the ripening process in cheese made with chloroform and normal cheese made without chloroform, found that the total water-soluble nitrogen was greater in the cheese made without chloroform. However, the enzymes contained in the chloroformed cheese were able to accomplish about 72 per cent as much decomposition of the casein as occurred in the unchloroformed cheese.

Working with raw milk cheddar cheese, Babcock, Vivian and Baer (9) found that the use of 3 ounces of rennet per 1000 lbs. of milk gave better results than using 6 or 9 ounces of rennet per 1000 lbs. of milk, when the ripening temperature employed was 60° F. However, the use of larger amounts of rennet at lower temperatures gave excellent results. These workers found that at 50° F. the high-rennet cheese cured faster than the cheese made with 3 ounces of rennet, and the flavor was much improved. Also, when the cheese was

ripened at 50° F., or under, the flavor of cheese made with 9 ounces of rennet was of superior quality, even when a year old, and the body and texture of such cheese were excellent.

Van Slyke and Hart (67) found that two-thirds of the soluble nitrogen compounds occurring in cheddar cheese were formed during the first three months of ripening, and over 90 per cent in the first nine months of an 18 month ripening period. Their data also indicated that between the limits of 32 and 70° F., there was an increase of 0.5 per cent in soluble nitrogen compounds for a temperature increase of 1° F. Proteolysis generally was increased by incorporation of more moisture into cheese, and the effect of a high salt concentration was to retard formation of soluble nitrogen compounds. Increasing the amount of rennet used in cheese making resulted in the production of increased quantities of water-soluble forms of nitrogen, such as paranuclein, caseoses and peptones.

Van Slyke and his coworkers (16) heated milk to destroy all milk enzymes and added chloroform to the milk to render all microorganisms inactive. They found that the protein degradation products in cheese made from such milk were mainly caseoses and peptones. Both rennet and commercial pepsin were used as setting agents in the cheese manufacturing process.

Working in New Zealand, Riddet, McDowall and Valentine (54) found that a more rapid breakdown of cheese in the early stages of ripening occurred when 5 ounces of rennet was employed, as compared to 3 ounces of rennet per 1000 lbs. of milk. These

workers noted that cheese made with 5 ounces of rennet per 1000 lbs. of milk had a tendency to develop a sticky body, and any off flavors were more pronounced in the mature cheese.

However, Davies, Davis, Dearden and Mattick (20) concluded that variations in the amounts of pepsin and rennin were without effect on flavor, body, texture or ripening of cheddar cheese. They found that 36 p. p. m. of copper added to cheddar cheese produced an abnormal flavor and markedly inhibited the ripening process. This quantity of copper inhibited protein degradation, particularly the formation of amino acids.

In this connection, Barnicoat (13) reported that 3 and 7 p. p. m. of copper added to milk which subsequently was made into cheddar cheese resulted in a product which did not mature with desirable cheddar cheese flavor. The milk containing 3 and 7 p. p. m. of copper resulted in cheese having 31 and 78 p. p. m. of copper, respectively. In 1949, Stine, Loos and Daume (62) reported similar results in connection with copper-contaminated cheddar cheese. They reported that a concentration of copper in excess of 2 p. p. m. either prevented or retarded normal flavor development. Copper tended to remain largely in the cheese curd, with only a small per cent passing into the whey.

Pepsin, trypsin, erepsin and rennet, separately or in combination, were used by Wojtkiewicz and Inikhoff (71),

in an attempt to accelerate ripening in cheddar cheese. Pepsin gave no increase in ripening, but rennet and trypsin gave increased proteolysis. Trypsin caused bitterness. Rennet at the level of 0.01 per cent of the quantity of milk used gave most satisfactory results.

Some interesting observations on the enzymes of commercial rennet were made by Davis, Davies and Mattick (22) in a paper published in 1937. They stated that the peptidase of rennet was markedly inhibited by an acid reaction, whereas the proteinase was not. The proteinase was active at pH 4.0 to 6.0 but the peptidase, which had its optimum at about pH 6.0, was much less active at pH 4.0 to 5.0.

Sherwood (57) made an attempt to determine the relative importance of rennet in the ripening of cheddar cheese made from flash-pasteurized milk by eliminating the bacterial factor through the addition of chloroform to the milk used for cheese-making. The general course of protein degradation, as measured by the determination of total soluble nitrogen and non-protein nitrogen, was almost identical with that occurring in normal control cheese, and he thus concluded that rennet was the only important agent attacking cheese protein during the ripening process. In 1935, Sherwood (58) found that the use of pepsin as a substitute for rennet in cheddar cheese gave a smaller amount of protein degradation than was found in control rennet cheese. Sherwood observed no direct correlation between the extent or type of protein

degradation and the commercial quality of the cheese. He concluded that rennet played a most important role in degradation of cheese protein, and that the proteolytic and the coagulating action of rennet apparently were due to separate enzymes.

A very interesting investigation of the chemical substances likely to affect the growth and metabolism of bacteria or the activity of the rennet enzymes in relation to protein degradation in cheddar cheese was that carried out by Davies, Davis, Dearden, and Mattick (21). The individual addition to cheese of cystine, sodium and ammonium citrates, sodium bisulphite, magnesium sulphate, calcium chloride, ferric ammonium citrate or autolyzed yeast gave no striking increase in non-protein nitrogen. The addition of iodoacetic acid inhibited the lactic acid fermentation.

Freeman and Dahle (26) found that additional amounts of pure rennin increased the rate of proteolysis in ripening cheddar cheese and produced an aged cheese with a slightly higher flavor score. The addition of pepsin to cheese increased the rate of proteolysis during ripening and produced an aged product with higher flavor score. Trypsin increased the rate of proteolysis but decreased the maximum flavor score.

Hanson, Arbuckle and Shepardson (32) found that when 2, 4, and 8 ounces of rennet extract per 1000 lbs. of milk were used for making cheddar cheese, the 4 ounce quantity gave the best results. Cheese made using 8 ounces of rennet per 1000 lbs. of milk was superior in flavor at all ripening

temperatures for a 12 week period. However, the body of this cheese was weak and sticky compared with cheese made with 4 ounces of rennet and so was considered to be objectionable.

Some of the latest work to be reported in connection with rennet extract and its role in the ripening process of pasteurized milk cheddar cheese is that of Babel (10), in 1948. He stated that there was a direct relationship between the amount of rennet extract used to coagulate the milk and the degree of protein degradation during ripening of the cheese. He also found that the replacement of part of the rennet by calcium chloride in the manufacture of cheese was not a desirable practice, since this resulted in cheese which ripened more slowly and was organoleptically inferior to cheese made with a normal amount of rennet extract.

Methods for Estimating the Activity of Proteolytic Enzymes

The purpose of this section is to review some of the methods for measuring the activity of proteolytic enzymes. Obviously, the general subject of proteolytic enzymes in all its ramifications is of such scope as to preclude complete review. Consequently, only those methods which were of most interest to the author in connection with the investigation at hand will be mentioned.

Probably the most common means of classifying proteolytic enzymes is on the basis of the degree of complexity of their substrates. Thus, proteolytic enzymes have been divided into two large groups, the proteinases being those enzymes active against high molecular weight proteins and the peptidases being those enzymes capable of hydrolyzing peptides of various degrees of complexity. Bergmann (15) and his coworkers have classified the proteolytic enzymes as proteinases or peptidases depending on the specificity effect rendered by certain chemical groups in the side chains and in the "backbone" of the substrate. The methods under consideration might best be separated into two rather arbitrary groups depending upon whether they are used to determine proteinase activity or peptidase activity. Those methods which commonly have been employed for the estimation of proteinase activity will be reviewed first, although it should be kept in mind that some methods have been used to estimate the activity of both proteinases and peptidases.

Of the proteinases, pepsin and trypsin probably have been studied most. Of the many methods for the estimation of these enzymes, the one which seems to have gained most common acceptance is the hemoglobin method of Anson (4). In this method denatured hemoglobin is digested under standard conditions with enzyme. The undigested hemoglobin is precipitated with trichloroacetic acid, and the unprecipitated products of hydrolysis are estimated with the phenol

reagent of Folin and Ciocalteau (24), which gives a blue color with tyrosine and tryptophane.

One of the advantages claimed for this method is that hemoglobin is a reproducible substrate and different batches of it are digested at the same rate by a given proteinase solution, while casein and gelatin are not as uniform from lot to lot. Also, under the experimental conditions established by Anson, the enzymatic hydrolysis of hemoglobin is due to the action of proteinase alone, so far as is known.

Some of the reasons for the popularity of the above method are as follows: (1) It is quite capable of detecting, accurately, rather small amounts of proteolysis. (2) Highly reproducible results are obtained with it. (3) It is a relatively fast method. (4) It is highly adaptable.

In addition to the colorimetric procedure for the estimation of pepsin, Anson (48) developed a method for the estimation of pepsin with hemoglobin by means of a quartz spectrophotometer.

The hemoglobin method has been used for the estimation of a wide variety of proteinases. In addition to pepsin and trypsin, the method has been used for papain and cathepsin (4). In some adaptations of the hemoglobin method of Anson, the colorimetric estimation of tyrosine and tryptophane has not been followed rigorously, since the phenol reagent reacts to give a blue color with other substances such as cysteine. When such a color-producing material must be included in the

digestion mixture, the practice, with some workers, has been to use the nitrogen content of the trichloroacetic acid filtrate, as determined by the Kjeldahl method, as a means of analysis. The increase in the nitrogen content of a trichloroacetic acid filtrate as a measure of enzyme activity when using substrates other than hemoglobin has been used rather widely.

The work of Winnick, Davis and Greenberg (70) with the proteolytic enzyme from the latex of the milkweed is an example in which the techniques mentioned above were employed successfully. Measurement of proteolysis by estimating the amount of non-protein nitrogen by the Kjeldahl method, and the tyrosine content colorimetrically in a trichloroacetic acid filtrate both were used.

An adaptation of the hemoglobin method of Anson has been used by Balls and Lineweaver (12) to determine the proteolytic activity of crystalline papain. These workers allowed the trichloroacetic acid precipitated hemoglobin to stand for 30 minutes before filtering.

Keith, Kazenko, and Laskowski (42) used essentially the same method for the determination of the enzymatic activity of a crystalline protein from beef pancreas, except that casein was used as the substrate. Still another application of the method is the work of Hecht and Civin (36) with a microbial enzyme. These workers have shown the presence in

yeast of a pepsin with an optimum pH at 1.8, using the formation of tyrosine from hemoglobin to determine the activity of their enzyme preparation.

Other methods for following the rate of digestion of proteins by proteinases embrace the determination of the increase in carboxyl groups or amino nitrogen, the decrease in protein nitrogen, or the changes in viscosity of the enzyme-substrate reaction mixture.

Probably the most commonly employed methods for measuring amino compounds have been the Sørensen (60) formol titration and the Van Slyke (63) procedure. A very good adaptation of the formol titration is that of Brown (18), and his procedure has been widely used for measuring the activity of proteolytic enzymes.

As for the determination of peptidase activity, there are several reliable methods which have developed into more or less standard procedures for this purpose. The earliest of these methods was the aforementioned Sørensen (60) titration of liberated carboxyl groups after the addition of formaldehyde. Later, other methods for titration of carboxyl groups were worked out by Foreman (25), in 1920, and Willstätter and Waldschmidt-Leitz (69), in 1921. These methods involved titration of carboxyl groups with sodium hydroxide, using alcohol as solvent.

In 1927, Linderstrøm-Lang (44) reported on a titrimetric method for the determination of amino nitrogen which since

has been used rather widely. This method involved titrating the amino groups in amino acids or related compounds with alcoholic HCl, upon the addition of acetone, and using benzene-azo-alpha-napthylamine (napthyl red) as an indicator. The author pointed out that the amino acids which could not be titrated by this method included those in which the amino groups were weak bases, such as the aromatic amino acids, and those in which the acid groups existed as strong acids, such as the sulfonic acids. Linderstrøm-Lang and Holter (45) have developed a method for the direct microtitration of amino groups; the method involves the acidimetric, acetone principle.

Another titrimetric method which has been widely used is the micro-method of Grassmann and Heyde (27) for the estimation of amino acids and peptides. This method involves an alcoholic titration of carboxyl groups, using thymolphthalein as an indicator.

In connection with the above methods, Van Slyke and Kirk (64) have presented a very excellent discussion of the theory of amino acid titrations, as well as a comparison of gasometric, colorimetric, and titrimetric determinations of amino nitrogen.

PART 1

A STUDY OF THE ENZYMES OF RENNET AND THEIR RELATION TO THE RIPENING PROCESS OF PASTEURIZED MILK CHEDDAR CHEESE

METHODS

Manufacture of Cheese

All of the experimental cheese was manufactured in the small 450-pound experimental cheese vats in the Dairy Industry Department. The milk was mixed herd milk, standardized to 3.4 to 3.6 per cent fat when necessary, and pasteurized by the holder method at 143° for 30 minutes. This milk was obtained and processed, held overnight in a storage cooler and used for cheesemaking the following morning.

In practically all cases, approximately 260 lbs. of milk per vat were used, and the method of Wilson (68) was followed quite closely for manufacture of the cheese. The milk was placed in the vats and heated to 86° F., a 1.0 per cent inoculum of S. lactis culture no. A-1 was added as starter, and the milk was ripened until an acidity increase of 0.01 to 0.02 per cent had occurred. This amount of starter culture gave approximately 0.50 per cent whey acidity 4.5 hours after setting. Annatto color was added at the rate of 30 ml. per 1000 lbs. of milk. The milk was set employing the experimental quantities of rennet or other enzymes as designated in the various experiments.

The curd was cut with one-quarter inch curd knives approximately 30 minutes after the addition of rennet. This time varied somewhat when using different quantities of rennet, and when larger quantities of rennet were used in setting the milk the curd could be cut in much shorter periods of time.

After cutting, the curd was stirred slowly for 15 minutes before cooking was started. The temperature was raised to 102° F. in 30 to 40 minutes. The curd was stirred and held at this temperature until an acidity increase of 0.025 to 0.030 per cent had been obtained and until the proper firmness of curd was obtained. The whey was drained from the curd, which then was piled about 10 inches high in one end of the vat and allowed to mat, with turning at 15 minute intervals for the duration of the cheddaring process. Milling took place when the whey acidity was approximately 0.50 per cent. After milling, the curd was forked by hand for 15 minutes and then salted at the rate of 2.5 per cent salt.

After salting, the curd was packed in longhorn cheese hoops holding approximately 13 lbs. per hoop. The hoops were placed in a hydraulic cheese press for 1 hour, removed from the press for dressing, and then replaced in the press where they remained for about 16 hours. When removed from the press, the cheeses were taken out of the hoops, dried for 3 to 4 days at 43° F., paraffined and placed in the curing rooms at either 43 or 50° F., as designated.

All rennet extract employed in this investigation was furnished through courtesy of Chr. Hansen's Laboratory, Inc., Milwaukee, Wisconsin. Several series of cheese were made (Tables 12, 13, 14 and 15) employing rennet extract, rennet powders and rennin. The rennet powder which is referred to as powder A was Hansen's commercial rennet powder. One gram of this powder was approximately equivalent to 7 ml. of Hansen's commercial rennet extract in milk-coagulating power. Powder B was Hansen's special rennet powder, 1 g. of which was approximately equivalent in its coagulating activity against milk to 46.5 ml. of rennet extract. In the manufacture of cheese these enzymes were used in quantities of equal coagulating power insofar as their abilities to coagulate mixed herd, pasteurized milk at a setting temperature of 86° F. were concerned.

Analyses of Cheddar Cheese

Analyses of the cheese for total nitrogen, soluble nitrogen, per cent moisture and pH were made at the intervals indicated in the tables.

Moisture

The moisture content of the cheese was determined using a Brabender semi-automatic moisture tester. A 10 g. sample of cheese was weighed into a test pan sitting on a torsion balance and the pan and sample transferred to the heating chamber of the Brabender apparatus. After heating for 50 minutes at 140° C.,

the per cent moisture in the sample was read directly on the scale. Heating was continued until two successive readings taken at 5 minute intervals checked with a difference of less than 0.2 per cent. The second of these two readings was recorded as the moisture content of the sample.

pH

The pH of the cheese was determined according to the method of Sanders (55) for the determination of pH of semi-plastic materials. A small portion of each cheese being analyzed was mixed well with a small amount of quinhydrone. The cheese-quinhydrone mixture was placed in the hollow of a small watch glass. A one inch portion of an ordinary cellophane drinking straw then was tamped full of the cheese-quinhydrone mixture, and the platinum needle of a stab-type electrode was inserted longitudinally through the cheese mixture. The pH was measured potentiometrically, using a saturated calomel reference cell.

Nitrogen

For analytical purposes, the cheese was sampled by removing two or more plugs taken diagonally from the top or bottom of the longhorn. These plugs were placed immediately in sample jars and removed to the laboratory. If the samples were to be held longer than 10 to 20 minutes, they were placed in a cold room in order to minimize moisture loss.

The cheeses were analyzed for total nitrogen according to the Kjeldahl-Gunning-Arnold method (6). About 1.5 g. of cheese was weighed in a weighing bottle on an analytical balance and transferred quantitatively to a 500 ml. Kjeldahl flask. Ten grams of sodium sulfate, 0.2 g. of copper sulphate and 25 ml. of concentrated sulfuric acid were added, and the mixture was digested until clear. After cooling, 200 ml. of distilled water and sufficient NaOH solution (450 g./l.) to make the digestion mixture alkaline were added in that order. The ammonia was distilled into 0.1 N HCl, and the excess acid titrated with 0.1 N NaOH solution.

The following procedure to determine water-soluble nitrogen was that used by Babel (10). A 25 g. sample of cheese was weighed quickly into a 50 ml. beaker. This sample was transferred to a Waring-type blendor with 240 ml. of distilled water and the cheese blended for a period of 10 minutes. At the end of this period the cheese suspension was transferred to a 300 ml. Erlenmeyer flask and digested for 1 hour in a water bath at 50° C. At 15 minute intervals the flask was removed from the bath and agitated by hand for several seconds to mix and redisperse the suspension. After 1 hour the flask was removed from the bath and the contents immediately filtered through a fluted Whatman no. 12 filter paper. The filtrate containing the water-soluble nitrogen compounds of the cheese was analyzed for nitrogen content by placing 25 ml. in a 500 ml. Kjeldahl flask and following the Kjeldahl-Gunning-Arnold method for determination of nitrogen

as described previously.

The per cent soluble nitrogen in the cheese was calculated on the basis of a 2.5 g. sample of cheese, 25 ml. being taken for analysis from an original 250 ml. volume containing 25 g. of cheese.

Organoleptic Examination and Scoring of Cheese

All of the cheese for which score data are presented were examined organoleptically at the intervals designated in the tables. The same two qualified judges examined all cheese, and, in addition to the author, a third or fourth individual occasionally rendered assistance in the evaluation of the cheese. The standard cheese score card which gave 45 points for flavor and 30 points for body and texture was used.

In general, the judges placed emphasis in scoring the cheese not so much on relative flavor development, or intensity of cheddar cheese flavor, but rather on defects in flavor and body. Consequently, it should be kept in mind that a mild cheese possessing no defects might very well score as high as a cheese with a larger amount of typical cheddar cheese flavor which also was lacking in defects.

Preparation of Pure Crystalline Rennin

The procedure of Hankinson (29) for the preparation and purification of rennin was followed. The pH of 1 gallon of

commercial rennet extract was adjusted to 5.0 with concentrated HCl. Enough NaCl was added to the rennet extract to saturate the material and the solution was allowed to stand at room temperature (about 22° C.) for 18 to 24 hours. The material which was salted out was thrown down by centrifuging at 3500 r. p. m. for 15 to 20 minutes. The supernatant liquid was decanted, and the precipitate was recovered and suspended in distilled water. The volume was made up to one-half of the volume of the original rennet extract. The reaction of this suspension was adjusted to pH 5.7 to 6.0, whereupon the precipitated material went into solution. The pH adjustment, salting out and recovering the rennin material, was repeated until the fourth precipitate was obtained.

The fourth precipitate was dialyzed for 24 hours in a cellophane membrane against distilled water. The dialyzed suspension was diluted with distilled water so as to obtain approximately 0.05 per cent solids concentration in the solution. The pH was adjusted to 5.7 to 6.0 with 0.1 N HCl, and the solution was filtered through Whatman no. 2 filter paper, using suction.

The rennin was crystallized from solution by adjusting the pH slowly with 0.1 N HCl. The solution was stirred slowly until the first definite turbidity appeared. The solution then stood about 10 minutes and the pH was decreased 0.1 unit by the addition of more acid. The crystallization process was continued by the addition of more acid at approximate

10 minute intervals until a heavy white turbidity was obtained. This suspension stood 3 hours at room temperature, and the crystallized material was recovered by centrifuging. The crystalline rennin was dissolved in 100 ml. of distilled water at pH 5.8 and preserved in the cold in the presence of a small amount of toluene.

Measurement of Relative Coagulating Activities of Rennet Extract, Rennet Powders and Rennin

Determination of Coagulating Activity

The various enzymes were tested for their relative coagulating activities by using as a substrate mixed herd milk. (3.4 to 3.5 per cent fat) which had been pasteurized and homogenized. During the course of the experiments, the pH of this milk approximated 6.60 to 6.70, measured by the glass-electrode potentiometer, and varied but slightly from day to day.

To determine coagulating activity, a 10 ml. sample of milk was placed in a 20 x 175mm. pyrex test tube and brought to 37° C. in a water bath. One ml. of suitably diluted enzyme solution was added, the contents of the tube mixed, and the tube returned to the water bath. For purposes of comparison, a standard enzyme solution was adopted. This standard enzyme solution was a dilution of enzyme in distilled water such that a 1 ml. quantity of it would just coagulate 10 ml. of the standard milk substrate in 10 minutes at 37° C. To test for

coagulation, the test tube containing milk and coagulating enzyme was tipped on its side and then held upright, allowing a film of milk to flow down the side of the tube. Looking through this film at a light source, it was possible to tell when the milk coagulated, as the film of milk broke into many discrete particles.

The above conditions for measuring coagulating activities were chosen in order to reproduce insofar as possible the conditions existing in a vat of milk at the time of addition of rennet extract. Probably the only experimental condition which was significantly different was the temperature employed. This was 37° C. in the laboratory, as compared with a setting temperature of 30° C. used in the manufacture of cheddar cheese.

Nitrogen Content and Salt-Free Dry Weight of Enzyme Preparations

Nitrogen determinations were performed on the rennet extract and solutions of rennet powders and rennin according to the Kjeldahl-Gunning-Arnold method, as described in the section on analysis of cheese. A 10 ml. sample usually was taken for analysis, although this varied with the concentration of the enzyme solution being tested.

Dry weight determinations also were carried out on these enzyme solutions. Using an analytical balance, 10 and 20 ml. quantities of the enzyme solutions were weighed into the weighing pan of a Brabender semi-automatic moisture tester. The pan was placed in the heating chamber, with the thermostat of the tester set at 100° C., and the sample was dried to a

constant weight. The pan and the residue were removed from the machine, cooled in a dessicator and weighed.

Since the enzymes being tested contained varying quantities of sodium chloride, total chloride determinations were carried out on the residues remaining after the drying operation. After the final weighing of the pan containing residue, approximately 25 ml. of distilled water were added to the pan and left to stand overnight at room temperature. The following day the contents of the pan were filtered through a Whatman no. 12 filter paper into a 300 ml. Erlenmeyer flask. The pan was rinsed with about 25 ml. of distilled water which was passed through the filter. The filter then was washed with 10 to 15 ml. of distilled water. In the case of rennet extract, the filtrate was made up to a volume of 100 ml. with distilled water, and a 10 ml. aliquot was taken for titration with silver nitrate. Since other enzymes contained much less salt, the filtrates were titrated directly. The chlorides present in the filtrate were titrated to a brick-red color with silver nitrate solution (1 ml. = 0.01 g. NaCl), using potassium chromate as an indicator. The results were expressed as parts by weight of the original enzyme solution as sodium chloride.

Calculation of Coagulating Activity

Knowing the concentration of enzyme present in the standard solution, and employing the data obtained in the above manner for nitrogen content and salt-free residue it was

possible to determine the relative coagulating activities of the separate enzymes. These activities have been expressed on a dry weight basis and a unit weight of nitrogen basis with commercial rennet extract considered to have an activity of unity in each case.

Measurement of Proteolytic Activity of Rennet Extract, Rennet Powders and Rennin

Preparation of Hemoglobin Substrate

For the preparation of the hemoglobin substrate, the amount of dry protein (Merck) required to make 1 liter of a 2.5 per cent solution was weighed and dispersed in about 100 ml. of distilled water in an Erlenmeyer flask. This suspension was transferred to cellophane tubing (0.75 inch diameter) and dialyzed for 24 to 36 hours at about 1.5° C. against distilled water. The hemoglobin suspension was washed from the cellophane tubing with distilled water. The suspension was made up to a volume containing slightly more than the desired amount of protein in solution. This hemoglobin preparation stood 2 to 3 hours at room temperature and then was centrifuged in an angle-head centrifuge for 5 minutes at 3000 r. p. m. The supernatant liquid containing hemoglobin in solution was decanted into a suitable glass container, and 1 mg. of merthiolate was added as a preservative to 40 ml. of solution.

A solids determination was carried out by weighing 20 ml. of the protein solution into the test pan of a Brabender semi-automatic moisture tester, using an analytical balance. The pan was placed in the heating chamber of the tester and heated

at 100° C. until the greater part of the moisture had been removed from the sample. The thermostat of the tester then was set at 140° C. and the sample dried to a constant weight. The pan with residue was removed from the machine, cooled in a dessicator and reweighed. The weight of hemoglobin per unit volume of solution was determined, and distilled water to make a stock solution containing 2.5 g. of hemoglobin per 100 ml. of solution was added to the solution obtained by centrifuging.

Preparation of Casein Substrate

Casein also was used to measure the proteolytic activity of the rennet enzymes. The casein solution was prepared by suspending the required amount of casein (Fisher, washed from milk) to make a 2 per cent solution in distilled water in an Erlenmeyer flask. The suspension was adjusted to approximately pH 10 by the addition of 1 N NaOH, and was heated in an Arnold steamer for a period of 10 to 15 minutes. The contents of the flask were swirled from time to time and the casein usually was completely in solution in about 15 minutes. The hot casein solution immediately was filtered through Whatman no.2 filter paper, using a large Buchner funnel and employing suction. After filtration, the solution was allowed to cool, the volume was checked and made up with distilled water if necessary. The pH of the solution was adjusted to 7.0 with 5 N sulfuric acid, and merthiolate was added as preservative at the rate of 1 mg. per 40 ml. of

solution. This solution was stored in a cold room at about 1.5° C. and used as needed. Solutions of a lesser concentration were made by dilution of this 2 per cent solution.

Colorimetric Determination

For the measurement of proteolysis by the rennet enzymes, a 5 ml. quantity of 0.8 per cent casein or 2.5 per cent hemoglobin solution was pipetted into a clean, dry 20 x 175 mm. pyrex test tube. One ml. of distilled water or a combination of hydrochloric acid or sodium hydroxide and distilled water to adjust the substrate to the desired pH was added, and 0.5 ml. of a composite buffer solution which was 0.5 M in acetate, 0.5 M in phosphate and 0.5 M in borate was added to make a volume of 6.5 ml. Aliquot stock solutions of the composite buffer solution were prepared and were adjusted with 5 N NaOH or HCl to pH values from 1.5 to 7.0.

Ten minutes prior to addition of enzyme the tubes containing substrate were brought to 37° C. in a water bath. Enzyme solution in a volume of 1 ml. was added and the tubes agitated for several seconds. The tubes were stoppered and replaced in the bath, where they remained for the duration of the reaction period. At the end of the reaction period, the tubes were removed from the bath, and 10 ml. of 0.4 N trichloroacetic acid was added to each tube from a burette.

After the addition of trichloroacetic acid, the reaction tubes were allowed to stand at room temperature for 1 hour

when hemoglobin was employed as substrate or 2 hours when casein was the substrate being used. Following this, the reaction tubes were placed in an angle-head centrifuge, and the trichloroacetic acid-precipitated protein was thrown down by centrifuging at 3500 r. p. m. for 15 minutes. Five ml. of the supernatant liquid was pipetted into a 125 ml. Erlenmeyer flask. Ten ml. of 0.5 N NaOH was added from a burette followed by 3 ml. of the phenol reagent of Folin and Ciocalteau (24) diluted 1:2 with distilled water. During the addition of the phenol reagent, the solution in the flask was swirled, and the rate of addition of reagent was standardized by adding the reagent as rapidly as possible and still have it come out of the burette as drops. The blue color which developed was read in a Klett-Summerson photo-electric colorimeter, using a filter having peak transmission at 600 millimicrons.

Blank determinations were carried out in the manner described above except that the corresponding quantity of enzyme was not added to the substrate until immediately after the addition of trichloroacetic acid.

Construction of Standard Curve and Expression of Enzyme Activity

Enzyme activity has been expressed as milliequivalent quantities of tyrosine present in 5 ml. of the trichloroacetic acid supernatant solution after 30 minutes digestion at 37° C.

In order to translate colorimeter readings into milliequivalent quantities of tyrosine, it was necessary to prepare a standard tyrosine curve. The tyrosine used was l (-) tyrosine (Merck), recrystallized and dried. A sample of this tyrosine which was checked for purity by determining its nitrogen content by the Kjeldahl-Gunning-Arnold method (6) was found to contain 98.8 per cent of the calculated theoretical quantity of nitrogen. The standard tyrosine solution contained 0.0008 milliequivalents of tyrosine per ml. of 0.1 N HCl, with 0.5 per cent formaldehyde added as preservative. Aliquots of the standard tyrosine solution were diluted with 0.1 N HCl so as to contain from 0.0001 to 0.0007 milliequivalents of tyrosine per ml. and used in construction of standard curves. One ml. quantities of these tyrosine-containing solutions were added to 5 ml. quantities of the supernatant liquid remaining after the centrifugation of a trichloroacetic acid precipitated blank substrate solution. The color was developed in the manner as stated, and the amount of color due to the added tyrosine was determined by comparing it with similar tests in which 1 ml. of distilled water was added in place of the 1 ml. quantity of tyrosine-containing solution.

The color values obtained in this manner were plotted as ordinates against the respective milliequivalent quantities of tyrosine as abscissas. It was found that the plotted points fell on very nearly a straight line. Fig. 5, page 74,

is representative of a number of curves obtained in this manner during the course of the experimentation.

In order to express enzyme activity in terms of milliequivalents of tyrosine, the difference in color values between an active enzyme determination and its corresponding blank was determined, and the value in milliequivalents of tyrosine corresponding to this color value was taken from the standard curve.

RESULTS

Effect of Different Quantities of Added Rennet Extract on Cheddar Cheese Made from Pasteurized Milk

Analyses of Cheese

Tables 1 to 9, inclusive, contain data for cheese made with different quantities of rennet extract.

Moisture

There was no evidence that the use of abnormally large quantities of rennet extract in the manufacture of cheddar cheese resulted in the incorporation of more moisture than found in cheese made with the normal amount of rennet extract. Within any one series of cheese the moisture content was quite constant. The majority of cheese contained from 37 to 39 per cent moisture. In a 180 day ripening period at 43° F., the paraffined cheese lost from 1 to 2 per cent of its weight through loss of moisture. In the case of the six series of cheese examined at a period in excess of 400 days, losses up to 5 per cent were noted, although 2 to 3 per cent losses were much more common.

pH

Although there were variations in the pH values of all cheese examined, the pH values among the cheese of any one

Table 1

The effect of different quantities of added rennet on the soluble nitrogen values and scores of cheddar cheese ripened at 43° F.

(Series A; made 8-20-46)

Cheese No.	Rennet Added (oz.)*	Age (days)	pH	Moisture (%)	Sol.N (% of Tot.N.)	Flavor Score	Remarks	Body, Text. Score	Remarks
A-1	1.5	42	4.95	37.4	18.1				
A-2	3.0	42	4.98	37.8	19.9				
A-3	5.0	42	5.00	36.9	23.0				
A-4	7.0	42	4.99	37.1	25.3				
A-1	1.5	90	4.97	37.0	19.7				
A-2	3.0	90	4.96	37.2	22.1				
A-3	5.0	90	4.95	36.3	23.8				
A-4	7.0	90	4.96	37.0	32.4				
A-1	1.5	180	4.97	36.5	23.6				
A-2	3.0	180	4.96	36.2	28.5				
A-3	5.0	180	4.98	36.4	36.8				
A-4	7.0	180	4.99	36.6	44.3				
A-1	1.5	510	5.11	34.8	38.4	38.0	Sl.unclean	28.5	Sl.mealy
A-2	3.0	510	5.06	34.4	40.8	37.5	Flat,Sl.bitter	28.0	Mealy,Sl.corky
A-3	5.0	510	5.12	32.3	47.8	37.5	Sl.bitter	28.0	Pasty
A-4	7.0	510	5.19	35.5	50.2	37.0	Fruity	27.5	Pasty

*Ounces of rennet extract per 1000 lbs. of milk.

Table 2

The effect of different quantities of added rennet on the soluble nitrogen values and scores of cheddar cheese ripened at 43° F.

(Series B; made 8-26-46)

Cheese No.	Rennet Added (oz.)*	Age (days)	pH	Moisture (%)	Sol.N. (% of Tot.N.)	Flavor Score	Remarks	Body, Text. Score	Remarks
B-1	2	42	5.09	37.8	18.7				
B-2	4	42	5.11	38.8	23.6				
B-3	6	42	5.04	38.4	24.0				
B-4	8	42	5.05	37.8	27.7				
B-1	2	90	4.93	37.7	15.7				
B-2	4	90	4.97	38.5	19.9				
B-3	6	90	4.97	38.5	27.6				
B-4	8	90	5.03	37.6	26.8				
B-1	2	180	4.91	37.7	26.1				
B-2	4	180	4.98	38.4	31.1				
B-3	6	180	4.98	38.2	37.4				
B-4	8	180	5.03	37.9	40.0				
B-1	2	510	5.11	35.3	39.2	39.0		29.5	
B-2	4	510	5.15	36.9	41.7	38.5	Sl.acid	28.5	Sl.weak
B-3	6	510	5.08	37.1	52.4	37.5	Sl.acid	28.0	Weak,pasty
B-4	8	510	5.17	35.3	55.6	37.5	Acid	27.5	Weak,pasty

*Ounces of rennet extract per 1000 lbs. of milk

Table 3

The effect of different quantities of added rennet on the soluble nitrogen values and scores of cheddar cheese ripened at 43° F.

(Series C; made 8-27-46)

Cheese No.	Rennet Added (oz.)*	Age (days)	pH	Moisture (%)	Sol.N. (% of Tot.N.)	Flavor Score	Remarks	Body, Text. Score	Remarks
C-1	1.5	42	4.99	39.7	21.9				
C-2	3.0	42	4.97	39.8	24.7				
C-3	5.0	42	4.90	39.1	28.8				
C-4	7.0	42	4.96	39.3	30.3				
C-1	1.5	90	4.99		20.6				
C-2	3.0	90	5.00		26.2				
C-3	5.0	90	4.98		29.6				
C-4	7.0	90	5.01		33.4				
C-1	1.5	180	5.10	38.6	29.1				
C-2	3.0	180	5.12	37.9	30.0				
C-3	5.0	180	5.07	36.7	37.8				
C-4	7.0	180	5.05	37.5	40.0				
C-1	1.5	503	5.21	36.7	44.7	38.5		29.5	
C-2	3.0	503	5.19	36.9	50.8	38.0	Sl.acid	28.5	Sl.weak
C-3	5.0	503	5.10	36.9	57.2	38.0	Sl.acid	28.0	Weak,pasty
C-4	7.0	503	5.17	37.0	57.3	37.0	Sl.acid,fruity	27.5	Weak,pasty

*Ounces of rennet extract per 1000 lbs. of milk.

Table 4

The effect of different quantities of added rennet on the soluble nitrogen values and scores of cheddar cheese ripened at 43° F.

(Series D; made 9-16-46)

Cheese No.	Rennet Added (oz.)*	Age (days)	pH	Moisture (%)	Sol.N. (% of Tot.N.)	Flavor Score	Remarks	Body, Text. Score	Remarks
D-1	2	42	4.85	37.1	14.0				
D-2	4	42	4.78	38.3	20.9				
D-3	6	42	4.87	38.4	24.2				
D-4	8	42	4.92	38.7	27.9				
D-1	2	90	4.88	35.9	18.5				
D-2	4	90	4.79	36.7	26.3				
D-3	6	90	4.90	37.1	30.6				
D-4	8	90	4.89	37.7	33.1				
D-1	2	180	5.00	36.9	24.2				
D-2	4	180	4.84	36.5	33.3				
D-3	6	180	4.94	37.0	41.8				
D-4	8	180	4.93	36.9	40.5				
D-1	2	480	5.05	35.1	39.3	38.0	Sl.acid,feed	28.5	Weak,Sl.mealy
D-2	4	480	4.99	35.4	49.5	37.0	Fruity,Sl.bitter	27.5	Mealy,Sl.corky
D-3	6	480	4.98	35.6	56.2	37.5	Sl.fermented	28.0	Pasty
D-4	8	480	5.08	35.9	57.2	37.0	Sl.fermented	27.5	Pasty

*Ounces of rennet extract per 1000 lbs. of milk.

Table 5

The effect of different quantities of added rennet on the soluble nitrogen values and scores of cheddar cheese ripened at 43° F.

(Series E; made 12-23-46)

Cheese No.	Rennet Added (oz.)*	Age (days)	pH	Moisture (%)	Sol.N. (% of Tot.N.)	Flavor Score	Remarks	Body, Text. Score	Remarks
E-1	1.5	42	4.96	39.6	11.2				
E-2	3.0	42	4.98	41.0	15.1				
E-3	5.0	42	5.04	39.4	22.3				
E-4	7.0	42	5.05	39.6	18.9				
E-1	1.5	90	5.17	38.5	20.6				
E-2	3.0	90	5.12	40.7	26.7				
E-3	5.0	90	5.13	39.0	31.2				
E-4	7.0	90	5.25	38.4	29.2				
E-1	1.5	180			23.4				
E-2	3.0	180			29.2				
E-3	5.0	180			34.7				
E-4	7.0	180			33.1				
E-1	1.5	420	5.12	36.9	29.5	38.5		29.0	
E-2	3.0	420	5.16	36.2	39.9	37.0	Fruity,musty	28.0	Weak
E-3	5.0	420	5.20	36.9	41.4	37.5	Fruity	27.5	Weak
E-4	7.0	420	5.26	37.4	42.4	37.5	Fruity	27.5	Weak

*Ounces of rennet extract per 1000 lbs. of milk.

Table 6

The effect of different quantities of added rennet on the soluble nitrogen values and scores of cheddar cheese ripened at 43° F.

(Series F; made 12-27-46)

Cheese No.	Rennet Added (oz.)*	Age (days)	pH	Moisture (%)	Sol.N. (% of Tot.N.)	Flavor Score	Remarks	Body, Text. Score	Remarks
F-1	2	42	4.95	40.1	17.9				
F-2	4	42	4.94	39.1	18.8				
F-3	6	42	4.96	39.7	20.2				
F-4	8	42	4.97	39.8	24.1				
F-1	2	90	4.98	39.6	20.5				
F-2	4	90	4.96	38.9	22.9				
F-3	6	90	5.02	39.2	25.5				
F-4	8	90	5.04	39.8	30.3				
F-1	2	200	5.01	37.4	23.9				
F-2	4	200	4.99	37.2	29.2				
F-3	6	200	5.03	36.7	31.7				
F-4	8	200	5.09	37.9	37.1				
F-1	2	420	5.09	35.2	33.4	38.0	Sl.acid	27.0	Weak
F-2	4	420	5.07	34.0	41.9	37.5	Sl.acid,fruity	26.5	Weak
F-3	6	420	5.12	34.7	50.1	39.0	Sl.fruity	27.5	Weak,Sl.mealy
F-4	8	420	5.20	36.2	51.8	37.5	Sl.bitter,fruity	27.0	Weak

*Ounces of rennet extract per 1000 lbs. of milk.

Table 7

The effect of different quantities of added rennet on the soluble nitrogen values and scores of cheddar cheese ripened at 43° F. and 50° F.

(Series G; made 12-17-48)

Cheese No.	Rennet Added (oz.)*	Age (days)	pH	Moisture (%)	Sol.N. (% of Tot.N.)	Flavor Score	Remarks	Body, Text. Score	Remarks
				Ripening Temp. 43° F.					
G-1	2	7	4.83	36.9	7.1				
G-2	4	7	4.88	37.5	9.1				
G-3	6	7	4.94	36.2	12.6				
G-4	8	7	4.90	37.4	11.1				
G-1	2	42	4.83		13.8				
G-2	4	42	4.90		18.5				
G-3	6	42	4.92		20.9				
G-4	8	42	4.92		23.0				
G-1	2	90	4.85		16.6	37.0	Acid,Sl.ferm.	28.0	Mealy
G-2	4	90	4.92		22.3	38.0	Acid	28.0	Weak
G-3	6	90	4.95		26.9	37.5	Sl.fermented	29.0	
G-4	8	90	4.93		27.9	37.5	Fermented	28.5	Sl.weak
G-1	2	180	4.90	35.0	25.7	37.0	Acid	27.0	Weak
G-2	4	180	4.94	35.6	31.0	37.0	Acid	27.5	Weak
G-3	6	180	5.04	35.2	34.0	37.5	Acid,Sl.bitter	27.0	Weak
G-4	8	180	5.00	35.4	37.1	36.5	Acid,Sl.bitter	27.0	Weak,mealy
				Ripening Temp. 50° F.					
G-1	2	7	4.95	37.0	7.2				
G-2	4	7	4.92	37.0	10.8				
G-3	6	7	4.97	36.1	13.2				
G-4	8	7	5.01	36.5	13.7				
G-1	2	42	4.90		14.3				

G-2	4	7	4.88	37.5	9.1				
G-3	6	7	4.94	36.2	12.6				
G-4	8	7	4.90	37.4	11.1				
G-1	2	42	4.83		13.8				
G-2	4	42	4.90		18.5				
G-3	6	42	4.92		20.9				
G-4	8	42	4.92		23.0				
G-1	2	90	4.85		16.6	37.0	Acid,Sl.ferm.	28.0	Mealy
G-2	4	90	4.92		22.3	38.0	Acid	28.0	Weak
G-3	6	90	4.95		26.9	37.5	Sl.fermented	29.0	
G-4	8	90	4.93		27.9	37.5	Fermented	28.5	Sl.weak
G-1	2	180	4.90	35.0	25.7	37.0	Acid	27.0	Weak
G-2	4	180	4.94	35.6	31.0	37.0	Acid	27.5	Weak
G-3	6	180	5.04	35.2	34.0	37.5	Acid,Sl.bitter	27.0	Weak
G-4	8	180	5.00	35.4	37.1	36.5	Acid,Sl.bitter	27.0	Weak,mealy
				Ripening Temp. 50° F.					
G-1	2	7	4.95	37.0	7.2				
G-2	4	7	4.92	37.0	10.8				
G-3	6	7	4.97	36.1	13.2				
G-4	8	7	5.01	36.5	13.7				
G-1	2	42	4.90		14.3				
G-2	4	42	4.95		21.3				
G-3	6	42	4.95		21.3				
G-4	8	42	4.98		22.1				
G-1	2	90	4.92		18.2	37.5	Acid,Sl.bitter	28.0	Mealy
G-2	4	90	4.95		23.7	38.0	Acid,Sl.bitter	28.0	Mealy
G-3	6	90	4.95		22.4	38.5	Sl.fermented	28.5	Mealy
G-4	8	90	4.98		30.3	38.0	Sl.bitter	28.0	Weak
G-1	2	180	4.90	33.1	25.6	37.5	Acid,bitter	27.0	Mealy,flaky
G-2	4	180	4.97	34.9	30.5	37.5	Bitter	27.0	Weak,mealy
G-3	6	180	4.98	34.2	35.0	38.0	Sl.bitter	27.5	Weak
G-4	8	180	4.98	33.8	36.5	37.0	Bitter	27.5	Mealy

*Ounces of rennet extract per 1000 lbs. of milk.

Table 8

The effect of different quantities of added rennet on the soluble nitrogen values and scores of cheddar cheese.

(Series H; made 12-20-48)

Cheese No.	Rennet Added (oz.)*	Age (days)	pH	Moisture (%)	Sol.N. (% of Tot.N.)	Flavor Score	Remarks	Body, Text. Score	Remarks
				Ripening Temp. 43° F.					
H-1	2	7	5.01	35.6	14.8				
H-2	4	7	5.10	35.1	13.5				
H-3	6	7	5.12	36.4	14.5				
H-4	8	7	5.06	35.0	14.7				
H-1	2	42	5.03		16.4				
H-2	4	42	5.12		16.1				
H-3	6	42	5.11		18.2				
H-4	8	42	5.10		21.9				
H-1	2	90			20.8	38.5	Flat	28.5	Open,acid
H-2	4	90			19.8	38.5	Acid	29.0	
H-3	6	90			23.8	39.5	Feed	29.0	
H-4	8	90			27.4	39.5	Feed	29.0	
H-1	2	180	5.12	35.2	27.9	37.0	Acid,Sl.bitter	27.0	Mealy
H-2	4	180	5.12	34.5	30.4	37.0	Acid	27.5	Weak
H-3	6	180	5.18	34.0	34.1	37.5	Acid,Sl.bitter	27.0	Weak
H-4	8	180	5.30	34.1	37.0	36.5	Acid,bitter	27.0	Mealy
				Ripening Temp. 50° F.					
H-1	2	7	4.99	34.5	14.9				
H-2	4	7	4.99	35.0	14.7				
H-3	6	7	5.06	35.2	14.8				
H-4	8	7	5.07	35.8	12.3				
H-1	2	42	5.01						
H-2	4	42	5.03						

H-3	6	7	5.12	36.4	14.5				
H-4	8	7	5.06	35.0	14.7				
H-1	2	42	5.03		16.4				
H-2	4	42	5.12		16.1				
H-3	6	42	5.11		18.2				
H-4	8	42	5.10		21.9				
H-1	2	90			20.8	38.5	Flat	28.5	Open,acid
H-2	4	90			19.8	38.5	Acid	29.0	
H-3	6	90			23.8	39.5	Feed	29.0	
H-4	8	90			27.4	39.5	Feed	29.0	
H-1	2	180	5.12	35.2	27.9	37.0	Acid,Sl.bitter	27.0	Mealy
H-2	4	180	5.12	34.5	30.4	37.0	Acid	27.5	Weak
H-3	6	180	5.18	34.0	34.1	37.5	Acid,Sl.bitter	27.0	Weak
H-4	8	180	5.30	34.1	37.0	36.5	Acid,bitter	27.0	Mealy
				Ripening Temp. 50° F.					
H-1	2	7	4.99	34.5	14.9				
H-2	4	7	4.99	35.0	14.7				
H-3	6	7	5.06	35.2	14.8				
H-4	8	7	5.07	35.8	12.3				
H-1	2	42	5.01						
H-2	4	42	5.03						
H-3	6	42	5.10						
H-4	8	42	5.06						
H-1	2	90	5.09		20.2	39.0	Sl.acid	28.5	Curdy
H-2	4	90	5.06		28.2	39.0	Feed	29.0	Sl.weak
H-3	6	90	5.15		26.0	40.0		29.5	
H-4	8	90	5.10		31.0	38.0	Acid,Sl.ferm.	28.5	Weak
H-1	2	180	5.15	34.1	28.4	37.5	Acid,bitter	27.0	Mealy,flaky
H-2	4	180	5.08	33.9	34.0	37.5	Bitter	27.0	Weak
H-3	6	180	5.27	33.4	33.8	38.0	Bitter	27.5	Weak
H-4	8	180	5.15	34.5	35.6	37.0	Bitter	27.5	Mealy

*Ounces of rennet extract per 1000 lbs. of milk.

Table 9

The effect of different quantities of added rennet on the soluble nitrogen values and scores of cheddar cheese.

(Series I; made 2-23-49)

Cheese No.	Rennet Added (oz.)*	Age (days)	pH	Moisture (%)	Sol.N. (% of Tot.N.)	Flavor Score	Remarks	Body, Text. Score	Remarks
				Ripening Temp. 43° F.					
I-1	2	7	5.02	37.8	8.3				
I-2	4	7	4.94	39.2	8.2				
I-3	6	7	5.02	37.8	10.4				
I-4	8	7	5.02	38.3	9.3				
I-1	2	42	5.05		12.4				
I-2	4	42	4.92		13.8				
I-3	6	42	5.06		14.5				
I-4	8	42	5.22		18.7				
I-1	2	90	5.07		18.1	38.5	Sl.acid	28.5	Weak
I-2	4	90	4.94		21.1	39.0	Feed	29.0	Sl.weak
I-3	6	90	5.01		23.7	39.0	Sl.acid	29.0	
I-4	8	90	5.16		28.9	39.0	Sl.acid	28.5	Pasty
I-1	2	180	5.09	36.0	21.8	38.0	Acid	28.0	Weak,mealy
I-2	4	180	5.00	37.0	26.1	38.5	Acid,feed	29.0	Weak
I-3	6	180	5.05	36.5	30.4	38.0	Acid,Sl.bitter	28.0	Weak,pasty
I-4	8	180	5.17	36.4	35.3	38.5	Sl.fermented	28.5	Weak
				Ripening Temp. 50° F.					
I-1	2	7	5.01	38.3	9.7				
I-2	4	7	4.98	38.5	8.7				
I-3	6	7	5.05	36.7	10.6				
I-4	8	7	5.01	37.9	11.9				
I-1	2	42	4.98		12.1				

I-2	4	7	4.94	39.2	8.2				
I-3	6	7	5.02	37.8	10.4				
I-4	8	7	5.02	38.3	9.3				
I-1	2	42	5.05		12.4				
I-2	4	42	4.92		13.8				
I-3	6	42	5.06		14.5				
I-4	8	42	5.22		18.7				
I-1	2	90	5.07		18.1	38.5	Sl.acid	28.5	Weak
I-2	4	90	4.94		21.1	39.0	Feed	29.0	Sl.weak
I-3	6	90	5.01		23.7	39.0	Sl.acid	29.0	
I-4	8	90	5.16		28.9	39.0	Sl.acid	28.5	Pasty
I-1	2	180	5.09	36.0	21.8	38.0	Acid	28.0	Weak,mealy
I-2	4	180	5.00	37.0	26.1	38.5	Acid,feed	29.0	Weak
I-3	6	180	5.05	36.5	30.4	38.0	Acid,Sl.bitter	28.0	Weak,pasty
I-4	8	180	5.17	36.4	35.3	38.5	Sl.fermented	28.5	Weak
				Ripening Temp. 50° F.					
I-1	2	7	5.01	38.3	9.7				
I-2	4	7	4.98	38.5	8.7				
I-3	6	7	5.05	36.7	10.6				
I-4	8	7	5.01	37.9	11.9				
I-1	2	42	4.98		12.1				
I-2	4	42	4.95		15.3				
I-3	6	42	4.99		16.9				
I-4	8	42	5.08		20.5				
I-1	2	90	5.00		18.3	38.0	Acid,musty	28.0	Mealy
I-2	4	90	4.96		26.5	38.0	Sl.bitter	28.0	Mealy
I-3	6	90	5.01		29.9	38.5	Acid,Sl.ferm.	28.0	Weak,pasty
I-4	8	90	5.04		31.9	38.0	Acid,Sl.bitter	28.0	Weak,pasty
I-1	2	180	5.03	36.0	24.3	37.5	Acid,Sl.bitter	28.5	Mealy
I-2	4	180	5.13	36.7	31.1	38.0	Sl.bitter	28.5	Sl.pasty
I-3	6	180	5.17	35.4	33.9	38.0	Sl.bitter	28.0	Weak,pasty
I-4	8	180	5.23	35.5	38.8	37.5	Sl.fruity Sl.bitter	28.0	Weak

*Ounces of rennet extract per 1000 lbs. of milk.

series were quite constant. As was to be expected, a slight but definite upward trend in the pH of the cheese during the course of the ripening period was noted. Also, within any one series of cheese at any one time, there was a slight tendency toward a higher pH value in that cheese which contained the larger quantities of rennet extract. This trend probably was due to increased proteolysis in that cheese made with the larger quantities of rennet extract.

Soluble Nitrogen

Increasing the quantity of rennet extract used in the manufacture of pasteurized milk cheddar cheese resulted in increases in the soluble nitrogen values; this was apparent at any of the sampling times. Only in a few cases were there exceptions to this relationship, and these probably were due to errors in sampling or analysis.

Unfortunately, there were no analyses made for soluble nitrogen as the cheese came from the press. However, an inspection of all available data indicated that the increases in soluble nitrogen brought about by increases in the amount of rennet extract employed were part of a gradual process throughout the ripening period. There was no evidence to assume that any but a very small amount of increase due to any added increment of rennet extract occurred during the first 7 days of ripening. Thus, the effect of the addition of increasing amounts of rennet extract, within the cheese

of any one series, was only beginning to be apparent at an interval of 7 days in the ripening period.

At 42 days of ripening the average soluble nitrogen values for all cheese made with 2, 4, 6 and 8 ounces of rennet extract were 15.6, 18.6, 20.4 and 24.0 per cent respectively. This general relationship was maintained throughout the duration of the ripening period.

Within any one series, the cheese made with the 8 ounce quantity of rennet extract had, in 90 days of ripening, reached a level of soluble nitrogen which was equal to, or greater than, that attained in the cheese made at the 2 ounce level of rennet extract after 180 days of ripening. This relationship was reflected to some extent in the body and texture characteristics of the cheese made at the higher test level of rennet extract when examined at 90 days. However, flavor development was not a direct function of the increase in water-soluble nitrogen brought about by the use of abnormally large quantities of rennet extract in the manufacture of cheddar cheese.

In an attempt to determine the relative importance of rennet extract in the hydrolysis of cheese protein, an analysis was made of the data for soluble nitrogen presented in the tables. Figures 1, 2 and 3 contain plots of the soluble nitrogen values obtained by analysis during the ripening period when 2, 4, 6 and 8 ounces of rennet extract were used. These data were taken from Tables 2, 4, 6, 7, 8 and 9, and represent only the cheese which was ripened at 43° F. The

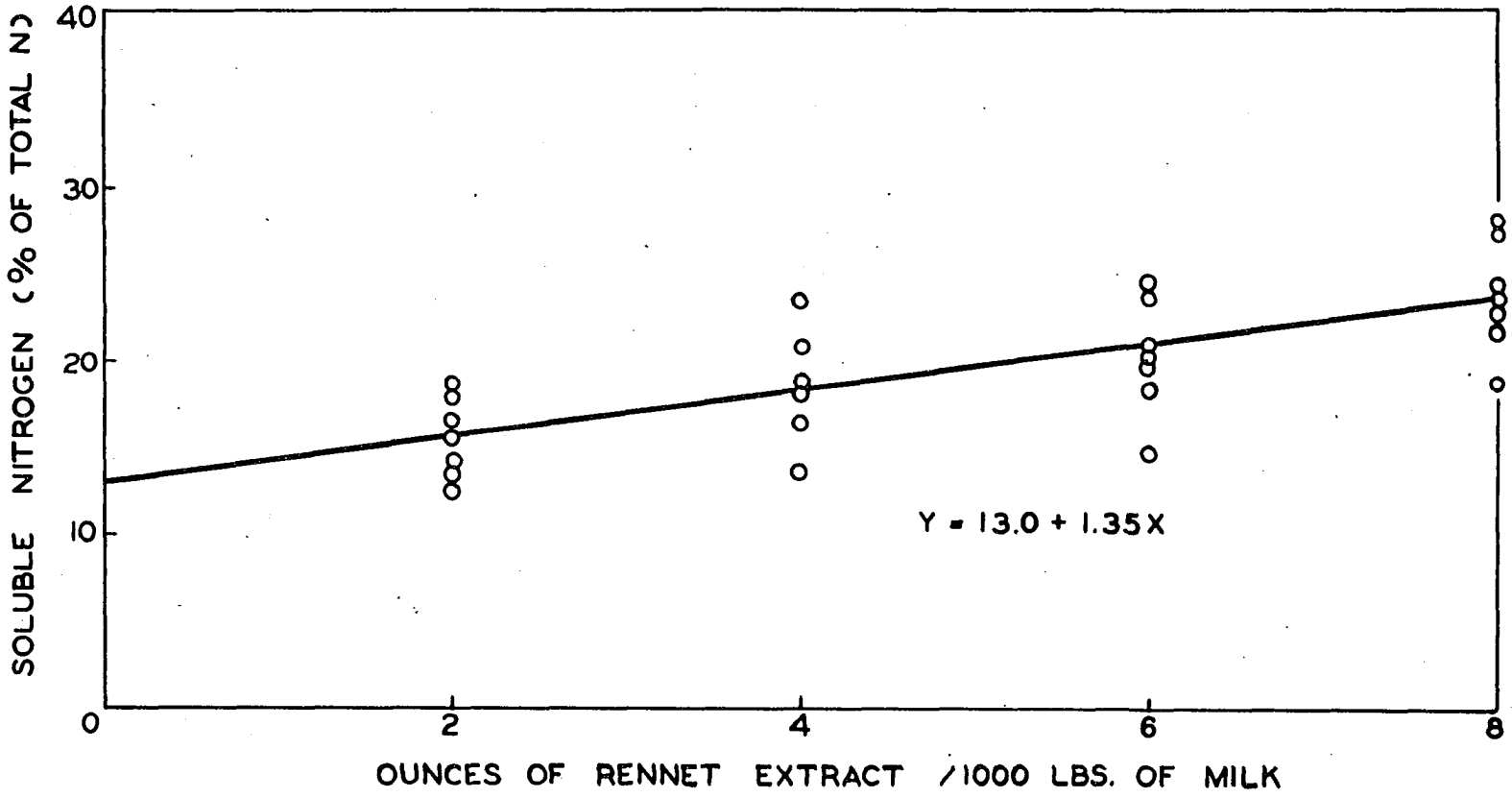

Fig. 1. Soluble nitrogen values for cheddar cheese made with 2, 4, 6 and 8 ounces of rennet extract after 42 days of ripening at 43° F.

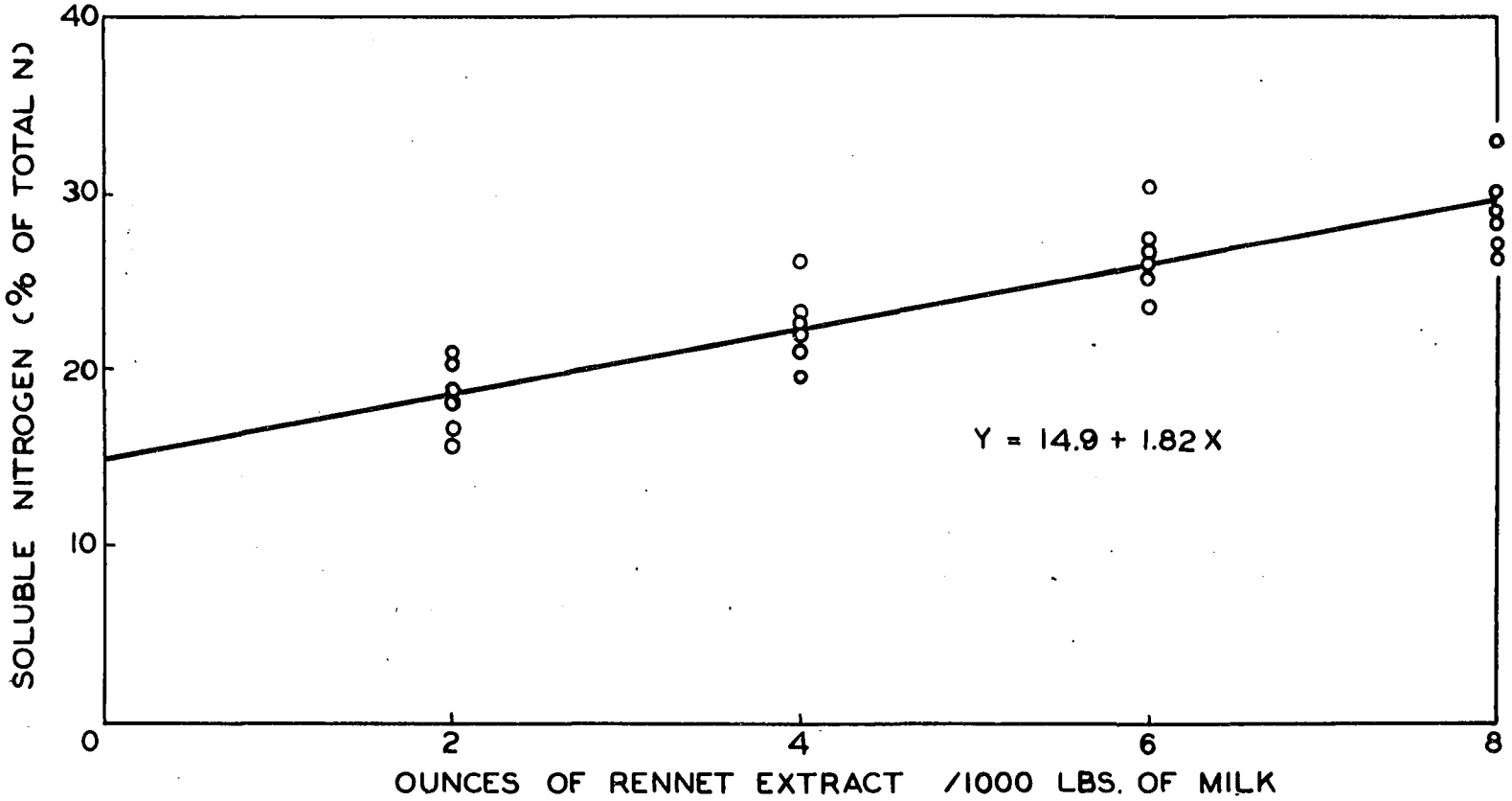

Fig. 2. Soluble nitrogen values for cheddar cheese made with 2, 4, 6 and 8 ounces of rennet extract after 90 days of ripening at 43° F.

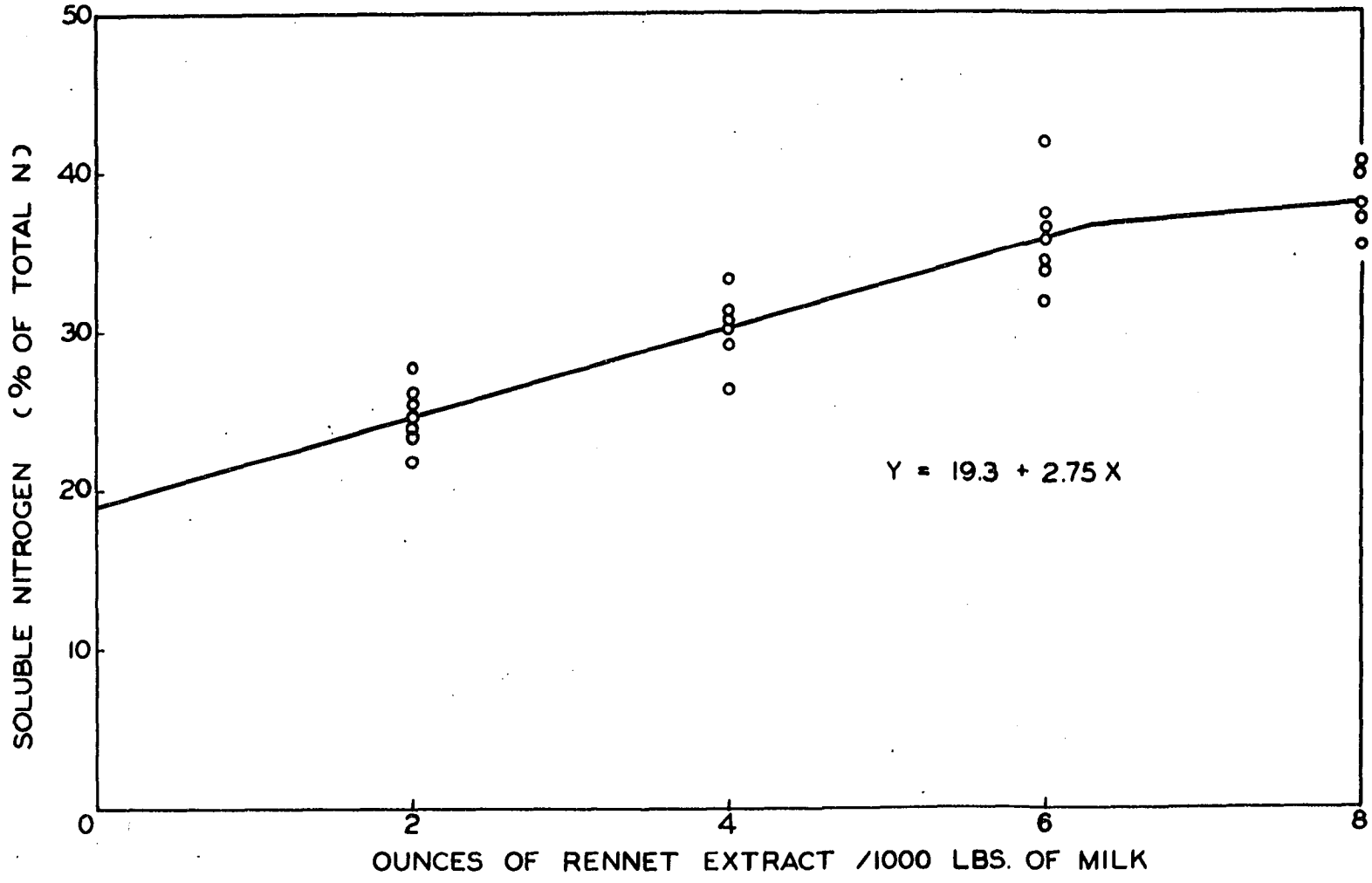

Fig. 3. Soluble nitrogen values for cheddar cheese made with 2, 4, 6 and 8 ounces of rennet extract after 180 days of ripening at 43° F.

average soluble nitrogen values plotted in Figures 1, 2 and 3 indicate that a linear relationship exists between soluble nitrogen production and the amount of rennet extract employed. This is apparent at all intervals during the ripening period except that at the 180 day interval the various lots of cheese made with 8 ounces of rennet have somewhat lower values for soluble nitrogen than would be expected if a linear relationship existed. The lines plotted in Figures 1, 2 and 3 are regression lines for soluble nitrogen values on the ounces of rennet extract employed per 1000 lbs. of milk. The regression in Figure 3 was computed only for the soluble nitrogen values obtained from the cheese made with 2, 4 and 6 ounces of rennet extract.

In each of Figures 1, 2 and 3, a theoretical value for soluble nitrogen which would result if the cheese could be manufactured without rennet extract was obtained by extending the regression line to the zero (Y) axis. These theoretical values after 42, 90 and 180 days of ripening are 13.0, 14.9 and 19.3 per cent soluble nitrogen, respectively.

Table 10 contains an analysis of the soluble nitrogen data plotted in Figures 1, 2 and 3. The theoretical values for soluble nitrogen obtained by extending the regression lines to the Y axis were used as bases from which to compute the increases in soluble nitrogen due to the added increments of rennet extract. The data in column 5 of Table 10 indicate the per cent of the total water-soluble nitrogen present in

Table 10

An analysis of the changes in the soluble nitrogen values of cheddar cheese (Tables 2, 4, 6, 7, 8, 9) made with different quantities of rennet extract. Cheese ripened at 43° F.

Age of Cheese (days)	Rennet Added (oz.)	Sol. N (% of Tot. N)	Sol. N Due to Action of Rennet (% of Tot. N)	Per Cent** of Sol. N Due to Rennet
42	0	13.0*	0	0
42	2	15.6*	2.6	16.7
42	4	18.6	5.6	30.1
42	6	20.4	7.4	36.3
42	8	24.0	11.0	45.9
90	0	14.9	0	0
90	2	18.4	3.5	19.0
90	4	22.1	7.2	36.6
90	6	26.4	11.5	43.5
90	8	29.1	14.2	48.8
180	0	19.3	0	0
180	2	24.9	5.6	22.5
180	4	30.2	10.9	36.1
180	6	35.9	16.6	46.2
180	8	37.8	18.5	48.9

*Average values for soluble nitrogen taken from data of Figs. 1, 2 and 3.

**Computed by dividing values in column 4 by values in column 3 and multiplying by 100.

the cheese which was attributable to the action of rennet extract. At an interval of 42 days, 2 ounces of rennet was responsible for 16.7 per cent of the total water-soluble nitrogen present in the cheese made with that quantity of rennet. The per cent of the total soluble nitrogen attributable to the action of rennet increased with time. It is seen that at 90 and 180 days the percentages of the total soluble nitrogen due to the action of 2 ounces of rennet were 19.0 and 22.5 per cent, respectively. Similar results may be observed for cheese made with 4, 6 and 8 ounces of rennet. However, the increases with time at each of these levels of rennet are not as pronounced as in the case of the cheese made with the 2 ounce quantity of rennet extract.

From the data of column 5, it may be observed that at any of the sampling times, the per cent of the total soluble nitrogen due to the action of rennet did not increase in a linear fashion with the increase in the amount of rennet extract employed.

From the data of column 3, Table 10, it is observed that well over one-half of the total soluble nitrogen formed during ripening and present in cheese after 180 days was formed after 42 days. It was computed that of the total soluble nitrogen present in cheese made with 2 ounces of rennet extract at 180 days, about 62 per cent of it was present after 42 days ripening, while almost 74 per cent of the total was present at an interval of 90 days. Similar data may be computed for cheese

made with 4, 6 and 8 ounces of rennet extract.

From the analysis of Table 10, it is evident that the enzymes of commercial rennet extract are responsible for a certain portion of the water soluble nitrogen existing in cheese. However, the data of Table 10 do not correctly interpret the hydrolysis of cheese protein from the standpoint of the total change in water-soluble nitrogen which occurs in the cheese during the entire extent of the ripening period. This is due to the fact that there is a certain amount of soluble nitrogen present in cheese at the time it is through draining in the press. This would correspond to zero time in the ripening period, and it is fairly evident that little of the soluble nitrogen present in the cheese at this time is due to the action of rennet extract.

Figure 4 contains a comprehensive plot of the soluble nitrogen values for cheese made with 2, 4, 6 and 8 ounces of rennet extract. The values plotted are those average soluble nitrogen values to be found in column 3 of Table 10. The average soluble nitrogen values at 7 days for three series of cheese made with 2, 4, 6 and 8 ounces of rennet extract (Tables 7, 8 and 9) were included in Figure 4. The theoretical values for cheese made in the absence of rennet extract also were plotted in Figure 4. It would be desirable to have an analysis of the changes in the soluble nitrogen values between 7 and 180 days in the ripening period similar to that found in Table 10. However, the few values obtained at an interval of

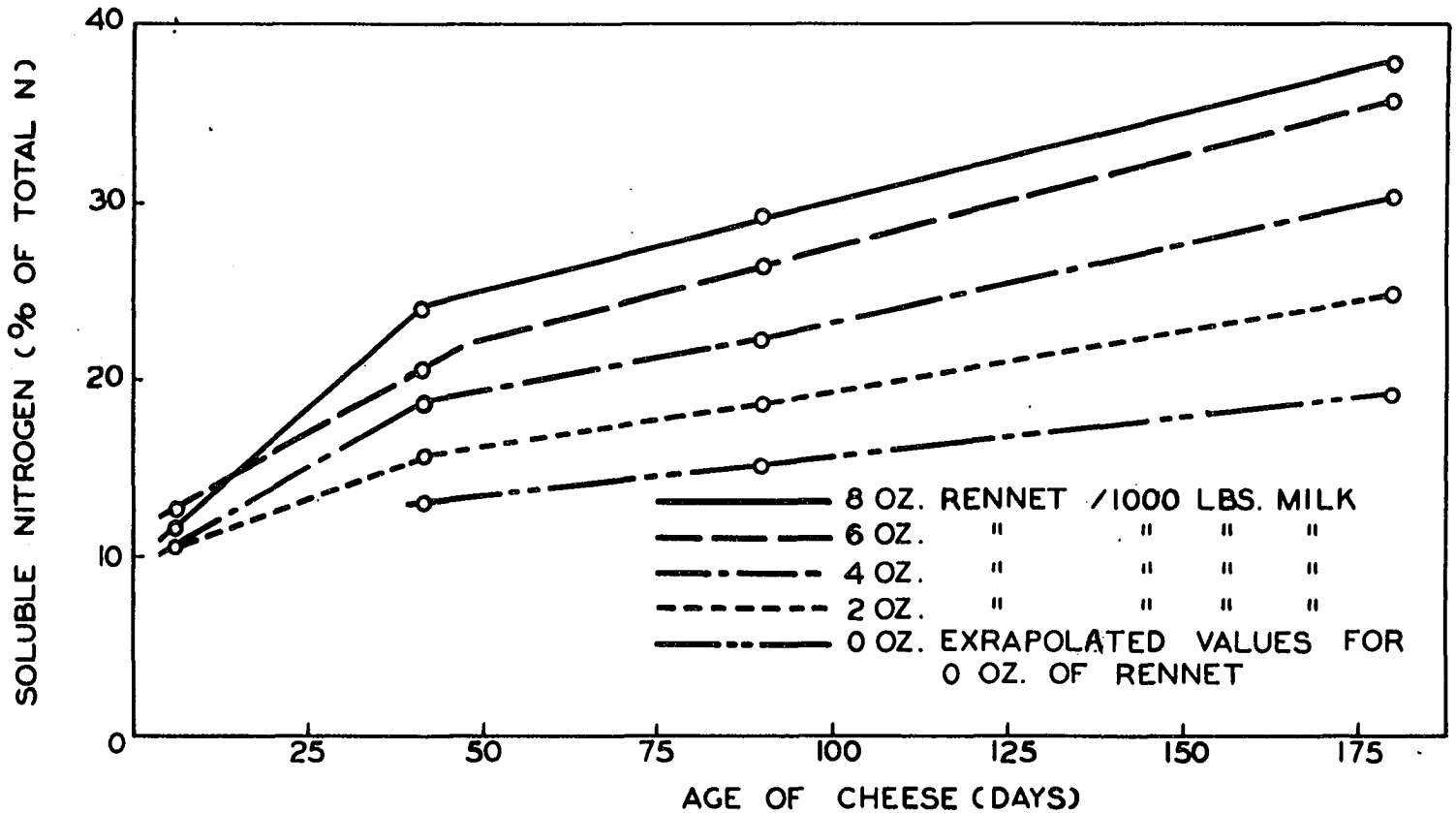

Fig. 4. Soluble nitrogen values during ripening for cheddar cheese made with different quantities of rennet extract. Cheese ripened at 43° F. (All data from Tables 2, 4, 6, 7, 8 and 9).

7 days would necessitate the use of certain assumptions which would be unjustified on the basis of the available data.

The approximate role of rennet extract between 7 and 180 days of ripening may be determined from Figure 4. It is obvious that rennet accounts for a greater proportion of the total increase in soluble nitrogen during this period than is apparent when a zero value for soluble nitrogen is used as a basis of calculation. This in no way changes the validity of the computed data of Table 10, but it merely indicates that after a certain interval in the ripening period (7 days) the rennet extract accounts for a proportionately greater part of the increase in soluble nitrogen than is apparent in the analysis of Table 10.

Organoleptic Examination and Scoring of Cheese

Table 11 contains the average scores for flavor, body and texture of the three series of cheese which were ripened at different temperatures (43 and 50° F.) and which were scored at two intervals during the ripening period. It is probable that an insufficient number of trials was included in this study to draw definite conclusions as to the effects on flavor, body and texture obtained by the use of increased quantities of rennet extract in the manufacture of cheddar cheese.

At both ripening temperatures employed and at both

Table 11

A comparison of average scores for flavor and body and texture of cheddar cheese made with different quantities of rennet extract. (data from Tables 5, 7, 8, 9)

Rennet Added (oz.)	Flavor Score*				Body and Texture Score*			
	43°		50°		43°		50°	
	90 Days	180 Days	90 Days	180 Days	90 Days	180 Days	90 Days	180 Days
2	38.0	37.3	38.2	37.5	28.3	27.4	28.2	27.2
4	38.5	37.5	38.3	37.7	28.7	28.0	28.3	27.2
6	38.7	37.7	39.0	38.0	29.0	27.4	28.7	27.7
8	38.7	37.2	38.0	37.2	28.7	27.2	28.2	27.7

*Average of 3 cheeses at each ripening temperature

examination times, the cheese containing a 6 ounce quantity of rennet extract had the highest average flavor score. Cheese made with the 4 ounce quantity of rennet extract possessed the second highest average flavor score in three out of four samples, while there would seem to be little to choose between cheeses containing 2 and 8 ounces of rennet extract.

Cheese containing 6 ounces of rennet extract also would appear to be slightly superior in body and texture on the basis of the results contained in Table 11. In all cases except one, the cheese with 6 ounces of rennet were equal to, or better than, the other cheese in body and texture. Here again, the differences noted were slight. However, in these particular lots of cheese the use of increased quantities of rennet extract up to 6 and possibly 8 ounces per 1000 pounds of milk had no deleterious effects on the body and texture of the resulting cheese. In all of the cheese which was made using different quantities of rennet extract, it was concluded that the effect of the increased quantities of rennet extract on body and texture development was relatively more important than the effect on flavor development. This was particularly true in the first 3 to 4 months of the ripening period.

In the remaining series of cheese which were scored after a very extended ripening period, somewhat different results were obtained. The use of increased quantities of rennet extract resulted in decreases in the flavor, body and texture scores of the resulting cheese. Fermented and fruity

flavors were noted in the cheese made with the larger quantities of rennet extract. The body and texture of these cheeses were critized as being weak and pasty. This might be expected since the soluble nitrogen values for most of the cheese made with the larger quantities of rennet were over 50 per cent of the total nitrogen present in the cheese. In general, these results are of a rather impractical nature since the cheese, although in very good condition, was from 420 to 510 days old at the time it was examined.

There seemed to be little difference between the average flavor scores of cheese ripened at 43° F. and that ripened at 50° F. at an interval of 90 days. At 180 days the cheese ripened at 50° F. had slightly higher flavor scores than that ripened at 43° F. for cheese made with 2, 4 and 6 ounces of rennet, while the flavor scores of the cheese made with 8 ounces of rennet extract were the same at 43 and 50° F. On six out of eight occasions the cheese ripened at 43° F. scored higher in body and texture than did the cheese ripened at 50° F.

A Comparison of Rennet Extract, Rennet Powders and Rennin in Cheddar Cheese Made from Pasteurized Milk

Analyses of Cheese

Moisture

Little variation in the moisture content of the cheese made with the different enzyme preparations was observed. Most of the cheese contained between 36 and 37 per cent

moisture 7 days after manufacture. (Tables 12, 13, 14 and 15.)

pH

In contrast to the cheese of Series G, H and I, most of the cheese made in this particular phase of the investigation tended to be slightly above pH 5.0 after 7 days ripening. Consequently, the flavor, body and texture criticisms arising from the slightly acid character of the former cheese were lacking in the cheese made in this phase of the investigation.

Soluble Nitrogen

Tables 12, 13, 14 and 15 contain the soluble nitrogen data for four series of cheese made with rennet extract, rennet powders and rennin. The data for soluble nitrogen calculated as per cent of total nitrogen show no consistent differences in the cheese made with the four different enzyme preparations. Probably an insufficient number of trials was included in this phase of the investigation to warrant far-reaching conclusions. The results strongly indicate that rennin or rennet powders when used in concentrations equal in coagulating activity to rennet extract were able to bring about approximately the same amount of proteolysis in cheese as was rennet extract. The techniques used to obtain enzymes of varying coagulating abilities have not changed the ratios of proteolytic and coagulating abilities. In other words, it appears that a certain definite amount of proteolytic activity is associated

Table 12

A comparison of rennet extract and rennet powders in cheddar cheese made from pasteurized milk and ripened at 43° F.

(Series J; made 3-26-48)

Cheese No.	Addenda	Age (days)	pH	Moisture (%)	Sol.N. (% of Tot.N.)	Flavor Score	Remarks	Body, Text. Score	Remarks
J-1	Rennet*	7	5.02	36.4	7.35				
J-2	Powder A**	7	5.01	36.5	7.24				
J-3	Powder B***	7	5.07	35.9	10.0				
J-1	Rennet	42	5.10		17.9				
J-2	Powder A	42	5.16		19.3				
J-3	Powder B	42	5.17		13.7				
J-1	Rennet	90	5.10	36.2	20.9				
J-2	Powder A	90	5.12	36.2	20.4				
J-3	Powder B	90	5.18	35.7	22.1				
J-1	Rennet	180	5.15	36.1	22.4	38.0	Flat,Sl.ferm.	28.5	Weak
J-2	Powder A	180	5.10	36.2	22.6	39.0		29.0	
J-3	Powder B	180	5.21	35.8	21.6	38.5	Sl.acid,flat	28.5	Weak

*1.5 oz. of commercial rennet extract added per 1000 lbs. of milk.
**Equivalent quantity of rennet powder A.
***Equivalent quantity of rennet powder B.

Table 13

A comparison of rennet extract and rennet powders in cheddar cheese made from pasteurized milk and ripened at 43° F.

(Series K; made 4-11-48)

Cheese No.	Addenda	Age (days)	pH	Moisture (%)	Sol.N. (% of Tot.N.)	Flavor Score	Remarks	Body, Text. Score	Remarks
K-1	Rennet*	7	5.04	36.8	10.5				
K-2	Powder A**	7	5.02	36.7	10.2				
K-3	Powder B***	7	5.01	35.9	14.2				
K-1	Rennet	42	5.02		19.7				
K-2	Powder A	42	5.02		18.3				
K-3	Powder B	42	5.00		24.7				
K-1	Rennet	90	5.01	36.5	27.2				
K-2	Powder A	90	5.02	36.4	26.2				
K-3	Powder B	90	5.01	35.5	30.0				
K-1	Rennet	180	5.00	36.3	29.4	39.0	Feed	29.5	
K-2	Powder A	180	5.00	36.4	31.4	38.5	Sl.fruity	29.0	Sl.weak
K-3	Powder B	180	5.07	35.2	31.9	39.5		29.5	

*3 oz. commercial rennet extract added per 1000 lbs. of milk.
**Equivalent quantity of renner powder A.
***Equivalent quantity of rennet powder B.

Table 14

A comparison of rennet extract, rennet powders and rennin in cheddar cheese made from pasteurized milk and ripened at 43° F.

(Series L; made 5-23-48)

Cheese No.	Addenda	Age (days)	pH	Moisture (%)	Sol.N. (% of Tot.N.)	Flavor Score	Remarks	Body, Text. Score	Remarks
L-1	Rennet*	7	5.07	36.4	13.1				
L-2	Powder A**	7	5.06	36.4	8.9				
L-3	Powder B***	7	5.12	37.1	11.3				
L-4	Rennin****	7	5.10	37.8	9.5				
L-1	Rennet	42	5.07		17.6				
L-2	Powder A	42	5.09		16.9				
L-3	Powder B	42	5.15		21.7				
L-4	Rennin	42	5.12		15.4				
L-1	Rennet	90	5.15		20.5				
L-2	Powder A	90	5.20		22.1				
L-3	Powder B	90	5.15		19.1				
L-4	Rennin	90	5.18		25.9				
L-1	Rennet	180	5.23	36.1	28.1	38.5	Acid	29.0	
L-2	Powder A	180	5.40	35.9	24.9	39.0		29.0	
L-3	Powder B	180	5.19	36.4	32.5	38.5	Sl.bitter	28.5	Weak
L-4	Rennin	180	5.32	37.1	28.1	39.0		29.0	

*3.0 oz. commercial rennet extract added per 1000 lbs. of milk.
**Equivalent quantity of rennet powder A.
***Equivalent quantity of rennet powder B.
****Equivalent quantity of rennin.

Table 15

A comparison of rennet extract, rennet powders and rennin in cheddar cheese made from pasteurized milk.

(Series M; made 3-2-49)

Cheese No.	Addenda	Age (days)	pH	Moisture (%)	Sol.N. (% of Tot.N.)	Flavor Score	Remarks	Body, Text. Score	Remarks
				Ripening Temp. 43° F.					
M-1	Rennet*	7	4.94	36.5	13.3				
M-2	Powder A**	7	5.01	36.9	15.5				
M-3	Powder B***	7	4.91	36.2	14.5				
M-4	Rennin****	7	4.86	35.9	10.3				
M-1	Rennet	42	4.94		20.0				
M-2	Powder A	42	5.00		19.0				
M-3	Powder B	42	4.95		18.9				
M-4	Rennin	42	4.94		17.4				
M-1	Rennet	90	4.94		20.5	38.0	Sl.acid	28.5	Weak
M-2	Powder A	90	5.00		20.3	38.5	Acid	28.5	Weak
M-3	Powder B	90	5.03		19.5	38.5	Sl.ferm.	28.5	Weak,mealy
M-4	Rennin	90	5.00		18.7	38.0	Sl.ferm.	28.5	Mealy
M-1	Rennet	180	4.96	35.2	29.2	38.0	Acid,Sl.fruity	28.5	Weak
M-2	Powder A	180	4.98	35.9	28.1	38.5	Acid	29.0	Weak
M-3	Powder B	180	5.00	35.0	28.1	38.5	Acid,Sl.bitter	28.5	Weak
M-4	Rennin	180	4.96	34.9	27.9	38.0	Acid	28.5	Weak
				Ripening Temp. 50° F.					
M-1	Rennet	7	4.97	37.5	14.2				
M-2	Powder A	7	4.93	36.6	16.8				
M-3	Powder B	7	4.92	36.3	15.1				
M-4	Rennin	7	4.84	36.3	15.3				
M-1	Rennet	42	4.95		19.0				
M-2	Powder A	42	4.98		19.5				

M-1	Rennet	42	4.94		20.0				
M-2	Powder A	42	5.00		19.0				
M-3	Powder B	42	4.95		18.9				
M-4	Rennin	42	4.94		17.4				
M-1	Rennet	90	4.94		20.5	38.0	Sl.acid	28.5	Weak
M-2	Powder A	90	5.00		20.3	38.5	Acid	28.5	Weak
M-3	Powder B	90	5.03		19.5	38.5	Sl.ferm.	28.5	Weak,mealy
M-4	Rennin	90	5.00		18.7	38.0	Sl.ferm.	28.5	Mealy
M-1	Rennet	180	4.96	35.2	29.2	38.0	Acid,Sl.fruity	28.5	Weak
M-2	Powder A	180	4.98	35.9	28.1	38.5	Acid	29.0	Weak
M-3	Powder B	180	5.00	35.0	28.1	38.5	Acid,Sl.bitter	28.5	Weak
M-4	Rennin	180	4.96	34.9	27.9	38.0	Acid	28.5	Weak
			Ripening Temp. 50° F.						
M-1	Rennet	7	4.97	37.5	14.2				
M-2	Powder A	7	4.93	36.6	16.8				
M-3	Powder B	7	4.92	36.3	15.1				
M-4	Rennin	7	4.84	36.3	15.3				
M-1	Rennet	42	4.95		19.0				
M-2	Powder A	42	4.98		19.5				
M-3	Powder B	42	4.90		19.3				
M-4	Rennin	42	4.91		18.2				
M-1	Rennet	90	5.00	35.0	24.4	38.0	Acid,Sl.ferm.	28.5	Mealy,weak
M-2	Powder A	90	5.00	35.7	23.9	38.0	Acid,Sl.ferm.	28.0	Mealy,pasty
M-3	Powder B	90	5.01	35.3	24.1	37.5	Musty,Sl.bitter	28.0	Pasty
M-4	Rennin	90	4.98	35.0	22.0	38.5	Acid	28.0	Flaky,mealy
M-1	Rennet	180	4.99	34.9	30.8	38.0	Acid,Sl.ferm.	28.5	Mealy
M-2	Powder A	180	4.95	34.3	31.9	38.0	Acid,Sl.bitter	28.0	Mealy,weak
M-3	Powder B	180	4.98	34.3	34.0	38.5	Acid	28.5	Mealy,weak
M-4	Rennin	180	4.96	33.9	29.5	37.5	Acid,Sl.bitter	28.0	Mealy,weak

*3 oz. rennet extract per 1000 lbs. of milk.
**Equivalent quantity of rennet powder A.
***Equivalent quantity of rennet powder B.
****Equivalent quantity of rennin.

with a certain concentration of coagulating enzyme.

Organoleptic Examination and Scoring of Cheese

All of the cheese made in this part of the investigation scored very well. The differences in flavor, body and texture among the cheese made with the different enzyme preparations were very slight. It would be impossible to say that one enzyme gave better results than another. It has been shown that any one of the preparations can be used satisfactorily. However, if a choice were to be made, rennet extract would probably be preferred by most cheesemakers because of the greater ease in handling.

Relative Coagulating Activities of Rennet Extract, Rennet Powders A and B and Rennin as a Measure of Enzyme Purity

The coagulating activities of commercial rennet extract, powder A, powder B and a solution of rennin were determined on a total nitrogen basis. The activities also were determined on a salt-free dry weight basis. The results presented in Table 16 are the average of duplicate analyses of single stock solutions of rennet extract, rennet powders and rennin. Rennet powders A and B were maintained in the powdered form in the cold. In this state they were extremely stable, retaining almost 90 per cent of their coagulating activity after being stored for 24 months at approximately 1.5° C. Two per cent solutions of rennet powders A and B were prepared by carefully weighing the required amounts of powdered enzyme into Erlen-

Table 16

Relative coagulating activities* of rennet extract, rennet powders and rennin

Preparation	Salt-Free Dry Weight (g./100 ml. solution)	Nitrogen (g./100 ml. solution)	Relative Coagulating Activity per Unit Wt. of:	
			Salt-Free Dry Matter	Nitrogen
Rennet Extract	8.4	0.53	1.00**	1.00**
Rennet Powder A (2% solution)	1.82	0.0123	1.10	7.4
Rennet Powder B (2% solution)	1.90	0.078	5.1	8.1
Rennin Solution	0.335	0.015	9.9	12.9

*Nitrogen and dry weight determinations in duplicate on one sample of each enzyme preparation.

**Value of 1.00 assigned arbitrarily to rennet extract.

meyer flasks. The solutions were made up to obtain the correct concentrations with distilled water. Rennet powders A and B also were used as standard coagulating enzymes. Whenever necessary, any sample of milk could be checked for irregularities in its behavior as a substrate for determining the activity of coagulating enzymes.

The stock solution of rennin which was used in determining the results presented in Table 16 resulted from a single crystallization procedure, and further purification of the enzyme was not attempted.

In Table 16, the relative coagulating activities per unit weight of nitrogen and per unit weight of dry matter are expressed, with commercial rennet extract considered to have an activity of unity. On a dry weight basis powder A had about the same relative activity as rennet extract, while powder B was about 5 times as active as rennet extract. The solution of rennin was approximately ten times as active as the rennet extract on a dry weight basis.

The relative activities of powder A and powder B per unit weight of nitrogen were 7.4 and 8.1 times the activity of rennet extract. The rennin preparation had about 13 times the activity of rennet extract per unit weight of nitrogen.

Measurement of the Proteolytic Activity of Rennet Extract, Rennet Powders and Rennin on Hemoglobin and Casein

An investigation of the proteolytic activities of commercial rennet extract, rennet powder A, rennet powder B and rennin prepared according to Hankinson was made. The

method used was essentially the method of Anson (4) for the determination of pepsin.

The above enzymes were tested in concentrations of approximately equal coagulating activity against milk. In preparing the enzyme dilutions for testing, it was felt that a time error of 5 per cent (30 seconds in 10 minutes) was rarely exceeded in obtaining solutions of equal coagulating power. For proteolysis of hemoglobin, as well as casein, by rennet extract, rennet powders and rennin, standard enzyme solutions were prepared. These standard solutions were of such concentration that 1 ml. quantities of them would just coagulate 10 ml. of the standard milk substrate in 10 minutes at 37° C. Whenever activity of a 1 ml. quantity of enzyme solution is referred to in this section of the dissertation, it indicates 1 ml. of the standard enzyme solution. All quantities of enzyme tested have the standard enzyme solution as a basis of concentration. For example, 2 ml. of enzyme solution indicates a concentration of enzyme in distilled water which is twice that of the standard solution. However, it should be noted that only 1 ml. quantities of suitably diluted enzyme solution were employed in the analytical procedure.

For comparative purposes, it is noted here that the quantity of enzyme used in the standard solution, on a proportionate basis, would be approximately equivalent to 4.5 ounces of rennet extract per 1000 pounds of milk.

Figure 5 contains the standard tyrosine curve which was used to relate Klett-Summerson colorimeter values to milliequivalent quantities of tyrosine.

Proteolysis of Hemoglobin

Concentration of Enzyme in Reaction Mixture

Figure 6 contains the results of the proteolysis of 5 ml. of 2.5 per cent hemoglobin solution with 0.5 to 2.0 ml. quantities of the respective enzyme solutions. A fairly linear relationship existed for proteinase activity when quantities up to 1.0 ml. of the respective enzyme solutions were employed. When 2 ml. quantities of enzyme solutions were employed the curves tended to fall slightly toward the horizontal, although still ascending quite rapidly. Quantities of enzyme solution larger than 2 ml. were not tested, since they would have resulted in values in excess of 300 units on the Klett-Summerson colorimeter scale.

Effect of pH on Proteolysis of Hemoglobin

Data for proteolysis of hemoglobin by rennet extract, rennet powders and rennin at pH values from 1.5 to 5.0 are plotted in Figure 7. The data used to plot the curves are the average of duplicate values obtained during the course of a single experiment. For each of the enzyme preparations tested, a maximum value for proteolysis of hemoglobin was

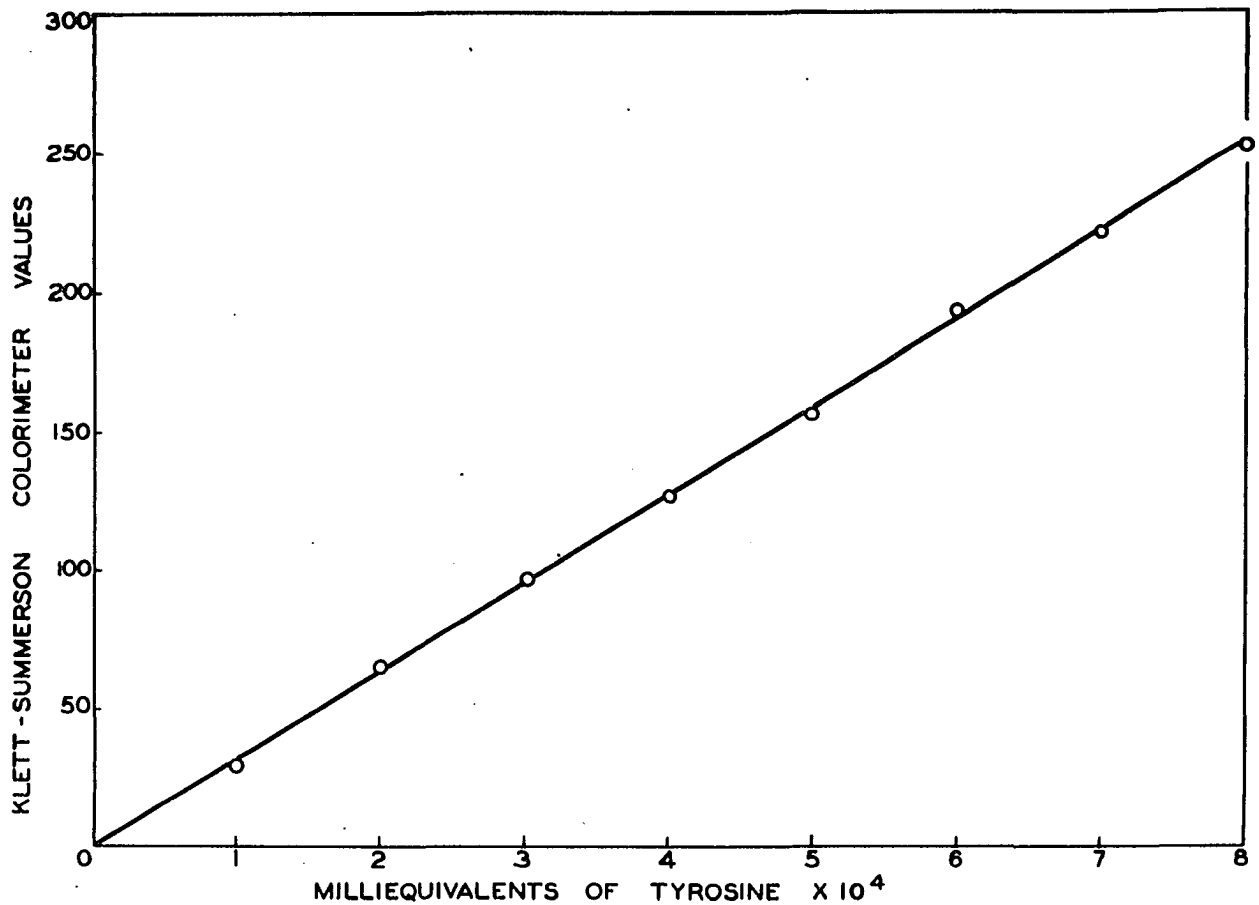

Fig. 5. Standard curve relating milliequivalent quantities of tyrosine to color produced by Folin-Ciocalteau reagent.

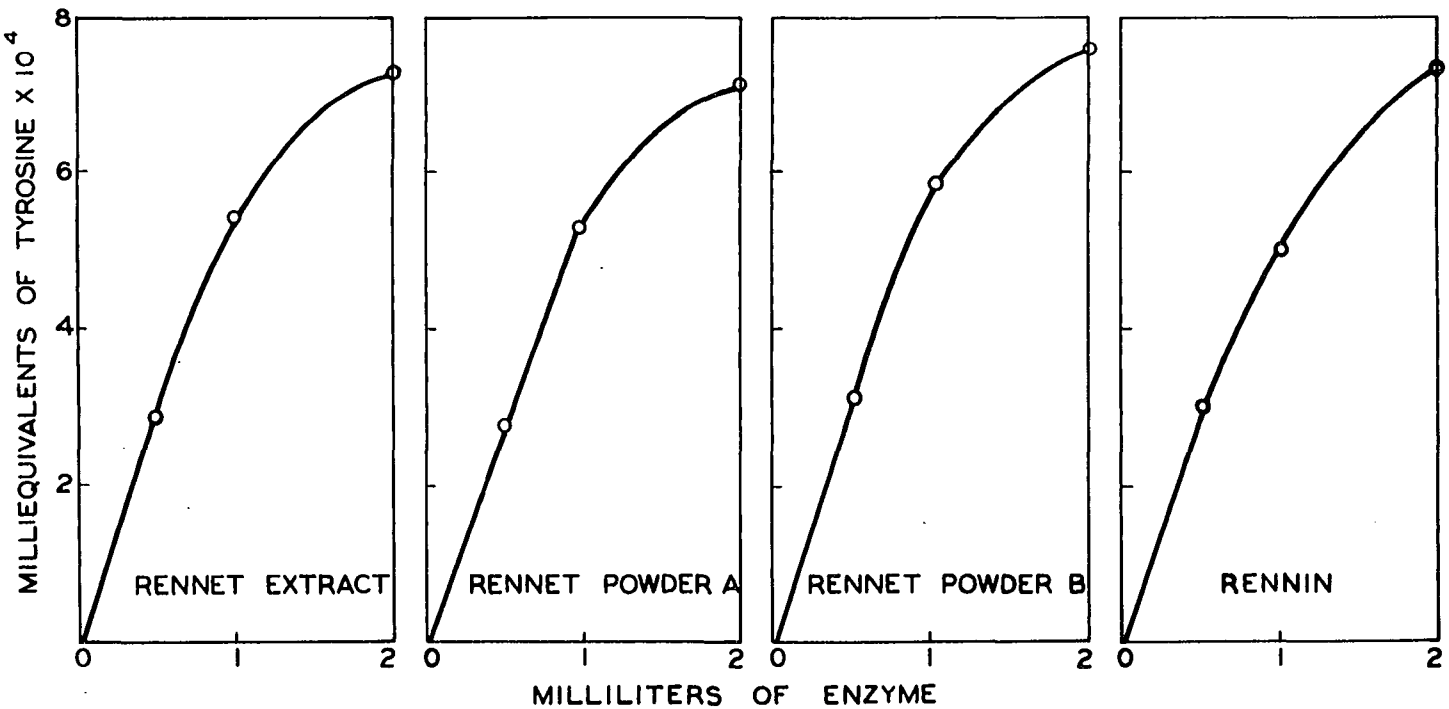

Fig. 6. Digestion of hemoglobin at pH 3.7 by equal coagulating quantities of rennet extract, rennet powders A and B and rennin. (30 minutes incubation at 37° C.)

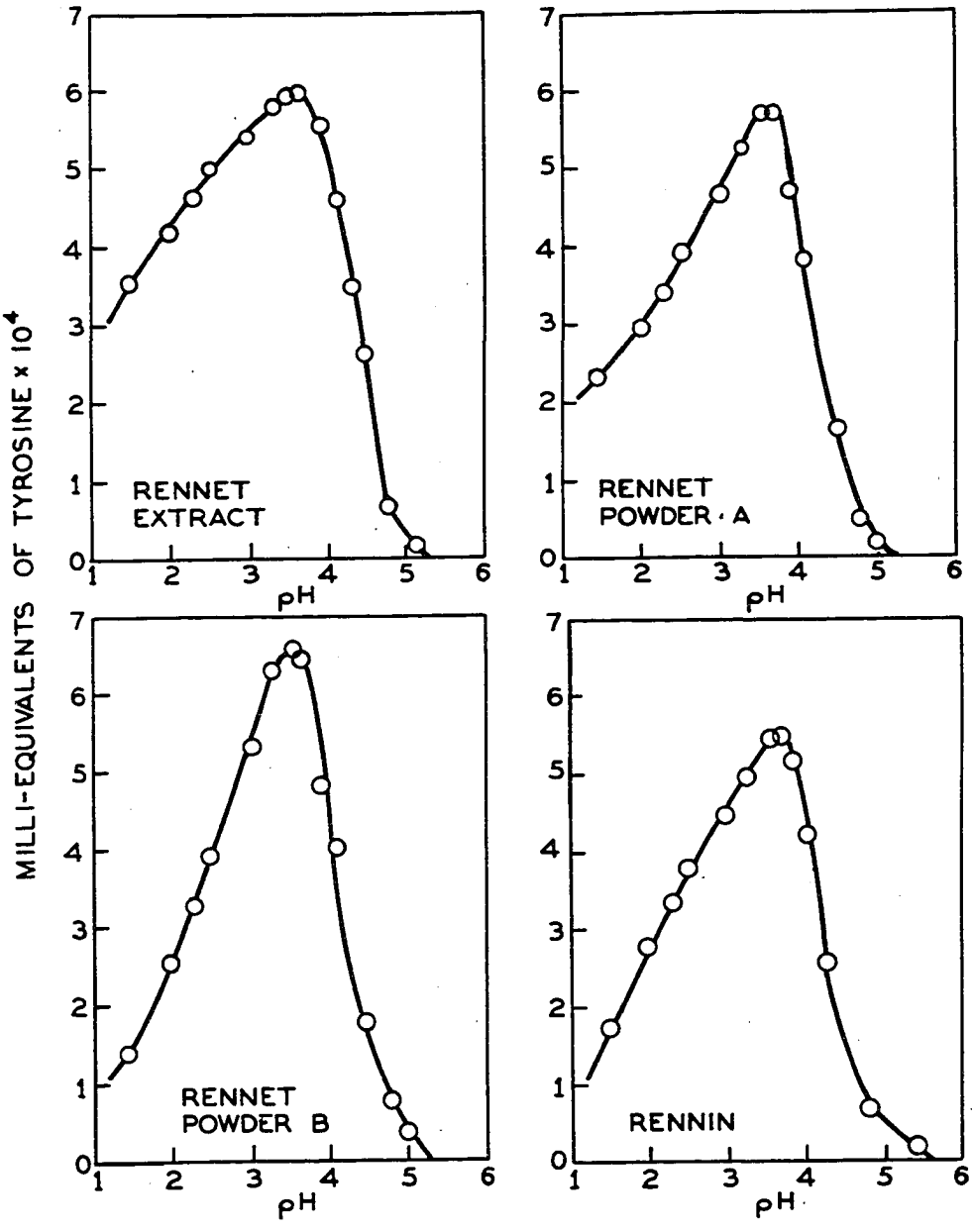

Fig. 7. Influence of pH on digestion of hemoglobin by rennet extract, rennet powders and rennin. (30 minutes digestion with standard enzyme solution at 37° C.)

obtained at pH values from 3.5 to 3.7. There seemed to be little difference in the optimum pH for proteolysis by the four enzymes. For each of the enzymes, proteolysis of hemoglobin at pH 5.0 was practically negligible. Activity of the preparations fell very rapidly between pH 3.9 and pH 5.0. The chief point of difference in the proteolysis of hemoglobin by these enzymes lay in the values obtained from pH 1.5 to pH 2.5. At pH values of 1.5, 2.0 and 2.5, proteolytic activity exerted by rennet extract was considerably greater than that of rennet powder A, rennet powder B, or rennin. This phenomenon was observed a number of different times during the experimentation.. These results indicate that commercial rennet extract contains pepsin, which has its optimum activity against hemoglobin at approximately pH 1.8 (16), and rennin, which has a pH optimum against hemoglobin at 3.7 (16). The smaller values for proteolysis of hemoglobin with the rennet powders and rennin at pH values from 1.5 to 2.5 indicated that, to some degree, pepsin had been removed during their preparation. If a comparison were to be made between powder A, powder B and rennin, it would appear that powder B and rennin contained less pepsin than did powder A. Rennet powder A probably resembled rennet extract more nearly than did the rennin, insofar as proteolysis at pH values of 1.5, 2.0 and 2.5 were concerned.

These results in regard to the identity of the separate enzymes tend to confirm the finding of Berridge (16) that rennin is a protease (proteinase) acting optimally on

hemoglobin at pH 3.7. In addition to rennet extract and rennin, Berridge presents data for the proteolysis of hemoglobin by pure pepsin. His results show that the activity of pure pepsin at pH 3.7 on hemoglobin is quite small in comparison to the activity of rennin. This is a strong indication that most of the activity of rennet extract at pH 3.7 on hemoglobin is due to the action of rennin rather than to the pepsin contained therein.

Proteolysis of Casein

The results of the manufacture of cheese (Tables 12, 13, 14 and 15) with commercial rennet extract, rennet powder A, rennet powder B and rennin, in concentrations of equal coagulating power, showed no consistent differences among the enzyme preparations insofar as soluble nitrogen production from cheese protein was concerned. Although casein was a less desirable substrate with which to work when using the hemoglobin method of Anson (4), a limited study of proteolysis was made with it as a substrate.

Measurements of proteolysis of casein at pH 2.0 and 5.4 were made with rennet extract, rennet powders and rennin, using preparations of equal coagulating strength. In Figure 8 are found the data obtained with the four enzyme preparations. These results are the average of duplicate analyses. For quantities of rennet extract from 1 to 3 ml., there were no differences in the results obtained at pH 2.0 and 5.4. With

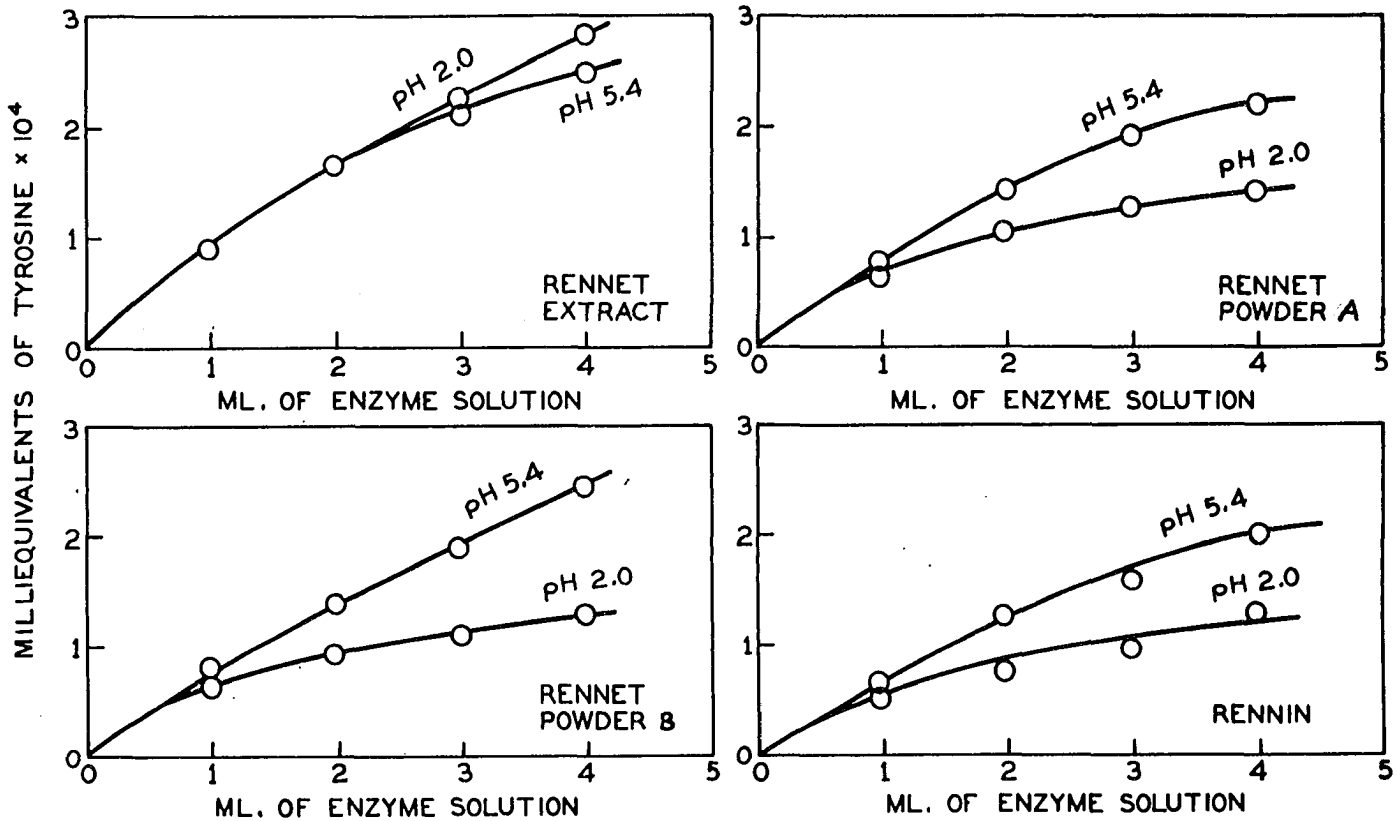

Fig. 8. Proteolysis of 0.8 per cent casein solution at pH 2.0 and 5.4 by rennet extract, rennet powders A and B and rennin. (30 minutes digestion at 37° C.)

a 4 ml. concentration of rennet extract, the values for proteolysis at pH 2.0 were slightly larger than those at pH 5.4.

In the case of rennin, the values at pH 5.4 were larger at all concentrations of enzyme from 1 to 4 ml. than those obtained at pH 2.0. Similar data for rennet powders A and B are found in Figure 8. With both of the rennet powders, the proteolytic activity at pH 5.4 was greater than that at pH 2.0. The rennet powders much more resembled the rennin in their relative activities at pH 2.0 and 5.4 than they did rennet extract. In comparing the proteolytic activities of the enzymes at pH 2.0, rennet extract exerted stronger proteolytic action against casein at all concentrations of enzyme tested than did any of the other enzymes. This was especially true at the 4 ml. concentration of enzyme, where proteolysis by rennet extract was approximately twice that of the other enzymes.

At pH 5.4 the enzymes were more nearly uniform in their action on casein. At pH 5.4 the curves for powder A, powder B and rennin tended to retain much more linearity than was the case at pH 2.0. These results indicate, as do those with hemoglobin, that the rennet powders and rennin have been purified considerably as far as freedom from pepsin was concerned. The work of Berridge (16) with a supposedly pure rennin preparation indicated a proteolysis against hemoglobin which was about one-third that obtained with commercial rennet

extract at pH 2.0. Some preliminary results in this study with an earlier rennin preparation (according to Hankinson) indicated that the rennin possessed from one-third to one-half the activity of commercial rennet extract at pH 2.0 on hemoglobin. However, these results were obtained with a different lot of rennet extract and a different hemoglobin substrate solution. It is known that all rennet extracts do not have the same characteristics. This probably is one reason why direct comparisons between various studies made using different rennet extracts may not give directly comparable results.

PART 2

AN INVESTIGATION OF THE PROTEOLYTIC ENZYMES PRESENT IN A CELL-FREE EXTRACT OF *S. LACTIS*

METHODS

Preparation of Cell-Free Extract of *S. lactis*

Since many workers in the past have indicated that enzymes liberated through autolysis of bacteria contained in cheddar cheese play an important role in the ripening process of that cheese, it was decided to study the endocellular proteolytic enzymes of one of the predominant microorganisms in cheddar cheese.

Preparation of Medium and Growth of Organisms

The particular strain of *S. lactis* used for production of the extract was culture no. A-1. The individual responsible for the isolation and characterization of the culture which has been referred to as *S. lactis* culture no. A-1 did not recognize *S. cremoris* as being a separate and distinct species. Subsequent to the beginning of this investigational work, culture no. A-1 was identified by other workers as being *S. cremoris*. In the great majority of the work which has been carried out in the past relative to these organisms, *S. lactis* has been the prevailing nomenclature regardless of the true identity of the organism being studied. For this reason, and because the biochemical characters which serve

to differentiate these organisms apparently are relatively unimportant in a study of this nature, this organism has been referred to as S. lactis in this study.

This culture was a single strain isolated from cream in 1946. It subsequently was carried as a laboratory culture in the Dairy Industry Department at Iowa State College and was used as the starter culture in all of the cheese for which data are herein reported.

A broth medium was used for growth of the organism, since it was virtually impossible to separate the bacterial cells from a milk culture of S. lactis. The following medium was employed:

Peptonized milk	0.7%
Yeast extract	0.7%
Beef extract	0.2%
Tomato juice	75 ml./l.
Skim milk	20 ml./l.
Disodium phosphate	0.8%

This medium was prepared and sterilized in 12-liter Florence flasks. The medium was inoculated with 100 ml. of an 18 to 20 hour culture of S. lactis. The inoculum was prepared in the same broth which had been sterilized in 6-ounce prescription bottles. The inoculation into these bottles was in turn made from a 24 hour old milk culture.

The 12-liter flasks containing sterile media were placed

in a 30° C. incubator 6 to 8 hours before inoculation. After inoculation the pH of the medium was maintained above 6.0 by neutralization of the developed acidity with sterile 5 N NaOH, and shortly before harvesting the bacteria the pH of the medium was adjusted to 7.0. A 30 hour incubation period was used for the cultures prepared in this manner.

Harvesting and Grinding the Cells

The cells were harvested in a Sharples supercentrifuge run at 24,000 r. p. m. An average of 4 g. of cells (wet weight) per liter of medium regularly was obtained by the above procedure. Immediately following centrifugation, the cells were placed in 50 ml. beakers, covered and held in a freezer unit overnight. The cells were frozen and thawed 4 times in 48 hours. At the end of this time the thawed cells were mixed with powdered pyrex glass in the proportion of 1 g. of cells to 4 g. of glass, according to the directions of Kalnitsky et al. (41). The powdered glass-cell mixture was ground in a small iced mortar once, and then passed twice through the grinding mill.

The powdered glass was prepared by grinding broken pieces of clean pyrex glass in a small ball mill and sieving the resulting product through a No. 40 mesh screen. The powdered glass was sterilized in a hot air oven before using.

Preparation of Extract for Testing

After grinding, the glass-cell mixture was frozen and

thawed 4 times in 48 hours in the freezing compartment of a refrigerator. Two ml. of water per gram of cells originally present was added to the thawed mixture, and it was allowed to extract for 24 hours at approximately 1.5° C. The glass, cells and cellular debris were removed by centrifuging the mixture in an angle-head centrifuge run at 4200 r. p. m. for 45 minutes. The supernatant liquid was removed by pipetting and was a transparent, yellowish-colored material.

This aqueous extract was found to contain products which produced a rather large amount of color with the Folin-Ciocalteau reagent. Most of these products could be eliminated by dialysis in a cellophane membrane without loss of enzymic activity. Consequently, the extract was dialyzed in cellophane tubing (0.75 inch diameter) for 24 hours in a cold room against distilled water.

The stock enzyme solution which was prepared in the above manner was kept in the frozen condition in a deep freeze cabinet. Portions of it were thawed 24 hours before use and held at approximately 1.5° C.

Estimating the Yield of Cells

In order to estimate the total number of bacteria harvested from the growth medium, a direct counting procedure was employed on the growth medium before separation of the cells and on the harvested cells. Shortly before harvesting the crop of cells a sample of culture medium was

removed from the flask and held in the cold for counting. A 1 ml. sample of this medium was dispersed in a 99 ml. water blank. A 0.01 ml. quantity of this dilution was removed with a pipette and spread over a surface of one square centimeter on a glass slide. This preparation was dried and stained with methylene blue. The microscope used in counting was standardized to give a microscopic factor of 600,000 with the 10X ocular and 1.8 mm. oil immersion objective. Thirty fields of this preparation were examined, counting the individual cells. From the average number of cells per field, a numerical estimate of the bacterial population per milliliter of growth medium was computed.

A second count was obtained by dispersing 0.3 g. of the mass of harvested cells from the growth medium in a 99 ml. water blank. A 1 ml. portion from this dilution was dispersed in a second 99 ml. water blank, and 0.01 ml. of this dilution was treated in the same manner as described above for the direct count on the growth medium.

A further count was obtained by plating quantities of the growth medium in serial dilution. Samples of the culture medium were taken shortly before harvesting the cells and held in the cold until plated. Counts were made on T. G. E. M. agar, and the plates were incubated at 30° C. for 48 hours.

Manufacture of Cheese

Two series of cheese consisting of four vats in each series were made in this phase of the investigation dealing

with a study of the endo-cellular enzymes of S. lactis. The two series of cheese were duplicate series, and each of the four vats in each series contained 260 pounds of pasteurized milk. Each of the vats received commercial rennet extract at the rate of 3 ounces per 1000 lbs. of milk. In the case of vats 2 and 3, the extract from 100 g. of cells was divided into two portions. One portion was added to vat 2 shortly before the addition of rennet, and the second portion was added to vat 3 in a similar manner.

In these series of cheese, vat 3 and 4 each received a solution (100 ml.) containing 4 g. of cysteine hydrochloride which was added to the milk before the addition of cell-free extract or rennet. Previous experiments had indicated that cysteine added to milk at a rate in excess of 4 g. per 260 lbs. of milk gave a very decided off-flavor to the resulting cheese. It should be noted that this concentration of cysteine in milk is only a very small fraction of the concentration usually taken for activation of proteolytic enzymes.

One of the longhorns prepared from each vat was ripened at 43° F. and the remaining longhorn was ripened at 50° F.

Analyses of Cheese

The cheese were analyzed at the intervals designated in Tables 17 and 18. Analyses were made for pH, moisture, total nitrogen and soluble nitrogen in the same manner as described beginning on page 26.

Organoleptic Examination and Scoring of Cheese

The organoleptic evaluation of the cheese was carried out in the same manner as that described on page 29 of this dissertation.

Testing Cell-Free Extract for Proteinase Activity

It was immediately apparent that in order to measure the activity of the cell-free extract of S. lactis it would be necessary to use a method capable of detecting a very small degree of protein hydrolysis. Hull (39) used a modification of the hemoglobin method of Anson (4) and was successful in measuring very small amounts of enzymatic protein degradation in milk.

The following method, which was adopted after much experimentation, was essentially the same as that used for measuring small amounts of proteolysis by the enzymes of rennet.

Preparation of Hemoglobin Substrate

A 2 per cent solution of hemoglobin was used as a substrate for the cell-free extract. This substrate solution was prepared according to the directions on page 34 in Part 1 of this dissertation.

Preparation of Casein Substrate

A 2 per cent solution of casein was prepared as described under methods, Part 1. This solution was diluted with distilled

water to obtain an 0.8 per cent solution of casein for the estimation of proteolysis by the cell-free extract of S. lactis.

Colorimetric Estimation of Proteolysis by Cell-Free Extract of S. lactis

The method employed here was the same as that described beginning on page 34 with the following exceptions. The concentration of trichloroacetic acid used to precipitate the unaltered protein after the reaction with enzyme was increased to 1.2 N. In general, a much longer incubation period was necessary. Twelve hours was a commonly employed incubation period.

Measurement of the Peptidase Activity of the Cell-Free Extract from S. lactis

It was decided to do a limited amount of work with the crude, cell-free extract in an attempt to determine if the S. lactis cells contained enzymes capable of hydrolyzing synthetic peptide substrates into their constituent amino acid molecules. For this purpose, glycyl-L-leucine and DL-alanylglycine were obtained from Amino Acid Manufacturers, U. C. L. A., Los Angeles, California. The Linderstrøm-Lang (44) method for titration of amino groups was used to measure enzyme activity, and the complete method is described below.

The substrate was made up as needed in M/30 concentration for glycyl-L-leucine or M/15 concentration for Dl-alanyglycine. Merthiolate was added to the substrate solution at the rate of

1 mg. of merthiolate to 40 ml. of solution, this concentration of merthiolate having no adverse effect on the enzymatic reaction.

The substrates were adjusted to the desired pH in the following manner. A weighed amount of substrate was put into solution in slightly less than the required amount of distilled water and buffer solution. Using the glass electrode potentiometer, the solution was brought to the desired pH with concentrated HCl or NaOH. The substrate solution was made up to the desired volume, usually by the addition of 1 to 2 ml. of distilled water.

Although buffers have not been used in some of the work to be found in the literature where the Linderstrøm-Lang titrimetric method for amino groups was used, the pH drifts of the unbuffered substrate during the course of the reaction sometimes were considerable, and so buffers were employed in this work. Each 3 ml. reaction sample of enzyme and substrate contained 0.6 ml. of 0.05 M acetate buffer in the pH range 4.0 to 5.5, 0.6 ml. of 0.05 M phosphate buffer in the range 5.6 to 8.0 and 0.6 ml. of 0.05 M borate buffer in the pH range 8.1 to 10.0.

The original method of Linderstrøm-Lang was varied slightly in that the reaction was carried out in 20 x 175 mm. pyrex test tubes instead of Erlenmeyer flasks. When working with small quantities of material, this has the advantage that the color change when approaching the endpoint in the

titration is more gradual, proceeding from top to bottom in the test tube, and secondly, the color of the endpoint may be observed through a depth of solution, which serves to make slight color differences more detectable.

To estimate peptidase activity, a 2 ml. quantity of buffered substrate solution was placed in a clean dry test tube and the volume made up to 3.0 ml. with enzyme and water. A 1 ml. quantity of this enzyme-substrate mixture was titrated immediately and a second titration was performed upon an identical quantity after incubation for the desired period of time in a water bath at 37° C.

The indicator used was 4-benzene-azo-1-napthylamine (napthyl red), 0.1 g. of the indicator being dissolved in 100 ml. of 96 per cent alcohol, and 2 drops of this solution being used for each titration.

The acetone used was the C. P. product, and a 15 ml. quantity was used for the 1 ml. sample under investigation.

To carry out the titration, two drops of indicator were added to 1 ml. of enzyme-substrate mixture, and N/20 HCl in 90 per cent alcohol was added from a microburette until the water solution was distinctly reddish in color. A part of the acetone was added, whereupon the indicator changed to its basic color, which is yellow. The titration was continued until an orange-red color was obtained. The remainder of the acetone was added and the solution was titrated to the orange color of the endpoint, as represented by napthyl red at pH 5.1 in water. When necessary, a control tube was set up consisting

of 1 ml. of water, two drops of indicator solution and 15 ml. of acetone. This control was brought to the color of the endpoint and used as a color guide in performing subsequent titrations.

The color of the endpoint in the titration may be reproduced with an accuracy of 0.01 to 0.02 ml. of N/20 HCl. This is equivalent to approximately 2 to 4 per cent hydrolysis of the substrate present.

The results of this enzyme-substrate reaction are expressed as per cent hydrolysis of one linkage (for the racemic peptide, per cent of one linkage of one optical component).

RESULTS

An Approximate Estimate of the Number of Cells Represented by the Cell-Free Extract Added to Cheese

When contemplating the addition of cell-free extract to cheese, it was considered desirable to know approximately what the quantity added would represent in terms of the number of cells from which it was obtained and what this quantity would be in relation to the normal bacterial content of cheddar cheese. The data obtained were useful only in a general way, since it was not possible to measure several rather important factors which were of concern. One of these unknown factors was the efficiency with which the cells were ground and the extract recovered. An arbitrary figure of 50 per cent recovery for these operations was assumed, based on rather general observations. A second unknown factor which would be extremely difficult to measure was the degree of retention of cell-free extract in the cheese curd.

The results of the counting procedures on one lot of harvested cells and on the growth medium before the separation of those cells indicated that 1 g. of the harvested cells (wet weight) contained about 24×10^{10} cells. On this basis 50 g. of cells would contain 12×10^{12} cells. Employing the correction factor mentioned above, it was calculated that the cell-free extract which was added to 260 lbs. of milk for

making the cheese of Series A and B (Tables 17 and 18) represented 6×10^{12} cells.

If the extract representing this number of cells were all retained in the cheese curd, it would represent 500,000,000 cells per gram of cheese. This assumes that 26 lbs. of cheese is approximately equivalent to 12,000 g. of cheese. This figure compares very well with data reported by Hammer (28) for plate counts on cheddar cheese early in the ripening period. Counts of more than a billion organisms per gram of cheese have been reported; however, the average counts per gram are somewhat lower than this.

Effect of the Addition of the Cell-Free Extract of *S. lactis* and Cysteine Hydrochloride, Alone and in Combination, on Cheddar Cheese Made from Pasteurized Milk

Tables 17 and 18 contain data for the cheese made employing the cell-free extract of *S. lactis* and cysteine hydrochloride.

Moisture

The initial moisture content of these cheese was approximately 36 per cent. This moisture content might preferably have been 1.5 to 2 per cent higher than was the case. The combination of slightly high acid and low moisture resulted in cheese which was a little difficult to evaluate organoleptically for typical cheddar cheese flavor development.

Table 17

The effect of the addition of a cell-free extract of *S. lactis* and cysteine hydrochloride on cheddar cheese made from pasteurized milk. (3 oz. rennet extract per 1000 lbs. milk used in all cheese.)

(Series A; made 2-4-49)

Cheese No.	Addenda	Age (days)	pH	Moisture (%)	Sol.N. (% of Tot.N.)	Flavor Score	Remarks	Body, Text. Score	Remarks
				Ripening Temp. 43° F.					
A-1	None	7	4.87	36.6	15.1				
A-2	Extract*	7	4.85	36.4	13.6				
A-3	Extr.* + cyst.**	7	4.99	35.8	13.7				
A-4	cysteine**	7	4.90	35.9	13.4				
A-1	None	42	4.90		17.8				
A-2	Extract	42	4.90		18.8				
A-3	Extr. + cyst.	42	4.98		18.4				
A-4	cysteine	42	4.92		16.7				
A-1	None	90	4.96		19.9	37.5	Acid	28.0	Mealy
A-2	Extract	90	4.95		24.5	38.5	Acid	28.5	Mealy
A-3	Extr. + cyst.	90	5.06		25.7	38.5	Sl.musty	28.0	Pasty
A-4	cysteine	90	4.87		23.7	38.5	Acid	28.0	Mealy
A-1	None	180	5.12	34.3	29.3	38.0	Sl.acid	28.5	Crumbly
A-2	Extract	180	4.85	34.7	30.3	37.5	Acid	28.5	Weak
A-3	Extr. + cyst.	180	4.95	35.4	32.2	38.5	Sl.bitter	29.0	
A-4	cysteine	180	4.99	34.7	30.9	38.0	Acid,Sl.bitter	28.5	Weak
A-1	None	260	5.11	33.9	34.5				
A-2	Extract	260	4.89	34.0	35.4				
A-3	Extr. + cyst.	260	5.09	33.8	36.4				
A-4	cysteine	260	4.96	33.6	35.7				
				Ripening Temp. 50° F.					
A-1	None	7	4.95	35.2	11.7				
A-2	Extract	7	4.80	36.8	13.3				

A-2	Extract	42	4.90		18.8				
A-3	Extr. + cyst.	42	4.98		18.4				
A-4	cysteine	42	4.92		16.7				
A-1	None	90	4.96		19.9	37.5	Acid	28.0	Mealy
A-2	Extract	90	4.95		24.5	38.5	Acid	28.5	Mealy
A-3	Extr. + cyst.	90	5.06		25.7	38.5	Sl.musty	28.0	Pasty
A-4	cysteine	90	4.87		23.7	38.5	Acid	28.0	Mealy
A-1	None	180	5.12	34.3	29.3	38.0	Sl.acid	28.5	Crumbly
A-2	Extract	180	4.85	34.7	30.3	37.5	Acid	28.5	Weak
A-3	Extr. + cyst.	180	4.95	35.4	32.2	38.5	Sl.bitter	29.0	
A-4	cysteine	180	4.99	34.7	30.9	38.0	Acid,Sl.bitter	28.5	Weak
A-1	None	260	5.11	33.9	34.5				
A-2	Extract	260	4.89	34.0	35.4				
A-3	Extr. + cyst.	260	5.09	33.8	36.4				
A-4	cysteine	260	4.96	33.6	35.7				
			Ripening Temp. 50° F.						
A-1	None	7	4.95	35.2	11.7				
A-2	Extract	7	4.80	36.8	13.3				
A-3	Extr. + cyst.	7	4.87	35.2	12.5				
A-4	cysteine	7	5.01	36.0	12.9				
A-1	None	42	4.91		18.6				
A-2	Extract	42	4.91		19.1				
A-3	Extr. + cyst.	42	5.09		21.5				
A-4	cysteine	42	4.91		18.9				
A-1	None	90	4.96		23.8	38.0	Acid	28.0	Mealy
A-2	Extract	90	4.98		25.1	37.5	Acid,Sl.bitter	27.5	Mealy,weak
A-3	Extr. + cyst.	90	5.00		24.9	38.5	Sl.acid	29.0	
A-4	cysteine	90	4.95		25.2	38.0	Acid	28.0	Mealy,weak
A-1	None	180	4.99	34.3	30.9	37.5	Acid	28.0	Weak,pasty
A-2	Extract	180	5.03	34.3	31.6	37.0	Acid,bitter	28.0	Weak
A-3	Extr. + cyst.	180	5.13	34.3	33.9	38.0	Sl.acid	28.5	Weak,mealy
A-4	cysteine	180	5.05	34.2	31.3	37.5	Sl.acid,bitter	28.0	Weak
A-1	None	260	4.98	33.8	34.9				
A-2	Extract	260	4.90	34.1	35.0				
A-3	Extr. + cyst.	260	5.06	33.6	38.4				
A-4	cysteine	260	5.00	33.9	35.6				

*Cell free extract from 50 g. (wet wt.) of S. lactis cells/260 lbs. milk.
**4 g. cysteine hydrochloride/260 lbs. milk.

Table 18

The effect of the addition of a cell-free extract of *S. lactis* and cysteine hydrochloride on cheddar cheese made from pasteurized milk. (3 oz. rennet extract per 1000 lbs. milk used in all cheese.)

(Series B; made 2-12-49)

Cheese No.	Addenda	Age (days)	pH	Moisture (%)	Sol.N. (% of Tot.N.)	Flavor Score	Remarks	Body, Text. Score	Remarks
				Ripening Temp. 43° F.					
B-1	None	7	4.92	36.1	10.6				
B-2	Extract*	7	4.89	36.7	10.6				
B-3	Extr.* + cyst.**	7	4.92	36.5	10.0				
B-4	cysteine	7	4.89	35.8	9.1				
B-1	None	42	4.90		21.4				
B-2	Extract	42	4.88		19.0				
B-3	Extr. + cyst.	42	4.87		15.1				
B-4	cysteine	42	4.89		16.1				
B-1	None	90			21.6	38.0	Acid	28.5	Mealy
B-2	Extract	90			21.1	38.5	Sl.acid	28.5	Mealy
B-3	Extr. + cyst.	90			19.7	38.5	Sl.acid	28.5	Sl.pasty
B-4	cysteine	90			18.7	38.0	Flat	28.5	Crumbly
B-1	None	180	4.94	34.7	25.1	38.5	Sl.acid	28.5	Weak
B-2	Extract	180	4.92	35.2	27.8	38.5	Acid	29.0	Mealy
B-3	Extr. + cyst.	180	4.95	35.4	26.6	38.5	Acid	28.5	Mealy
B-4	cysteine	180	4.93	34.7	25.0	38.0	Acid, Sl.bitter	28.5	Weak
B-1	None	275	4.99	34.2	29.7				
B-2	Extract	275	4.96	34.7	30.9				
B-3	Extr. + cyst.	275	4.98	34.4	31.6				
B-4	cysteine	275	4.95	34.0	28.9				
				Ripening Temp. 50° F.					
B-1	None	7	4.86	35.6	14.7				
B-2	Extract	7	4.94	36.3	10.0				
B-3	Extr. + cyst.	7	4.89	36.3	10.6				

B-1	None	90			21.6	38.0	Acid	28.5	Mealy
B-2	Extract	90			21.1	38.5	Sl.acid	28.5	Mealy
B-3	Extr. + cyst.	90			19.7	38.5	Sl.acid	28.5	Sl.pasty
B-4	cysteine	90			18.7	38.0	Flat	28.5	Crumbly
B-1	None	180	4.94	34.7	25.1	38.5	Sl.acid	28.5	Weak
B-2	Extract	180	4.92	35.2	27.8	38.5	Acid	29.0	Mealy
B-3	Extr. + cyst.	180	4.95	35.4	26.6	38.5	Acid	28.5	Mealy
B-4	cysteine	180	4.93	34.7	25.0	38.0	Acid,Sl.bitter	28.5	Weak
B-1	None	275	4.99	34.2	29.7				
B-2	Extract	275	4.96	34.7	30.9				
B-3	Extr. + cyst.	275	4.98	34.4	31.6				
B-4	cysteine	275	4.95	34.0	28.9				
				Ripening Temp. 50° F.					
B-1	None	7	4.86	35.6	14.7				
B-2	Extract	7	4.94	36.3	10.0				
B-3	Extr. + cyst.	7	4.89	36.3	10.6				
B-4	cysteine	7	4.91	36.8	10.6				
B-1	None	42			18.5				
B-2	Extract	42			18.5				
B-3	Extr. + cyst.	42			16.1				
B-4	cysteine	42			16.5				
B-1	None	90			22.0	38.0	Acid	28.0	Mealy,weak
B-2	Extract	90			21.4	38.0	Acid	27.5	Mealy,weak
B-3	Extr. + cyst.	90			23.1	38.0	Acid,Sl.bitter	27.5	Mealy,weak
B-4	cysteine	90			21.3	38.0	Acid	28.0	Weak
B-1	None	180	4.96	34.0	30.2	38.0	Acid	28.5	Weak
B-2	Extract	180	4.97	34.2	29.1	38.5	Acid	29.0	Weak
B-3	Extr. + cyst.	180	4.99	34.2	31.3	38.0	Acid	28.0	Weak
B-4	cysteine	180	4.98	34.9	29.1	38.0	Acid,Sl.bitter	28.5	Weak
B-1	None	275	4.99	34.0	31.8				
B-2	Extract	275	4.98	33.6	33.9				
B-3	Extr. + cyst.	275	5.03	33.9	33.8				
B-4	cysteine	275	5.00	33.0	32.0				

*Cell free extract from 50 g. (wet wt.) of S. lactis cells/260 lbs. milk.
**4 g. cysteine hydrochloride/260 lbs. milk.

pH

The pH values of the cheese in these series were slightly lower than would be desirable for cheddar cheese of the best quality. One-half of the cheese had pH values below 4.90 at the end of a 7 day ripening period. This rather low pH of some cheese may be reflected in some of the bitter flavors which were noted in some of the cheese.

Soluble Nitrogen

Tables 17 and 18 contain soluble nitrogen data for the two series of cheese made in this part of the investigation. The results show that in the quantities used the cell-free extract of S. lactis, alone, or in combination with cysteine hydrochloride exerted no great effect on the soluble nitrogen values of the cheese in which it was incorporated. Cysteine hydrochloride in the concentration used appeared to neither stimulate nor inhibit the ripening process of the cheese insofar as production of total soluble nitrogen was concerned.

In the cheese of Series A (Table 17) there was a trend toward larger soluble nitrogen values in the cheese containing both the cell-free extract and cysteine hydrochloride than in the corresponding control cheese. This was particularly true in the cheese of this series which was ripened at 50° F. However, in the case of the cheese of Series B (Table 18) the effect was not as pronounced.

In the overall picture, the cheese maintained at 50° F. appeared to ripen slightly faster than did the cheese held at 43° F. This effect was probably less pronounced than it would have been if the cheese were higher in moisture content and slightly less acid.

In view of the limited number of trials, it would be rather difficult to say whether any one treatment was consistently better than another. Generally speaking, none of the treatments effected more than a small amount of change in the resulting cheese.

Organoleptic Examination and Scoring of Cheese

Tables 17 and 18 contain the flavor, body and texture scores and criticisms of the cheese of these series. An acid flavor was noted in most of the cheese. This was a manufacturing defect and was not related to the experimental treatment received by the cheese. In most cases this was not considered to be too objectionable.

The cheese in Series A (Table 17) showed definite differences in flavor development after 90 and 180 days of ripening. Cheese A-2 tended to be more acid in character than the other cheese. Cheese A-3 was considered to be the best cheese of this series. This cheese developed more flavor at both ripening temperatures employed than did the three other cheese of this series. Cheese A-4 more closely resembled the control cheese in flavor, body and texture characteristics than it

did the two cheese containing cell-free extract. Differences in flavor, body and texture were less pronounced among the cheese of Series B.

Although some differences in flavor, body and texture characteristics among the cheese made employing the different treatments were noted, the lack of consistency in the results coupled with the relatively few number of trials make it difficult to come to a definite conclusion. In general, though, the cell-free extract of S. lactis, alone or with cysteine hydrochloride, did not exert any great effect upon the flavor or the body and texture of cheese in which it was incorporated.

Proteolytic Activity of the Cell-Free Extract of S. lactis Against Hemoglobin and Casein

Using the colorimetric method, it was determined that there was no measurable proteolytic activity against either casein or hemoglobin associated with the growth medium after the separation of the S. lactis cells.

There was a slight but consistent proteolysis against casein associated with the cells of S. lactis before grinding. This activity was determined by suspending approximately 1 g. of the harvested cells in 50 ml. of 0.85 per cent sodium chloride wash solution. The cells were separated from this wash solution by centrifuging in an angle-head centrifuge at 4000 r. p. m. for 30 minutes. The washed cells were resuspended in 20 ml. of 0.85 per cent sodium chloride solution. One ml. quantities of this cell suspension were tested against

5 ml. of 0.8 per cent casein solution. Small amounts of proteolysis were observed at pH 7.0 when an incubation period of 18 hours at 37° C. was used.

Several cell-free extracts were prepared as described under Methods in Part 2 of this dissertation. These extracts were tested rather extensively for proteolysis on casein and hemoglobin. Only a very feeble proteolysis was observed for these cell-free extracts against casein, and their activity, measured in milliequivalents of tyrosine present in 5 ml. of trichloroacetic acid filtrate, rarely amounted to more than 0.0001 milliequivalent of tyrosine and averaged much less.

The proteolytic activity associated with the cell-free extract of S. lactis was evident only when incubation periods of 12 hours or more were employed. This proteolytic activity which was observed both in the case of the intact cells and the cell-free extract was evident only when the amount of trichloroacetic acid used in the method was increased from 10 ml. of 0.4 N acid to 10 ml. of 1.2 N trichloroacetic acid. Other workers (52) have found it necessary to adopt a similar procedure. The reason for this probably is because the higher concentrations of trichloroacetic acid effect a more complete separation between undegraded protein and partially degraded protein. Anson (5) states that there are split products resulting from the enzymatic digestion of hemoglobin which are precipitated by concentrated trichloroacetic acid but not by dilute trichloroacetic acid. Anson (3) further stated

that, when strong concentrations (N/1) of trichloroacetic acid were used along with extended incubation periods (18 to 24 hours), the proteolysis which resulted may or may not have been due to the action of a proteinase.

Although it was possible consistently to demonstrate proteolysis of casein and hemoglobin with the cell-free extract of S. lactis, this activity was so small that it was impossible to obtain reproducible results from one experiment to another. From the results obtained, it was concluded that only a very feeble proteolytic activity against either casein or hemoglobin was present in the cell-free extract of S. lactis. On the basis of these results, it would be presumptious to make any definite conclusions as to the nature of the proteolytic system present in the cell-free extract of S. lactis.

Peptidase Activity of a Cell-Free Extract of S. lactis

Two separate cell-free extracts of S. lactis were used to determine peptidase activity. These extracts were prepared as nearly as possible in the same manner. Neither of the extracts was dialyzed for the determination of peptidase activity. These extracts are referred to subsequently as enzyme preparations I and II.

Concentration of Enzyme in Reaction Mixture

Figure 9 contains representative data showing hydrolysis of glycyl-L-leucine and Dl-alanylglycine with increasing

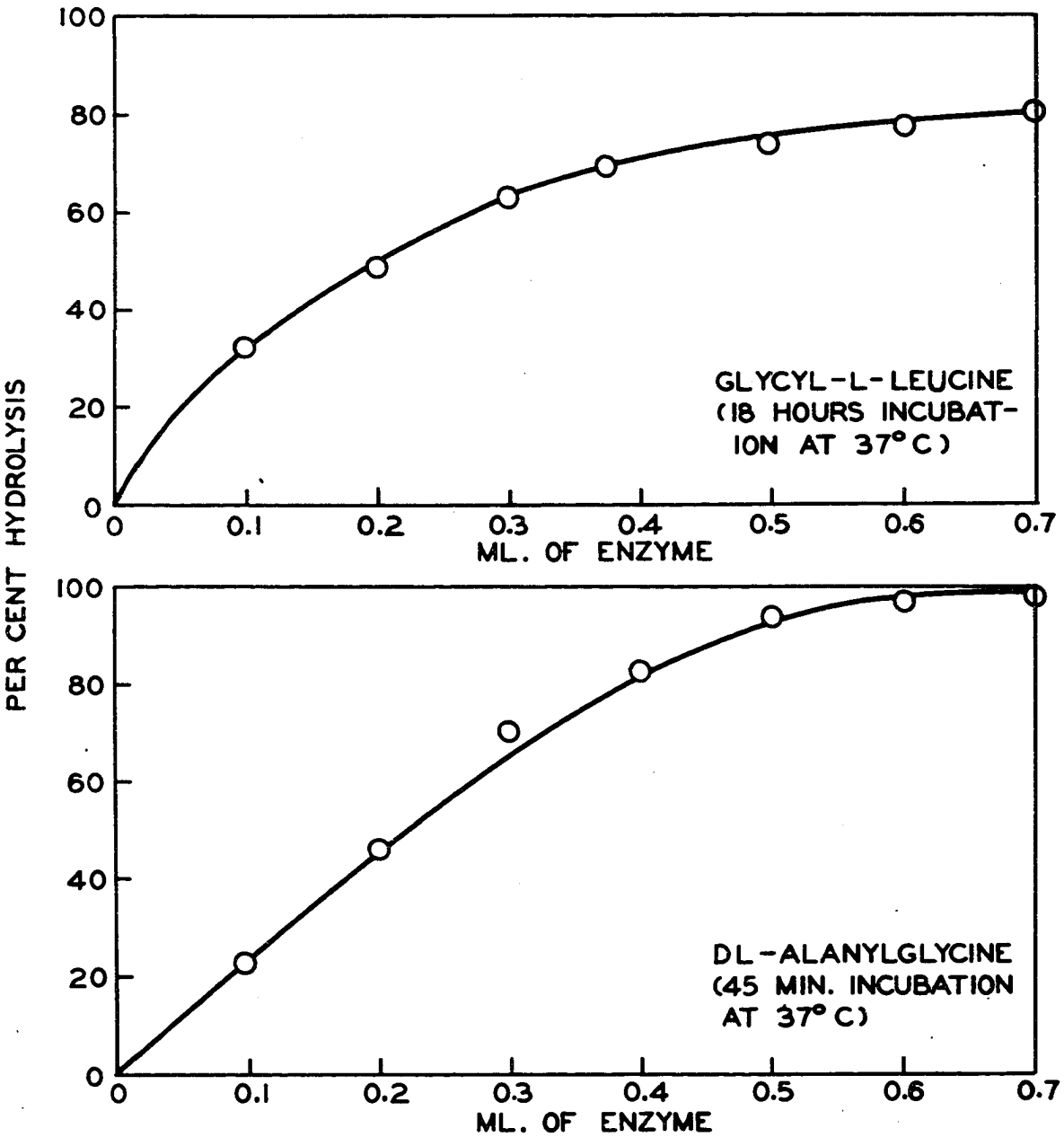

Fig. 9. Hydrolysis of peptides at pH 8.0 with increasing quantities of enzyme preparation II.

quantities of enzyme preparation II. For glycyl-L-leucine, a straight line relationship was observed for hydrolysis when enzyme quantities up to 0.2 ml. were employed. When 0.3 to 0.7 ml. quantities of enzyme were employed the curve tended toward the horizontal.

When DL-alanylglycine was used as a substrate, a linear relationship between per cent hydrolysis of substrate and quantity of enzyme preparation was evident for enzyme quantities up to 0.3 ml. The curve tended to fall toward the horizontal as quantities of from 0.4 to 0.7 ml. of enzyme preparation were tested.

Figure 9 also shows that the substrate DL-alanyglycine was hydrolyzed much more rapidly by enzyme preparation II than was glycyl-L-leucine. Approximately 50 per cent hydrolysis was obtained in 18 hours with 0.2 ml. of preparation II for glycyl-L-leucine. The same amount of hydrolysis of DL-alanylglycine was obtained in only 45 minutes with the same quantity of enzyme preparation II. Preliminary results using enzyme preparation I were similar to those in Figure 9.

Influence of pH on the Reaction

Some preliminary work with enzyme preparation I indicated that a pH optimum for this crude extract against glycyl-L-leucine existed at approximately 8.0. However, this work was done without the use of buffers in the enzyme-substrate reaction mixture. It was found that the changes in pH when using

unbuffered substrate were sometimes quite considerable. This was particularly true when working in the pH ranges 4.5 to 6.5 and 8.0 to 10.0. Consequently, it was decided to employ buffers, and all of the data reported in this section were obtained using buffers as described on page 91.

Figures 10 and 11 contain representative data for the hydrolysis of glycyl-L-leucine and DL-alanylglycine with enzyme preparations I and II at various pH values. The results for the hydrolysis of glycyl-L-leucine with preparation II at pH values from 5.0 to 9.5 are plotted in figure 10. This plot shows a distinct optimum at pH 7.6 to 7.8. There appears to be but a single optimum for the crude enzyme preparation against glycyl-L-leucine, and the curve rises and falls quite symmetrically about this point.

For the hydrolysis of DL-alanylglycine with the enzyme preparations from S. lactis, somewhat different curves are obtained when the results are plotted. It is seen from Figure 11 that with preparation I a rapid increase in activity occurs from pH 5.0 to approximately pH 6.3. The curve flattens at this point and rises to its maximum value at approximately pH 8.0. The activity with preparation I then decreases rather rapidly as the reaction passes from pH 8.5 to pH 9.0.

In the case of preparation II, the results plotted in Figure 11 indicate somewhat the same relationship. Between pH 6.5 and pH 8.0 the curve tends to form a plateau, reaching

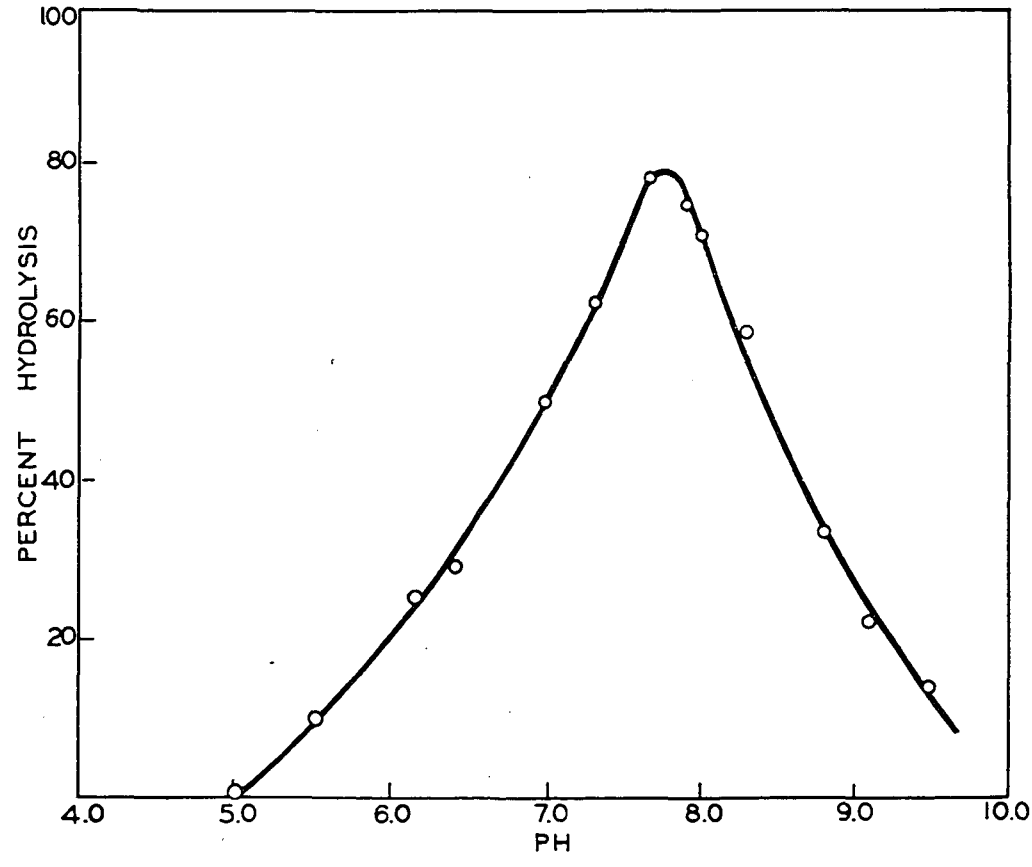

Fig. 10. pH optimum for enzyme preparation II against glycyl-L-leucine. (0.5 ml. of enzyme incubated for 18 hrs. with substrate at 37° C.)

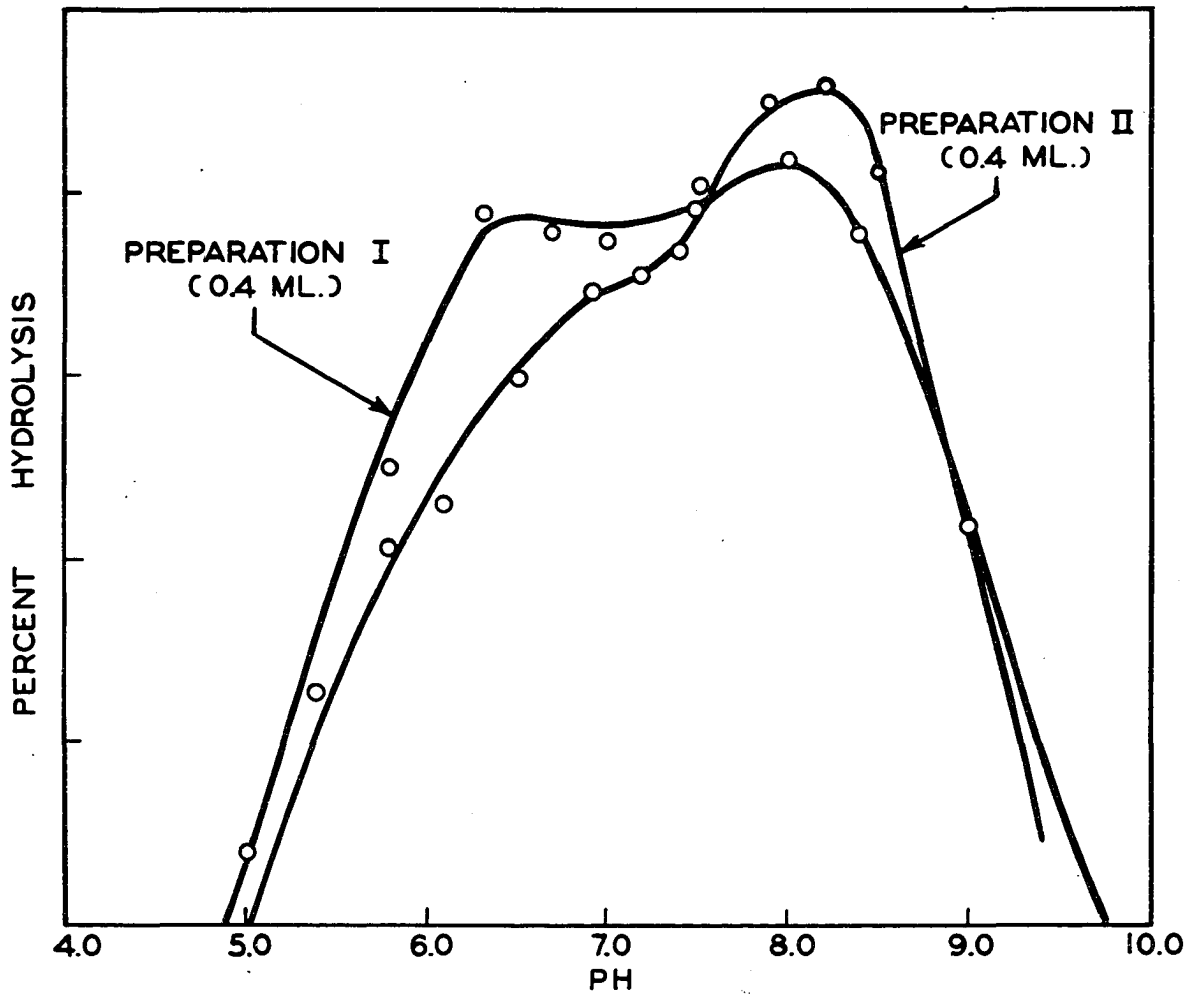

Fig. 11. pH optima for enzyme preparations I and II against DL-alanylglycine. (Incubation for 40 (I) and 45 (II) minutes at 37° C.)

its maximum height at pH 8.1 to 8.3. For the hydrolysis of DL-alanylglcine, it would appear that there was a somewhat wider pH range for activity of the enzyme preparation than was the case in the hydrolysis of glycyl-L-leucine.

Influence of Metallic Ions on the Peptidase Reaction

The effect of certain metallic ions on the peptidase activity of the crude enzyme preparations from S. lactis was studied as a final part of this investigation. The metallic ions chosen for study were Mn^{++}, Cu^{++}, Zn^{++}, Mg^{++} and Ni^{++}. These ions were used as sulfates, with the exception of nickel, which was used as the chloride.

The effect of these metallic ions, in several concentrations, on the hydrolysis of glycyl-L-leucine by preparations I and II are summarized in Table 19. It is seen that the manganese ion increased the hydrolytic activity. The manganese ion in concentrations from 10^{-2} to 10^{-5} M stimulated the activity of both enzyme preparations.

The effect of the ions of copper, zinc and nickel in concentrations of 10^{-2} and 10^{-3} M was to retard hydrolysis. The zinc ion appeared to inhibit hydrolysis most severely, while copper and nickel were somewhat intermediate in their inhibitory action. In the case of magnesium, little or no effect on the hydrolysis of glycyl-L-leucine was observed at any of the ion concentrations tested.

Table 20 gives the results of a similar series of investigations employing DL-alanylglycine as substrate. Only prepar-

Table 19

Effect of metallic cations on enzymatic hydrolysis of glycyl-L-leucine. (pH 8.0, 37° C.)

Enzyme Preparation	Metallic Ion*	Per Cent Hydrolysis at Metal Concentrations			
		10^{-2}M	10^{-3}M	10^{-4}M	10^{-5}M
I (0.18 ml., 17 hrs. incubation period.)	Control	49			
	$Mn^{++}(SO_4)$	-	81	83	-
	$Cu^{++}(SO_4)$	26	29	42	-
	$Zn^{++}(SO_4)$	5	10	45	-
	$Mg^{++}(SO_4)$	-	43	48	-
	$Ni^{++}(Cl_2)$	-	27	36	-
II (0.4 ml., 18 hrs. incubation period.)	Control	60			
	$Mn^{++}(SO_4)$	90	86	88	75
	$Cu^{++}(SO_4)$	20	46	55	85
	$Zn^{++}(SO_4)$	11	14	52	52
	$Mg^{++}(SO_4)$	54	61	62	59
	$Ni^{++}(Cl_2)$	9	46	58	57

*The respective salts in the stated concentrations were incubated with enzyme preparation for 2 hrs. at 37° C. prior to addition of substrate.

Table 20

Effect of metallic cations on enzymatic hydrolysis of DL-alanylglycine.* (pH 8.0, 37° C.)

Enzyme Preparation	Metallic Ion**	Per Cent Hydrolysis at Metal Concentrations			
		10^{-2}M	10^{-3}M	10^{-4}M	10^{-5}M
II (0.2 ml., 40 min. incubation)	Control	55			
	$Mn^{++}(SO_4)$	11	16	31	50
	$Cu^{++}(SO_4)$	26	52	59	58
	$Zn^{++}(SO_4)$	-	24	54	57
	$Mg^{++}(SO_4)$	40	59	56	59
	$Ni^{++}(Cl_2)$	16	26	46	50

*Per Cent hydrolysis of one optical isomer.

**The respective salts in the stated concentrations were incubated with enzyme preparation for 2 hrs. at 37° C. prior to addition of substrate.

ation II was tested in this case. The results with this substrate are somewhat different than those obtained using glycyl-L-leucine. Instead of increasing enzymatic hydrolysis, manganese quite severely inhibited hydrolysis in all concentrations except that of 10^{-5} M.

Copper, zinc and nickel also definitely retarded hydrolysis, but not as drastically as did manganese. Copper inhibited only in the relatively high concentration of 10^{-2} M. Zinc inhibited at 10^{-3} M concentration but not at 10^{-4} or 10^{-5} M concentrations. Nickel retarded hydrolysis in 10^{-2}, 10^{-3} and 10^{-4} M concentrations but had only slight effect at a concentration of 10^{-5} M. The magnesium ion apparently was inhibitory in a concentration of 10^{-2} M but was without appreciable effect at the lower concentrations.

DISCUSSION

It seems obvious from a review of the investigational work carried out relative to the proteolytic changes which occur in cheddar cheese that the responsible factors may be divided into two groups. These are the proteolytic enzymes of rennet extract added in the manufacture of the cheese and the proteolytic enzymes associated with the bacteria contained in the cheese. There no longer seems to be any doubt that the proteolytic enzymes of commercial rennet extract play a rather important role in the hydrolysis of cheese protein. However, it is apparent from this investigation that enzymes other than those of rennet extract must be involved. By a process of elimination, the proteolytic enzymes of bacteria contained in the cheese constitute a second group of enzymes probably responsible for proteolysis in cheddar cheese.

Although the different investigators have disagreed upon the relative importance of rennet extract in the hydrolysis of cheese protein, most workers have shown that rennet does contribute significantly to the observed proteolytic changes. It is felt that some of the conflicting results with rennet extract in cheese have arisen as a result of different methods of analysis of cheese protein during the

ripening period. Other conflicting results have come about through measurements of proteolysis under conditions which were not at all normal in the cheese. Thus, the use of chloroform (6) in an attempt to eliminate the bacterial factor in cheese ripening would not appear to be a desirable procedure. The main objection to the use of chloroform in a cheese ripening study is that it results in a product which is organoleptically almost impossible to evaluate.

While some of the different findings relative to the action of rennet extract in cheddar cheese may be explained on the basis of the different investigational procedures involved, it is difficult to explain some of the more or less opposite findings in this regard. An example of such is the work of Peterson, Johnson and Price (53), in 1948. These workers concluded that only a small portion of the total proteinase activity in cheddar cheese was contributed by the rennet extract. Their results indicated a very gradual increase in the proteinase content of cheddar cheese during a 300 day ripening period. The proteinase content of the cheese during the first 24 hours of the ripening period was extremely small and only about one-fortieth of what it was at the end of the ripening period. These results are incompatible with those obtained in the present investigation. This apparent anomaly may possibly be explained on the basis of the method which these workers used to prepare the cheese samples for the estimation of proteinase activity. These workers blended

a sample of cheese in distilled water in a Waring blendor run at low speed for 2 minutes and at high speed for an additional 7 minutes. Somewhat the same procedure was used in the present investigation, and it was found that the blending produced rather considerable amounts of foam. Schmidt-Nielsen and Schmidt-Nielsen (56) reported that rennin was irreversibly denatured through precipitation in the walls of a foam and vigorous shaking deactivated the enzyme for that reason.

In addition to the treatment given the cheese sample to be analyzed for proteinase content described above, the blending operation was followed by passing the sample through a hand homogenizer five to six times before addition of a portion of the sample to the substrate solution. Following the addition of the enzyme-containing suspension to the substrate solution, the resulting mixture was shaken vigorously for 30 seconds and then run quickly through a hand homogenizer two to three times. Probably the enzymes of rennet were impaired by this rather drastic treatment. Proteinase activity due to them may have been at a very low level if not completely lacking in the cheese samples handled as described by these workers.

As to the effect of rennet extract on flavor development in cheddar cheese, it is apparent that rennet is only one of several factors involved in this phenomenon. Most workers have agreed that the use of abnormally large quantities of rennet

extract in the manufacture of cheddar cheese leads to some improvement in flavor. However, increasing the amount of rennet extract employed in the manufacture of cheese does not solve entirely the problem of flavor development. An explanation of this may be that the products of digestion of cheese protein by the enzymes of rennet extract are confined to the higher decomposition bodies. Well-ripened cheese, on the other hand, has been associated with an increase in the amino nitrogen fraction during the ripening period.

The chief objection of some workers to the use of abnormally large quantities of rennet extract in the manufacture of cheddar cheese has been that the body and texture of the resulting cheese were of inferior quality. This effect was not observed in the present investigation. The use of 6 ounces of rennet extract per 1000 lbs. of milk gave better results for the body and texture of cheese in which it was contained than did either 2 or 4 ounces of rennet extract. It is possible that this effect may be due to the lower moisture content of the cheese which was made for this investigation. The effect on body and texture probably should be determined by each individual cheesemaker when contemplating the use of increased amounts of rennet extract as an aid in the ripening of cheddar cheese.

In the preparation of rennin according to Hankinson (29), some difficulty was encountered on one occasion in obtaining a fully active preparation at the end of the purification

procedure. Another worker (11) has stated that difficulty was encountered in getting the method of Hankinson to work satisfactorily. It was felt that the difficulty in the present investigation was caused by inadvertently adjusting the pH of the solution during the purification procedure to about 7.0. Hankinson states that the pH zone should be limited to 4.5 to 6.5, and another worker (47) has shown that rennin is slowly denatured at pH values in excess of 6.0.

From the known characteristics of rennin, it would not be expected that its action in cheddar cheese would be much different than rennet extract. Such was the case in the relatively few trials made in the course of this investigation. Freeman and Dahle (26) found that incorporation of pure rennin in addition to the usual amount of rennet extract in cheddar cheese resulted in greater proteolysis in that cheese. Also, the quantities of rennin added were of only small value in hastening the production of flavor. Although there was a rather small number of trials, the results attained were in accordance with those of the present investigation.

In some of the reported work comparing the action in cheese of pure rennin and pure pepsin with that of rennet extract, the interpretation of the results has been difficult because the enzyme quantities used were not equal in their effective concentrations. Sherwood (58) came to the conclusion that protein breakdown due to pepsin in cheese was much

less than that due to rennet extract. It may be noted that the milk coagulation time when pepsin was employed was longer than the time when rennet was employed. In fact, the difference in coagulating time was eliminated by ripening the milk to 0.195 per cent acid when pepsin was the coagulant employed. It is felt that the use of equal coagulating quantities of enzyme affords a much better basis of comparison than any other method which has been suggested.

The method which was used to measure proteolytic activity of the enzymes of rennet on hemoglobin is specific for proteinase activity, according to Anson (3). Anson states that when the hemoglobin method is used only the first stages of digestion are measured, for only a small amount of digestion is needed to make hemoglobin unprecipitable by trichloroacetic acid, and, therefore, only true proteinase is estimated. In the hemoglobin method as adopted for use in this investigation, a hemoglobin powder (Merck) was used with entirely satisfactory results. Other workers (50) recently have reported good results using almost the same method as reported herein. Centrifugation of the trichloroacetic acid-precipitated protein was employed in preference to filtration, since more reproducible results could be obtained.

The results of the present investigation in regard to the proteolysis of hemoglobin by rennin have shown that rennin possesses hydrolytic as well as coagulating properties. Not only has it been shown to have a hydrolytic action against

hemoglobin, but the results of this investigation have shown that it is able to hydrolyze casein at a pH not far removed from that of ripening cheddar cheese.

The action of the rennet enzymes on casein were studied at pH 5.4 since this has been reported by several workers (46) as the iso-electric point of rennin and the region at which it coagulates milk optimally. According to the results plotted in Figure 8, rennin has much more pronounced proteolytic activity at pH 5.4 than it does at pH 2.0. This would indicate that the rennin had been freed of pepsin to some extent. Whether or not rennin is able to hydrolyze casein at pH 2.0 would influence any conclusion as to the relative purity of the enzyme as determined on the above basis. The work of Berridge (16) with a rather pure rennin preparation indicated that hemoglobin was proteolyzed at pH 2.0 by rennin.

The action of rennet extract on casein was approximately the same at pH 5.4 as it was at pH 2.0. The data plotted in Figure 8 indicate that rennin possessed about 80 per cent as much activity against casein at pH 5.4 as did the rennet extract from which it was obtained. This is good evidence that the rennin which is contained in the rennet extract probably is responsible for the greater part of the protein hydrolysis of ripening cheese. This conclusion is based on the assumption that a separation between pepsin and rennin was effected in the preparation of rennin from commercial rennet extract.

A comparison of the proteolytic activities of rennet

extract and rennin against casein at pH 2.0 (Figure 8) indicated that rennin possessed about 50 per cent as much activity as rennet extract. This is additional evidence that commercial rennet extract is composed of pepsin and rennin, both of which are capable of hydrolyzing casein.

The work which has been reported herein has indicated a very strong relation between coagulating activity and extent of proteolysis by the respective enzymes. Increases in the quantities of enzyme tested resulted in increases in proteolysis of hemoglobin and casein. It was noted that these increases in proteolytic activity never were of an exactly linear nature, even when measured at pH values supposedly optimum for the enzymes being tested. However, neither rennin nor rennet extract obeyed the so-called law of Segelcke and Storch under the experimentally established conditions; the product of the time required for coagulation and the concentration of enzyme was not a constant but increased as the quantity of standard enzyme solution was increased from 1 to 4 ml. The evidence linking coagulation with proteolysis indicates that coagulation may be only the first step in the hydrolysis of casein, and that these two processes are not separate and distinct.

A critical review of the work dealing with proteolysis by S. lactis in milk and cheese leads one to several conclusions. That S. lactis organisms growing in pure culture in skim milk are able to attack the casein contained therein and degrade

it at least to the amino acid stage has been definitely established. A further fact in this connection is that these enzymes are inhibited to some extent by the acid which arises as a result of organism growth. Whether the inhibitory effect of the acid is a true inhibition of the proteolytic enzymes or whether the effect is a manifestation of the cessation of growth at pH values of approximately 5.0 is not known. That inhibition of proteolytic enzymes may be one of the reasons for growth stoppage is, of course, a possibility. It has been demonstrated in this investigation that hydrolysis of two synthetic peptides by a crude extract of S. lactis cells was very slight at pH values approximating 5.0.

Arranged alongside this evidence are the facts as they are known concerning the hydrolysis of cheese protein during the ripening period. There are enzymes present in cheese, other than those contained in rennet extract, which proteolyze the casein. These enzymes are capable of hydrolyzing the casein to amino acids and ammonia, while the extent of proteolysis in cheese by rennet extract has been shown to be largely confined to the higher protein decomposition products. Thus, the extent of proteolysis by the enzymes of rennet is not believed to be a critical factor in regard to the development of cheddar cheese flavor.

From the above discussion, and from the results obtained in the present investigation, one might predict that proteolysis in cheddar cheese due to the action of S. lactis would be much

less pronounced than that due to rennet extract. In addition, there is another factor which has not been mentioned before which would contribute to the apparent inactivity of the enzymes of S. lactis. If the enzymes which are concerned are endo-cellular in nature, their activity may have to wait upon death and autolysis of the bacteria in which they are contained.

In the light of the above discussion, it is not altogether strange that the cell-free extract of S. lactis added to the milk used for cheddar cheese manufacture in the present studies produced no consistent change. However, there are influencing factors in regard to the results obtained which were more or less impossible to control. Whereas the greater part of the bacteria contained in the milk are retained in the cheese-curd, it is not known what portion of the cell-free extract which was added to the milk was retained in the curd. It is not definitely known that the enzymes responsible for the proteolysis which has been ascribed to S. lactis are endo-cellular in nature. The work of Collins and Nelson (19), in 1949, indicated that a very substantial portion of the total soluble nitrogen produced by S. lactis in skim milk culture was formed during the first 24 hours of growth. This would seem to indicate that the activity of the enzymes responsible for the observed degradation of casein would not have to wait upon death and autolysis of the bacteria from which they were produced.

As to the effect of the cysteine added in the manufacture

of the cheese, it is possible that the quantity added was too small to effect activation of whatever enzymes in the cheese might have been cysteine-activated. It is pertinent to point out here that Peterson et al. (53) found that the greater portion of the active proteinase in cheddar cheese was of bacterial origin with only a small portion of the total activity being contributed by the milk and rennet. In addition, it was found that a portion of the proteinase of cheddar cheese could be activated by cysteine.

It also is not too surprising that the cell-free extract of S. lactis showed only a feeble hydrolytic action on hemoglobin and casein substrates under the experimentally established conditions. Unpublished data (23) by another worker in this laboratory have shown that the proteolytic enzymes of S. liquefaciens are adaptive in nature. It has been found that casein was not proteolyzed to any great extent by the exo-cellular enzymes of S. liquefaciens unless casein was included in the medium used for the growth of the organisms and elaboration of the enzymes. It is quite probable that the proteolytic enzymes of S. lactis may be similar to those of S. liquefaciens, and, if such is the case, some of the difficulty encountered in the present investigation may be explained on this basis.

Although this latter portion of the investigation concerned with proteolytic activities of S. lactis gave largely negative results, measurement of the peptidase

activity of the cell-free extract of S. lactis gave much more clearly definable results. This study of peptidase activity using synthetic peptides as substrates was intended to be only of a preliminary nature in this investigation. However, there are indications that a pursuance of this particular phase of the problem might yield valuable information relative to the cheese ripening problem. This should be true not only relative to the enzymes of S. lactis but also to the enzymes of the lactobacilli.

The results obtained in regard to the effect of certain metals on the hydrolysis of synthetic peptides by the cell-free extract of S. lactis possibly may provide evidence as to why very small amounts of copper are effective in retarding the development of normal cheddar cheese flavor. It is known that flavor development can be improved if the copper content of cheese be reduced below 2 p. p. m. by removing all exposed copper from the cheese factory. If copper alone is capable of exerting such an influence, it is possible that other metals may act in a similar fashion. This phenomenon seems worthy of investigation, and it would appear that investigations similar to that dealing with the peptidases of S. lactis would offer a point of attack.

SUMMARY AND CONCLUSIONS

1. Several series of cheese were manufactured employing quantities of from 1.5 to 8 ounces of rennet extract per 1000 lbs. of milk. The effect of these quantities of rennet extract on soluble nitrogen, and on the flavor, body and texture characteristics of the resulting cheese were determined. Rennet extract was shown to be a very important agent in the hydrolysis of cheese protein. However, rennet extract was not responsible for all of the soluble nitrogen produced in cheese during a ripening period of 180 days at 43° F. Of the total soluble nitrogen present in cheese at 42 days of ripening, approximately 16 per cent was formed by the action of 2 ounces of rennet extract. This value had increased to approximately 23 per cent at the end of a 180 day ripening period.

2. The use of 6 ounces of rennet extract per 1000 lbs. of milk resulted in cheese having slightly higher flavor scores than for cheese made with 2, 4 and 8 ounces of rennet extract.

3. In the cheese which was manufactured for this investigation, the use of quantities of rennet extract up to 8 ounces per 1000 lbs. of milk did not result in objectionable body and texture characteristics during a 180 day ripening period at 43° F. The use of quantities of rennet extract of from

1.5 to 8 ounces per 1000 lbs. of milk introduced no objectionable irregularities in the manufacturing process of the cheese.

4. In all of the cheese which was examined it appeared that the use of increased quantities of rennet extract contributed to both flavor and body and texture development. The differences in flavor scores were slight, however, the cheese containing the larger quantities of rennet extract consistently scored higher in flavor than the cheese with the smallest quantity of rennet. The effect on body and texture development probably was relatively more important than the effect on flavor development.

5. The effects of rennet extract, rennin and two rennet powders were compared in cheddar cheese made from pasteurized milk. These enzymes were used in amounts which were approximately equal in their coagulating activity against milk. No consistent differences in soluble nitrogen production or in the flavor, body and texture characteristics among the resulting cheese were observed.

6. A rennin preparation was determined to have about 13 times the milk-coagulating activity that the commercial rennet extract from which it was obtained had per unit weight of nitrogen and about 10 times as much activity per unit of salt-free dry weight. The relative milk coagulating activities of the rennet powders were from 6 to 8 times that of commercial rennet extract on a nitrogen basis. On a salt-free dry weight basis, rennet powder A had about the same coagulating activity as rennet extract, while rennet powder B was about 5 times as active as rennet extract.

7. Proteolysis by rennet extract, rennet powders A and B and rennin was studied using hemoglobin and casein as substrates.

Maximum values for proteinase activity of commercial rennet extract, rennet powder A, rennet powder B and rennin on hemoglobin were obtained from pH 3.5 to 3.7. For all of these enzyme preparations, the activity against hemoglobin at pH 5.0 was practically negligible.

8. The proteinase action of rennin was differentiated from that of commercial rennet extract. Smaller values for proteinase activity from pH 1.5 to 2.5 were obtained with rennin than with rennet extract. This indicated that pepsin was present in rennet extract, and that it was partially or completely removed in the rennin purification procedure.

9. The proteinase activity of rennet extract on hemoglobin at pH 3.5 to 3.7 was apparently due to the action of both pepsin and rennin but was considered to be largely due to rennin.

10. Powder A was intermediate between rennet extract and rennin in its proteinase activity against hemoglobin. Powder B more nearly resembled rennin in regard to its activity on hemoglobin.

11. The action of rennet extract on casein was approximately the same at pH 5.4 as it was at pH 2.0. Rennin possessed about 80 per cent as much activity against casein at pH 5.4 as did the rennet extract from which it was obtained. From these facts, it was concluded that rennet extract was composed of both pepsin and rennin and that the rennin which was contained in the rennet extract probably was responsible for

the greater part of the protein hydrolysis of ripening cheese. The results obtained in the hydrolysis of casein, using different concentrations of rennet extract, were parallel to the results obtained for soluble nitrogen in cheese employing different concentrations of rennet extract.

12. The action of the rennet powders on casein was more pronounced at pH 5.4 than at pH 2.0. In this respect they more nearly resembled the action of rennin than rennet extract.

13. Addition of cell-free extract of S. lactis to cheddar cheese did not cause any appreciable difference in rate of cheese ripening. Cysteine hydrochloride added in combination with the cell-free extract of S. lactis or by itself did not alter the rate of ripening of the cheese in which it was incorporated.

14. There was not enough proteolytic activity associated with the cell-free extract of S. lactis, as prepared in this study, to be measured accurately, using either hemoglobin or casein as substrate.

15. Measurements of hydrolysis of glycyl-L-leucine with the cell-free extract of S. lactis indicated an optimum at pH 7.6 to 7.8. For the hydrolysis of DL-alanylglycine with the cell-free extract of S. lactis, the pH range of optimum activity was less well defined. An optimum was indicated at about pH 8.0.

16. The hydrolysis of DL-alanylglycine proceeded much more rapidly than did the hydrolysis of glycyl-L-leucine.

17. Manganese ions increased the rate of hydrolysis of glycyl-L-leucine by the cell-free extract of S. lactis. Copper, nickel and zinc ions retarded hydrolysis, while magnesium ions had no apparent effect. When DL-alanylglycine was used as substrate, the presence of manganese ions inhibited hydrolysis, while copper, nickel and zinc ions inhibited but to a lesser degree than did manganese. Magnesium ions were without appreciable effect.

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