tives allows the zones to be visible without column extrusion, the *p*-phenylazobenzoates are not as well characterized as the acetates and are more difficult to prepare. Since the acetate group is relatively small, inherent differences in the carbohydrate structure are not so masked as when a larger substituent is employed. Thus, the sugar residue constitutes 44.9% of glucose pentaacetate but only 14.3% of glucose penta-*p*-phenylazobenzoate.

Experimental

The adsorbent employed was "Magnesol" (Westvaco Chlorine Products Co., South Charleston, West Virginia), a synthetic hydrated magnesium acid silicate ($2MgO \cdot 5SiO_2$).[12] It is necessary to examine the extrusive and adsorptive properties of each new lot of this material. "Celite" (No. 535, Johns-Manville Co., New York, N. Y.) was used as a filter aid. A mixture of 5 parts (by wt.) of "Magnesol" and 1 part of "Celite" was extracted at room temperature with a large volume of acetone and air-dried at room temperature. An amount of 50 g. of this mixture was packed in a chromatographic tube (35 mm. in diameter, 230 mm. in length) with the aid of suction. After the top of the column had been moistened with 10 cc. of benzene, there was added 5 cc. of a benzene solution containing *ca.* 250 mg. of a 1:1 mixture of the two sugar acetates to be separated. The amount and composition of the benzene (thiophene-free)–ethanol (absolute) solution then employed for developing each column is shown in Table II. The column was extruded and immediately brushed with a freshly prepared 1% solution of potassium permanganate in 2.5 *N* sodium hydroxide. The zones were detected by the appearance in the brush streak of yellow to orange bands separated by green interzones of adequate width. The brush mark exhibited a series of color changes from an initial green, through a yellow and orange, to a final light brown. The green interzones were caused by the relatively slower reaction of the solvent on the column. Eventually, the brush streak assumed a uniform, light brown color.

(12) U. S. Patent 2,076,545 (1937); *C. A.*, **31**, 4023 (1937); U. S. Patents 2,163,525, 2,163,527, 2,163,528 (1939); *C. A.*, **33**, 8049 (1939).

The average rates of appearance of the zones are shown in Table I. The zones were cut out and the brush marks removed with a scalpel. Elution of each zone was effected with 125 cc. of acetone and the acetone was removed by evaporation at room temperature. The properties and yields of the residues so obtained are shown in Table II. Adequate purity was present without any further purification.

In a typical separation, the chromatogram was developed in ninety minutes. The brush reagent located a β-maltose octaacetate zone (35 mm. broad) 25 mm. below the top of the column and beneath this, separated by a 60-mm. interzone, the β-D-glucose pentaacetate zone (22 mm. broad). Different lots of "Magnesol" may lead to variations in these data.

Preliminary experiments indicated that the differences in adsorption affinity between the following pairs would be such that rechromatographing would probably be required for their separation: α- and β-D-glucose pentaacetate; α-D-arabinose tetraacetate and α-D-glucose pentaacetate; α-D-arabinose tetraacetate and *keto*-D-fructose pentaacetate; D-mannitol hexaacetate and (*levo*)-sorbitol hexaacetate; β-D-glucose pentaacetate and (*levo*)-sorbitol hexaacetate.

Summary

The chromatographic brush method with aqueous alkaline permanganate as brush reagent and "Magnesol" as adsorbent has been employed successfully in the separation of a selected number of two component (equal portions) mixtures of fully acetylated sugars and sugar alcohols. The following separations were effected: β-D-glucose pentaacetate (I) from β-maltose octaacetate (II); I from sucrose octaacetate; I from *keto*-D-fructose pentaacetate; I from raffinose hendecaacetate; II from sucrose octaacetate; II from raffinose hendecaacetate; raffinose hendecaacetate from sucrose octaacetate; (*levo*)-sorbitol hexaacetate from L-rhamnitol pentaacetate. The components were recovered in nearly quantitative yield and in excellent purity.

COLUMBUS, OHIO RECEIVED NOVEMBER 20, 1944

[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY,[1] PHILADELPHIA, PENNSYLVANIA]

On the Presence of a Proteolytic Enzyme in Casein

BY ROBERT C. WARNER AND EDITH POLIS

The presence of a proteolytic enzyme in milk was first reported by Babcock and Russell.[2] They studied the decomposition of skim milk in the presence of antiseptics and the formation of soluble nitrogenous products during the ripening of cheese and attributed the observed proteolysis to an enzyme, "galactase." Others[3,4] have studied the same problem, and the general results of Babcock and Russell have been confirmed. No successful preparations of concentrates of the enzyme have been made.

We have found that almost all of the proteolytic activity in milk is precipitated along with the casein when milk is acidified. The activity is extremely difficult to separate from casein and is absent from the usual casein preparations only if they have been exposed for long periods to alcohol. It is thus present in both commercial casein and most purified laboratory preparations.

The fact that casein may undergo proteolysis in solution with the formation of products soluble at *p*H 4.6 has been noted by Robertson[5] and Walters.[6] However, they believed that their

(1) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture. Article not copyrighted.

(2) Babcock and Russell, *Ann. Rep., Wisconsin Agr. Exp. Sta.*, **14**, 161 (1897); Babcock, Russell and Vivian, *ibid.*, **15**, 77, 93 (1898).

(3) Van Slyke, Harding and Hart, N. Y. Agr. Exp. Sta. Bulletin 103, 215 (1901).

(4) Thatcher and Dahlberg, *J. Agr. Research*, **11**, 437 (1917).

(5) Robertson, *J. Biol. Chem.*, **2**, 317 (1906–1907).

(6) Walters, *ibid.*, **11**, 267 (1912); **12**, 43 (1912).

casein preparations were free from enzymes and that the "autohydrolysis" was an inherent property of casein.

The proteolysis caused by the enzyme may have considerable influence on the results of chemical studies on casein, especially those dealing with fractionation, depending on the conditions under which they are carried out. A knowledge of the presence and behavior of the enzyme is also of importance in the commercial applications of casein.

Experimental

Our attention was first drawn to this problem by the observation that a concentrated solution of commercial casein in borax rapidly decreases in viscosity with time. This effect has been noted in the literature,[7] but no particular attention appears to have been paid to it. It is accompanied by an increase in the concentration of nitrogenous products which are soluble at *p*H 4.6 and is abolished by heating the solution to 80° for ten minutes. There is thus presumptive evidence that the changes may be the result of the activity of an enzyme which slowly hydrolyzes casein.

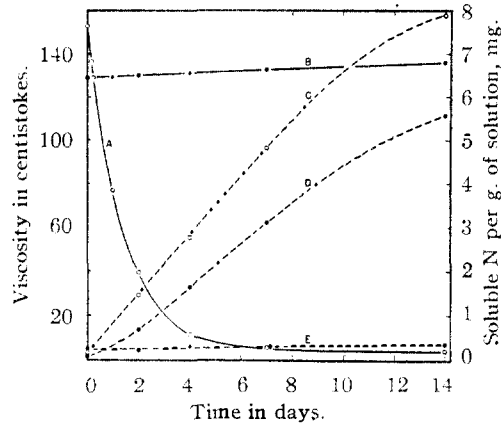

Fig. 1.—Changes in viscosity and soluble nitrogen in a casein solution at *p*H 8.6: A, viscosity of unheated solution; B, viscosity of heated solution; C, nitrogen soluble at *p*H 4.6 in unheated solution; D, nitrogen soluble in trichloroacetic acid in unheated solution; E, nitrogen soluble at *p*H 4.6 in heated solution.

These processes and their dependence on the *p*H of the solution are shown in detail by the following experiment. Reprecipitated casein prepared as previously described[8] was dissolved in borax (0.021 g. per g. of solution) and hydrochloric acid or sodium hydroxide was added to obtain the desired *p*H. The casein was made up to a concentration of 9.71% by weight. The kinematic viscosity was determined in a capillary viscometer at 25° at intervals after making up the solution. The viscosity drop during the first few hours was linear with respect to time and the viscosity at zero time, *i. e.*, at the time the alkali was added to the casein, was determined by extrapolation.

The increase in soluble nitrogen at 25° was followed by two methods. (1) A sample of one gram of the solution was weighed into a 25-ml. volumetric flask and was precipitated by adding 2.5 ml. of a 1 *M* acetate buffer of *p*H 4.6 plus sufficient hydrochloric acid to bring the sample to this *p*H. After being made to volume, the contents were filtered, and nitrogen was determined on an aliquot of the filtrate by the method of Koch and McMeekin.[9] (2) A similarly prepared sample was precipitated with trichloroacetic acid at a final concentration of 2.5%, and nitrogen was determined on the filtrate as before.

The changes in viscosity and soluble nitrogen with time are shown in Fig. 1 for an experiment at *p*H 8.6. The viscosity drop is rapid initially and gradually decreases in rate until it is very slow at the end of a week. The increase in soluble nitrogen continues even after the viscosity is almost constant. Data are also given for a sample of the same solution which had been heated to 80° for ten minutes immediately after the casein was dissolved. It is seen that the heated solution shows no drop in viscosity, but rather a very slow increase. This increase was followed in other cases for periods up to six months and was still continuing. The appearance of soluble nitrogen was almost completely abolished by the heating.

The influence of *p*H on the viscosity changes is shown in Fig. 2. The initial viscosity has an extremely sharp maximum at a *p*H of about 9.4. At all *p*H's below 9.8 the viscosity decreases with time. After one week it has reached its lowest value at *p*H 8.5, and this may be taken as the *p*H of optimum activity of the enzyme. At *p*H 10.3 the viscosity, although lower initially, increases with time at a rate somewhat greater than that of the heated solution at *p*H 8.6. This increase was also observed at *p*H 11.2 but was much slower. On the acid side of the optimum the viscosity drop proceeds more slowly than at *p*H 9 to 9.5, but is still evident at *p*H 5.8, the lowest *p*H at which satisfactory solutions can be prepared.

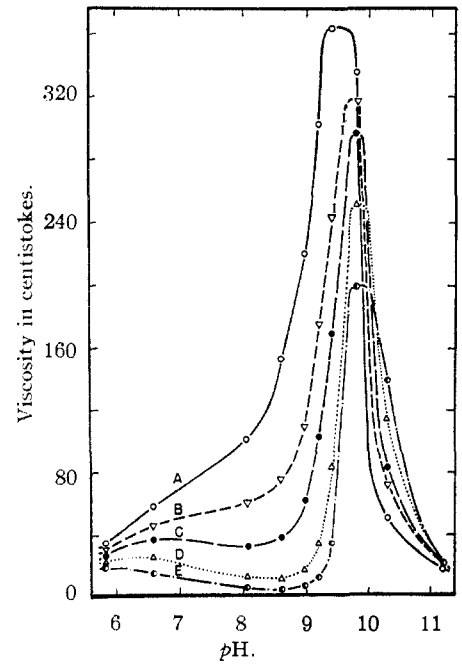

Fig. 2.—Viscosity of 9.71% casein solutions as a function of *p*H. Curves A, B, C, D and E show the viscosity at 0, 1, 2, 4, and 7 days, respectively, after the solutions were made up.

The changes in soluble nitrogen in these solutions (Fig. 3) were consistent with the viscosity data. A definite maximum was found at *p*H 8.5 with a decrease in the rate of formation of soluble nitrogen on either side of this *p*H

(7) Zoller, *Ind. Eng. Chem.*, **12**, 1171 (1920).

(8) Warner, THIS JOURNAL, **66**, 1725 (1944).

(9) Koch and McMeekin, *ibid.*, **46**, 2066 (1924).

The amount of nitrogen soluble in trichloroacetic acid was somewhat more than half of that soluble at *p*H 4.6 in the region of activity of the enzyme. The points on the curves at *p*H 4.6 were obtained on suspensions of casein which were allowed to stand at this *p*H in a borate buffer in the presence of chloroform. At *p*H 11.2 the increase in the rate of formation of soluble nitrogen can be attributed to alkaline hydrolysis of the casein. After seven days there was a noticeable growth of mold at *p*H 5.8 and 6.6. The points at fourteen days in these solutions are therefore too high.

Similar experiments have been carried out on a number of samples of commercial casein. All of the results were consistent with the above interpretations, although there was a wide variation in the magnitude of both the initial viscosity and the viscosity changes with time. The variation in initial viscosity in the region of *p*H 8 is probably due largely to the heat used in commercial practice in precipitating and drying the casein. By heating these dry samples further at 75° the viscosity of their solutions could be made to increase several-fold. This procedure did not abolish the decrease in viscosity with time.

Casein samples which had been exposed to ethanol at room temperature in drying contained less enzyme activity than the samples used in the above experiments in which the treatment with ethanol was of very short duration and was carried out at 2°. By treatment of a wet casein precipitate with ethanol for several hours at room temperature, particularly with concentrations around 50%, it was possible to reduce the enzyme activity to a very low level.

While there was no marked bacterial or mold growth in the solutions during the period of observation (except at *p*H 5.8 and 6.6 as noted above) because of the bacteriostatic action of borax, only those solutions above *p*H 9 were sterile. In order to eliminate bacterial activity as the cause of the casein hydrolysis, the increase in nitrogen soluble at *p*H 4.6 was followed on a solution sterilized by filtration through a Seitz filter. The solution used had a *p*H of 8.5 and was made up to the same borax concentration but to one-half the casein concentration used in previous experiments. The filtered solution was placed in a series of sterile test-tubes and one tube was used for each determination of soluble nitrogen. The sterility of each of the samples was checked by plating with nutrient agar. Half of the solution was retained unfiltered as a control and was sampled at the same time as the sterile solution. The results are shown in Table I.

TABLE I

COMPARISON OF SOLUBLE NITROGEN FORMED IN STERILE AND NON-STERILE SOLUTIONS

Time sampled, days	Unfiltered solution			Filtered solution[b]
	Bacteria per ml.	Molds per ml.	Soluble N,[a] %	Soluble N,[a] %
0	5300	0	1.7	1.8
2	80	20	8.6	8.1
4	10	100	18.4	18.0
7	0	130	31.4	31.5

[a] Nitrogen which is soluble at *p*H 4.6 expressed as percentage of the total nitrogen. [b] All samples of the filtered solution were sterile.

In the fractionation of casein by the method reported previously,[8] some enzyme activity is found in both the α- and β-fractions after the first precipitation. In the α-fraction a small degree of activity remained even after nine precipitations, but none was present in the purified β-fraction.

Only a trace of enzyme remains in whey after precipitating the casein. The enzyme was not destroyed when casein was exposed for one hour to a *p*H of 1.5 or 11. Chloroform was found to have an inhibitory effect on the enzyme, but none was shown by borax.

Preparation of an Enzyme Concentrate.—A number of unsuccessful attempts were made to separate the enzyme from casein. However, it was found possible to separate the enzyme from the hydrolysis products after allowing the enzyme to act on casein. Several concentrates were prepared by the following method:

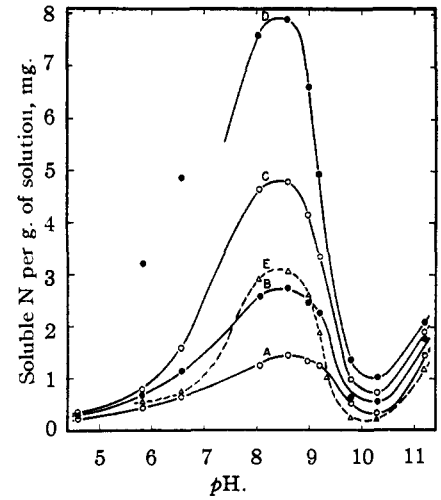

Fig. 3.—Increase in soluble nitrogen as a function of *p*H. Curves A, B, C and D show the nitrogen soluble at *p*H 4.6 at 2, 4, 7 and 14 days, respectively, after the solutions were made up. Curve E shows the nitrogen soluble in trichloroacetic acid after seven days. Total nitrogen in all cases was 15.2 mg. per g. of solution.

A kilo of commercial casein was made up as a 15% solution at *p*H 8.5 by dissolving in borax. Toluene was added, and the solution was allowed to stand at room temperature for two weeks. It was then dialyzed against a solution of borax for an additional two weeks. The dialyzed solution was precipitated by adjusting to *p*H 4.5 with hydrochloric acid. The filtrate from this precipitation was salted out with ammonium sulfate, and the fraction appearing between 15 and 23% salt was retained. It was dissolved at a *p*H of 5.5, dialyzed, and then precipitated by adjusting the *p*H to 4.5. The precipitate was dissolved at *p*H 5.5 to give about 25 ml. of a 0.6% solution of protein.

The various fractions of the digest were tested for enzyme activity by adding them to a solution of enzyme-free casein dissolved in borax at *p*H 8.5 and following the viscosity change with time. If the sample being tested does not contain sufficient protein to increase the viscosity of the solution appreciably, the concentration of enzyme is proportional to the initial slope of the viscosity–time curve. The enzyme-free casein was prepared by heating a solution of casein to 90° for half an hour and then precipitating, washing and drying the casein. A solution of this casein showed a very slow rise in viscosity with time similar to that for the heated solution in Fig. 1.

Several of the fractions in the above separation, especially the first precipitate at *p*H 4.5, showed some enzyme activity, so the fractionation is not very efficient. The final preparation described contained more activity per gram of nitrogen than any other fraction and had on this basis about 150 times the activity of the original casein.

The enzyme appears to be insoluble at *p*H 4.5 in the absence of salt, but is somewhat soluble at this reaction in the presence of salt or the hydrolysis products of casein.

The concentrate was examined electrophoretically by the Tiselius method. The pattern obtained showed the presence of four boundaries, the activity migrating with one of the two slower boundaries. Although it is evident that the concentrate contained much protein in addition to the enzyme, no further attempts at purification were made.

Discussion

The proteolysis that occurs in casein solutions is attributed to the presence of an enzyme on the

basis of the following evidence: (1) The activity is destroyed by heat; (2) it has a definite *p*H optimum; (3) the activity can be concentrated; (4) the proteolysis proceeds in sterile solutions. The enzyme is presumably the same as that studied by Babcock[2] and Van Slyke[3] and their coworkers in their investigations on the proteolysis in skim milk and cheese. The changes observed in their experiments were very slow, and the period of observation sometimes continued for as long as a year. However, they were carried out at a *p*H considerably below the optimum for the enzyme. Since the enzyme occurs in rennet casein as well as in acid-precipitated casein, it would also be present in cheese.

Babcock and Russell attempted to purify the enzyme and found that an aqueous extract of chloroform-treated separator slime showed more proteolytic activity than milk. Separator slime contains very large numbers of bacteria and, in addition, leucocytes and other cellular debris. Chloroform will cause lysis of much of this material, and at least a part of the proteolytic activity may come from this source. Their results with this preparation have a doubtful bearing on the properties of the enzyme occurring in milk.

It is not easily settled as to whether the enzyme is secreted in milk as such or whether it is of bacterial origin. Babcock decided in favor of the first alternative because of the invariable presence of the enzyme in milk in fairly uniform concentrations. Appreciable bacterial activity subsequent to milking was excluded in the preparation of the casein used in this work. However, bacteria are normally present in the udder of the cow, and, while the bacterial counts made on milk immediately after milking are usually low, these bacteria cannot be excluded *a priori* as the source of the enzyme.

Carpenter[10] has studied the appearance of nitrogen soluble in trichloroacetic acid in solutions of casein as a function of *p*H. He did not give any details on the preparation of the casein used, but it is evident from his data that it did not contain any of the enzyme. Our data on soluble nitrogen by the trichloroacetic acid method at *p*H's 10.3 and 11.2 (Fig. 3), under which conditions the enzyme is inactive, are in fair agreement with Carpenter's data in this *p*H range, when both are calculated as percentage of the total nitrogen.

Walters[6] reported 8% hydrolysis of casein in 137 hours at 37.5° in a solution neutral to phenolphthalein. This hydrolysis, which is greater than that reported by Carpenter but less than that in Fig. 3, can doubtless be attributed to the enzyme. A similar observation was made by Pertzoff.[11]

The influence of *p*H on the initial viscosity of casein solutions, as shown in Fig. 2, is essentially in agreement with the data of Zoller.[12] He reported viscosity determinations as a function of *p*H in solutions of various alkalies and alkaline salts. All of his solutions exhibited maxima at a *p*H of about 9.2, except the ones made up with borax. The maximum at *p*H 8 rather than at *p*H 9.2, in the presence of borax, can probably be attributed to the high concentration of borax that must be used to attain a *p*H approaching 9. In our series of measurements at a constant concentration of borax, the viscosity maximum occurred at about the same *p*H as was found by Zoller for other alkalies. The change in initial viscosity from that at *p*H 7 to 8 to that at the maximum is considerably greater in Fig. 2 than in Zoller's data.

An interesting point resulting from the viscosity data was the slow increase in the viscosity of casein solutions in which the enzyme had been destroyed by heat or rendered inactive at a high *p*H. The effect was not studied systematically as a function of *p*H, but was at a maximum at *p*H 10.3 and was much smaller in the range of *p*H 8 to 9 and at *p*H 11.3. This increase in the viscosity may be related to a slow structural change in the casein.

Acknowledgment.—The authors are indebted to Joseph Naghski for carrying out the bacteriological work and to T. L. McMeekin and R. W. Jackson for their advice and interest in this investigation.

Summary

Casein contains an enzyme that causes its slow hydrolysis accompanied by a decrease in the viscosity of its solutions and the appearance of nitrogenous products soluble at *p*H 4.6 and in trichloroacetic acid. The enzyme was present in all samples of commercial casein examined and in all laboratory preparations made by the usual methods.

PHILADELPHIA, PA. RECEIVED DECEMBER 11, 1944

(10) Carpenter *J. Biol. Chem.*, **67**, 647 (1926).

(11) Pertzoff, *J. Gen. Physiol.*, **11**, 239 (1927–1928).

(12) Zoller, *ibid.*, **3**, 635 (1920–1921).