Precipitation of Protein During Mashing: Evaluation of the Role of Calcium, Phosphate, and Mash pH[1]

M. J. Lewis and N. Nelson Wahnon, *Department of Food Science and Technology, University of California, Davis 95616*

ABSTRACT

In a temperature-programmed mash, malt protein first dissolved during the protein rest, then decreased in concentration as the mash temperature approached 70° C. With a technique called grain-out mashing, a precipitate containing about 62% protein was isolated. This accounted for the entire decrease in dissolved protein observed in normal (grain-in) mashes. Addition of calcium significantly affected mash pH, which was not constant during the mash period, but clear evidence of reaction of calcium with malt phosphate could not be gained. Calcium did not react directly with protein or affect protein precipitation, although it somewhat reduced the dissolution of protein from malt particles. Although wort pH, calcium, and phosphate concentration changed during mashing in parallel with protein solution and precipitation, a causal relation among these factors could not be established.

Key words: Calcium, Mashing, pH, Phosphate, Precipitation, Protein

Proteins of 4,000 daltons and above can be conveniently measured by a method based on the binding of Coomassie Brilliant Blue G-250 (3). When Lewis et al (5) applied this method to samples taken from a temperature-programmed malt mash, they observed a massive and rapid solution of proteins during the "protein rest" (about 40° C); as the mash temperature approached 70° C, the dissolved protein substantially decreased, presumably by precipitation. Moll et al (7) observed a rise and fall in the total soluble nitrogen of wort during mashing, although Lewis et al (5) had not found this. The suggestion that protein dissolves, then precipitates in mashing is contrary to the traditional view of mashing, which assumes protein breakdown by proteolysis occurs during the "protein rest." Substantial protein precipitation, in contrast, is taken to be a primary function of the kettle boil, although Lewis et al (5) observed relatively little protein precipitation during boiling.

Because of the importance of proteins in, for example, beer foam and haze, it seemed worthwhile to confirm protein solution during mashing and to demonstrate unequivocally whether protein decrease during mashing results from proteolysis or protein precipitation. We expected the behavior of protein in the mash to be influenced by mash pH and that this factor could even cause the large changes in wort protein observed during mashing (5). Because mash pH in turn is affected by calcium, probably through reaction with malt phosphate (4), we also measured these ions in the mash. Although changes in mash pH and calcium and phosphate concentration often parallel protein solution and removal during mashing, we could not establish a causal relation among these factors. Indeed, our results lead us to question the assumptions we used in deciding to investigate the interrelationships of these factors.

EXPERIMENTAL

Mashing Systems

Laboratory-scale mashes were conducted according to ASBC method MALT-4 for determining extract (1), except where otherwise noted. The malt used was two-rowed Klages and was milled finely in a Miag cone mill. Mashes were conducted with deionized water; where indicated, Ca^{++} was added as calcium sulfate. The water:malt ratio was 8:1 (50 g of malt in 400 ml of water) unless otherwise specified. All analyses were done on samples at room temperature after filtering through Whatman No. 2 paper if necessary.

For "grain-out" mashes, the standard method and temperature profile was followed in all details except that the mash comprised a cold water extract of malt. Finely milled malt was extracted for 30 min with deionized water (to which Ca^{++} was added if required) at 24° C. After filtering, the clear liquid was "mashed."

Similarly, for isolation of large amounts of proteinaceous

[1] Presented at the 49th Annual Meeting, Nashville, TN, April 1983. First presented orally at the Master Brewers Association of the Americas District Venezuela Meeting, Caracas, 1982.

precipitate, 10 kg of milled malt was extracted with 40 L of cold deionized water and filtered in the lauter vessel of our Pilot Brewery. After the cold water extract was twice filtered through cellulose pads in a plate filter, the clear liquid was mashed as above but with use of the Pilot Brewery vessels. The protein precipitate was allowed to settle, collected, then washed repeatedly with cold water by centrifuging to remove occluded wort substances. The material was freeze-dried to a fine, gray powder.

Analytical Methods

Protein was determined by the method of Bradford (3) and of Kjeldahl and of Lowry as previously described (5). Total phenols were measured by the method of Singleton and Slinkard (8), with gallic acid as the standard. Ca^{++} was determined by atomic absorption spectrophotometry using a Perkin Elmer instrument, model 5000.

To determine total orthophosphate, 4.0 ml of ammonium molybdate reagent was added to a clear wort sample (0.5 ml) diluted to exactly 100 ml. The reagent comprised 25.0 g of ammonium molybdate dissolved in 175 ml of water plus 280 ml of pure sulfuric acid and diluted to 1 L. After mixing, 0.5 ml of a 2.5% solution of stannous chloride in glycerol was added. After exactly 10 min, the color was measured at 690 nm and compared to a standard curve made with known solutions of KH_2PO_4 anhydrous. Total phosphates were recorded as phosphorus (P, μg/ml).

Total carbohydrate was measured in the proteinaceous precipitate by an anthrone-sulfuric acid method (9) after the precipitate was redissolved in warm 2*N* NaOH.

Haze was determined in a Klett-Summerson colorimeter with a blue (No. 42) filter using filtered cold water extract of malt as blank; the values are arbitrary but linear over the range required in this work.

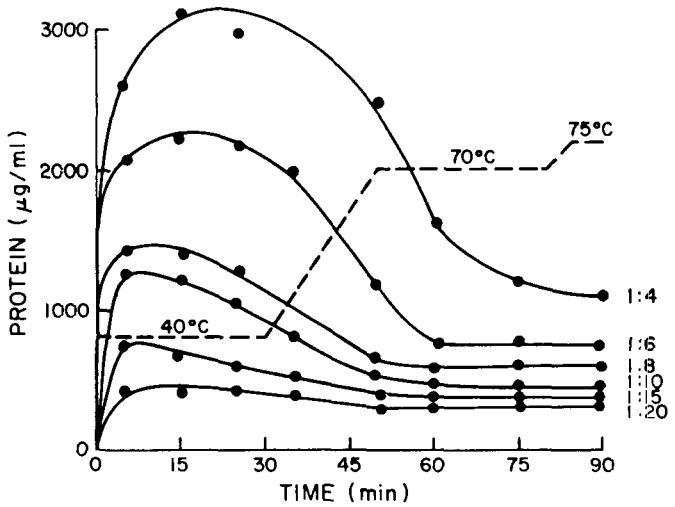

Fig. 1. Effect of mash thickness expressed as a malt:water ratio (w/v) on the solution and precipitation of protein in mashing. Mash volume was 400 ml in each case.

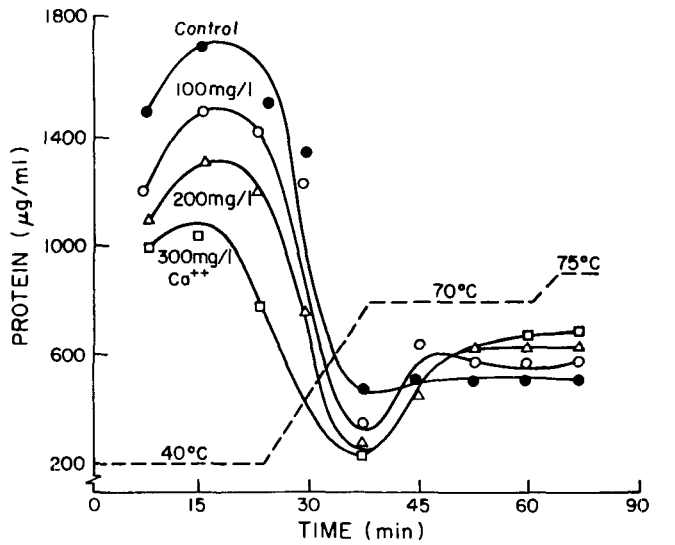

Fig. 2. Effect of added Ca^{++} at the concentrations shown on the solution and precipitation of protein.

RESULTS

Solution of Protein in Mashing

Protein dissolved during the low temperature stage (40° C) of the mash temperature program. More protein dissolved in thick mashes than in thin ones (Fig. 1), but the efficiency of protein solution was rather constant at about 12.5 mg of protein per gram of malt regardless of mash thickness. In contrast, protein decreased more efficiently in thick mashes (up to 70% of the dissolved protein) than in thin ones (50% or less). Although some decrease in protein occurred at relatively low temperature, especially with thin mashes, the major decrease in protein content of the wort was a function of increasing mash temperature; protein removal was complete after the mash reached the conversion temperature of 70°C, although this was slower in thick mashes.

Addition to the mash of calcium, an element that has frequently been associated with the phenomenon of protein precipitation, reduced protein solution but did not increase protein removal (Fig. 2). Indeed, added calcium marginally, but consistently, increased the amount of protein remaining in solution at the end of the mashing cycle. When malt was mashed throughout at 70°C (infusion mashing) without added Ca^{++} (Fig. 3, left), the initial massive solution of protein did not occur and the final protein concentration (about 600 μg/ml) in the wort was the same as in wort from a temperature-programmed mash of equal thickness (Fig. 3). Addition of 50 mg/L of calcium (Fig. 4) resulted in less protein removal than in the control mashes (Fig. 3) and an increase in polyphenol concentration at the end of the mash period. In these mashes, the solution and removal of calcium roughly paralleled the solution and removal of protein, but it is not clear whether removal of Ca^{++} was a function of high temperature, protein precipitation, or some other phenomenon.

Malt milling (Table I) and, to a lesser extent, mash pH (Table II) also affected protein solution and its removal from wort. Both variables influenced the amount of protein dissolved more than the

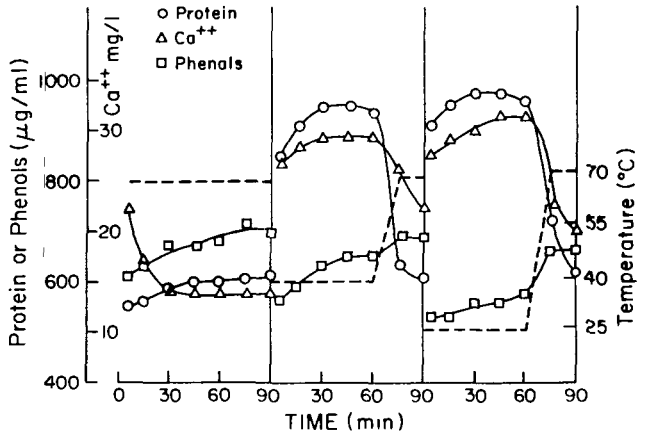

Fig. 3. Effect of the temperature program of the mash (----) on the solution and precipitation of protein, Ca^{++}, and total phenols. No calcium added.

amount remaining in wort after mashing (which was similar in all cases, about 600 μg/ml).

Protein in "Grain-out" Mashes

We could not directly observe precipitation of protein in normal mashes because of the solid matter present. Therefore, decrease in protein (eg, Figs. 1–4) could not be ascribed unequivocally to either precipitation or proteolysis. However, protein dissolved rapidly from finely milled malt at low temperature; at this stage we could remove the mash particles and conduct the remainder of the mash temperature program using only the clear supernatant liquid. We called this a "grain-out" mash. When a grain-out mash was done, the initial amount of protein in solution was the same as in a normal (grain-in) mash at the end of the "protein rest" (about 1.0 mg/ml). As mash temperature increased toward 70° C, protein precipitated from solution in a grain-out mash in the same proportion (about one-half) as in a grain-in mash of equal thickness. Furthermore, precipitation coincided with a mash temperature of 70° C and was unaffected by added Ca^{++} (Fig. 5). Thus, the pattern of events vis-à-vis protein was the same in grain-in and grain-out mashes. More importantly, however, we observed the precipitation of protein directly in grain-out mashes. First, a haze appeared in the originally clear mash, the intensity of which was roughly inversely proportional to the amount of protein in solution; then when 70° C was reached, rapidly settling flocs appeared that caused the haze to decrease, leaving a clear supernatant (Fig. 6). The concentration of Ca^{++} and total phenols remained constant.

When a large-scale grain-out mash was conducted, the precipitate was easily collected and washed. We recovered approximately twice as much material by weight as was needed to account for the decrease in protein during mashing measured by the Bradford method. The protein content of the isolated precipitate,

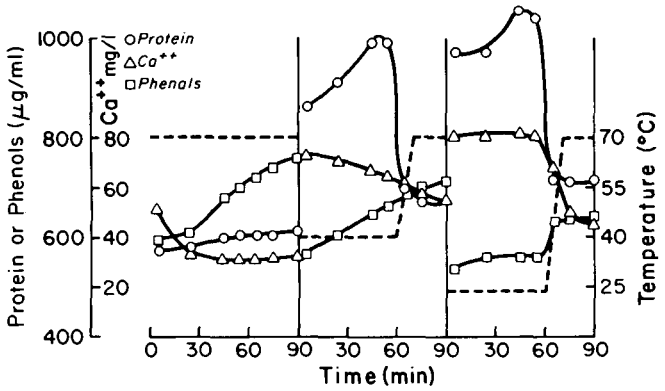

Fig. 4. As for Fig. 3, but with Ca^{++} added (50 mg/L).

TABLE I
Effect of Malt Milling on Protein Solution and Removal During Mashing

Malt Grind	Protein in Wort (μg/ml) Very Fine	Fine	Semi-coarse	Coarse	Very Coarse
Maximum protein concentration during mashing[z] (a)	1,475	1,245	970	930	810
Protein remaining in wort after mashing (b)	650	625	605	600	590
Protein removed during mashing relative to that dissolved $\left(\frac{a-b}{a} \cdot 100\%\right)$	56	50	38	35	27

[z] Peak of protein solution curve during each mash.

TABLE II
Effect of Initial Mash pH on Protein Solution and Removal During Mashing

Mash pH	Protein in Wort (μg/ml) 5.15	5.63	5.65	5.72
Maximum protein concentration during mashing[z] (a)	1,145	1,370	1,420	1,245
Protein remaining in the wort after mashing (b)	590	600	615	650
Amount of protein removed during mashing relative to that dissolved $\left(\frac{a-b}{a} \cdot 100\%\right)$	48	56	57	48

[z] Peak of protein solution curve during each mash.

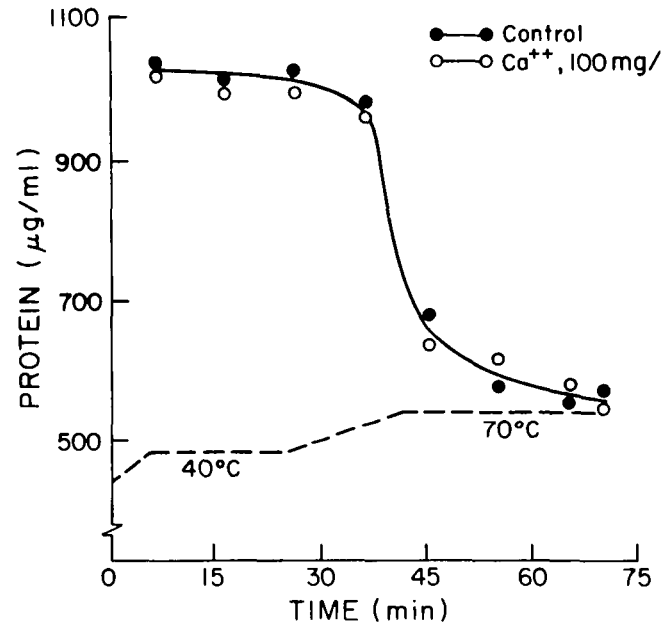

Fig. 5. Precipitation of protein during the course of a grain-out mash in the presence or absence of Ca^{++} added after extraction and before mashing.

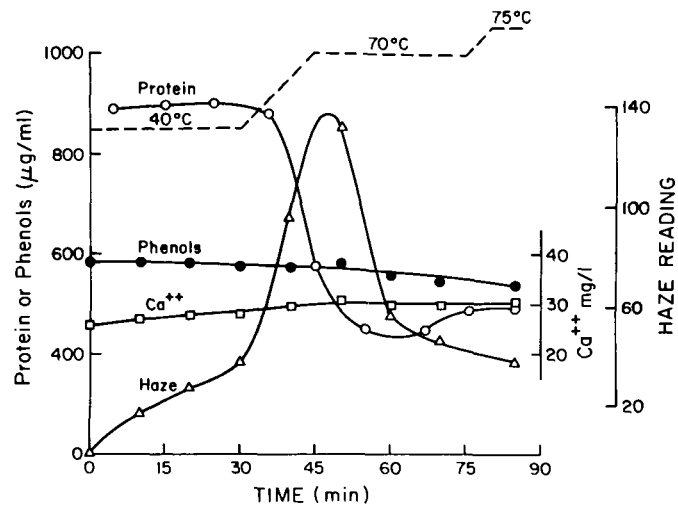

Fig. 6. Precipitation of protein in a grain-out mash and the formation of haze material. The concentration of Ca^{++}, total phenols, and phosphate and wort pH (not shown) remained constant throughout the mash cycle.

determined by the Kjeldahl, Lowry, or Coomassie Brilliant Blue method, was between 60 and 64% (Table III). With this correction, the weight of precipitate recovered from a grain-out mash was still sufficient to account for all of the decrease in protein measured in a grain-in mash of equal thickness. Although proteolysis is indubitably active in mashing, no proteolysis is required to explain the decrease in dissolved protein that we observe during mashing.

The Effect of Calcium

Although calcium did not increase protein precipitation in grain-in mashes (Figs. 2 and 3), this ion decreased in such mashes roughly in parallel with protein precipitation (Figs. 3 and 4). It appeared that calcium reacted directly with protein and may even have caused protein precipitation. In contrast, in grain-out mashes, calcium native to the wort (or added calcium, 100 mg/L) did not decrease in concentration during the precipitation of protein but remained constant throughout the mash cycle (Fig. 6). Thus, calcium did not react and precipitate with the wort protein. Furthermore, the amount of ash in the protein precipitate recovered from grain-out mashes (even assuming all the ash to be calcium; Table III) accounted for barely one-tenth of the loss of calcium observed in grain-in mashes. In addition, there was no measurable amount of calcium associated with the proteinaceous portion of wort in grain-in mashes after the protein rest or after the conversion stage of mashing (Fig. 7). We conclude that Ca^{++} reacts strongly with mash components but not with those that dissolve during the protein rest. Ca^{++} reacts with insoluble mash particles.

Mash pH and Phosphate

In grain-in mashes, mash pH varied somewhat with mash thickness and was not constant during the mash period (Fig. 8). Mash pH first increased to a maximum during the low-temperature stage, then decreased as mash temperature increased; the final (and lowest) mash pH was achieved during the conversion stage (70° C). Added calcium lowered initial and final mash pH to an extent that depended on Ca^{++} concentration but did not substantially alter the shape of the time/pH curve (Fig. 9). From 0 to 300 mg/L of added Ca^{++}, there was a roughly linear relationship between the initial, minimum, or maximum mash pH and the amount of Ca^{++} added. Generally, at each concentration of Ca^{++} tested, mash pH decreased by 0.2 pH units from the maximum to the minimum. Ca^{++} is supposed to react with phosphate to effect a pH change (4). When we measured orthophosphate in the same samples, the curve for this ion followed a shape very similar to that for pH and Ca^{++} (Fig. 10). In contrast to its effect on pH, however, added calcium had little influence on the amount of phosphate in the wort at the end of mashing (approximately 300 μg/ml). Addition of Ca^{++}

TABLE III
Proximate Analysis of Proteinaceous Fraction Precipitated in Mashing

Analysis	%[a]
Moisture	7.06
N (Kjeldahl dry basis)	10.28
Protein (N × 6.25)	64 20
Protein (Lowry)	62.70
Protein (Bradford)	60.40
Total (phenols)	9.10
Total carbohydrates (as glucose)	5.70
Fat	0.05
Fiber	0.22
Ash	0.73
Unaccounted	12.94

[a] Values are averages of analyses by triplicate

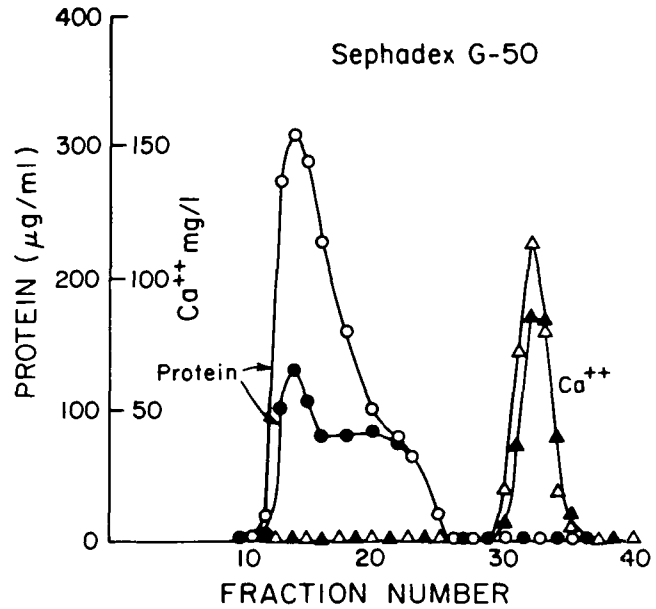

Fig. 7. Separation of protein and Ca^{++} on Sephadex G-50. O and △ = mash sampled at the end of the protein rest; ● and ▲ = mash sampled after mash-off.

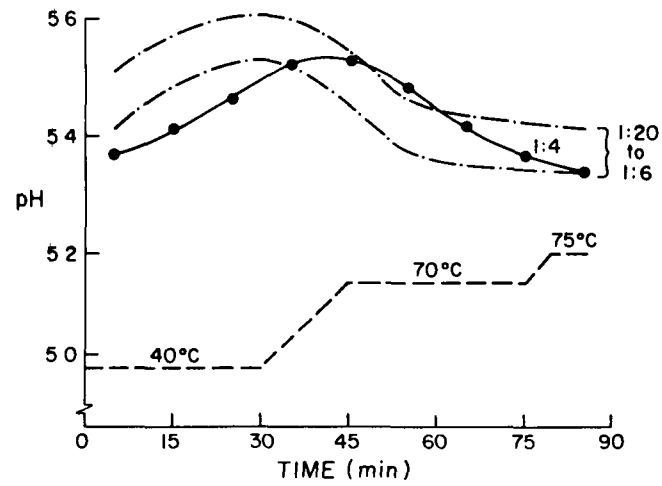

Fig. 8. Effect of mash thickness on mash pH during mashing.

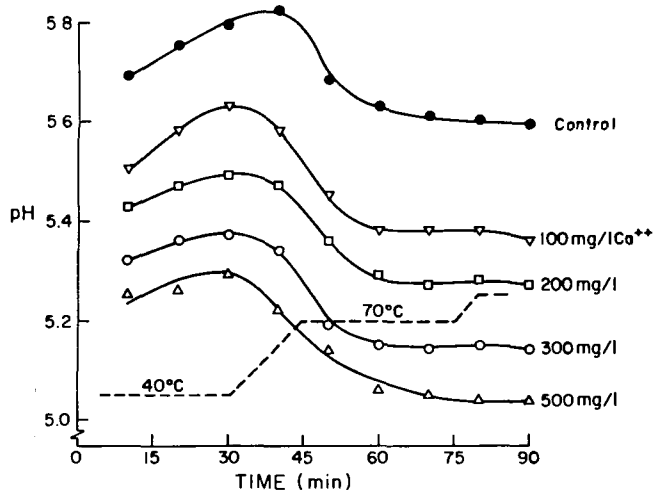

Fig. 9. Effect of added Ca^{++} at the concentrations shown on mash pH during mashing.

considerably decreased the amount of phosphate dissolved during the low-temperature stage of the mash, but this effect, unlike pH change, was largely independent of Ca^{++} concentration. In grain-out mashes, pH and phosphate concentration remained constant throughout the mash period.

In grain-in mashes, wort pH and the concentration of protein, calcium, and phosphate in solution first increased during the protein rest, then decreased as the conversion temperature was reached. In contrast, in grain-out mashes, these components, except for protein, were unaffected by mash temperature, even though they were present at the same concentration in solution. We conclude that the solid particles of the mash play an important role in the changes that occur in Ca^{++} and phosphate concentration and in wort pH during mashing.

DISCUSSION

The amount of protein dissolved in mashing was about 1.25% of malt dry weight. The "protein rest" stage of a temperature-programmed mash may therefore more aptly be called the "protein solution" stage because (depending on particle size of the malt and length of the protein rest) malt protein has great potential for becoming wort protein during this time. This could significantly affect wort quality because proteins are important as enzymes in mashing and as foam and haze precursors in beer. After the mash approached the conversion temperature, the protein that dissolved during the protein rest significantly decreased in the wort. Although proteolysis is undoubtedly active in mashing, by isolating a proteinaceous precipitate we have demonstrated conclusively that this decrease is entirely the result of protein precipitation.

The final protein content of wort produced by the infusion (single-temperature) mashing method was about the same as for wort from a temperature-programmed mash. The precipitated protein is located differently in the two cases, however. In an infusion mash, the protein never dissolved from the mash particles but precipitated in situ. In a temperature-programmed mash, protein first dissolved, then precipitated *among* the malt particles. This may have practical effects. First, precipitated protein may clog the filter bed of a lauter vessel (2) and promote slow runoff of wort. High mash pH, frequently caused by mashing water that is low in calcium, is commonly reported to have this effect; we observed that a low calcium concentration permitted maximum solution of protein from the mash particles (Fig. 2), which may partially explain the practical observation. Second, dissolved protein has much greater opportunity to react with other soluble malt components than does protein that never dissolves from the malt particles. The presence of polyphenolic material in the precipitated protein (Table III) is an example of such a reaction (6).

In the presence of mash particles, calcium and phosphate in solution and wort pH increased as protein dissolved, then decreased roughly in parallel with protein precipitation as the mash temperature approached 70° C. Although it is tempting to assume that events taking place in concert are directly linked, our results suggest that these events are under the control of mash temperature and are influenced by the presence of mash solids but are otherwise unconnected. For example, we found no evidence that Ca^{++} reacts either directly with protein in solution or indirectly through affecting mash pH to promote protein precipitation.

In grain-out mashes, Ca^{++} and protein behaved independently; protein precipitated but Ca^{++} remained in solution. Thus, Ca^{++} did not react and precipitate with protein in the mash or with any other component that dissolved during the low-temperature stage of the mash; this includes phosphate, which reached its maximum concentration during the protein rest. In grain-out mashes, phosphate and calcium concentration and mash pH remained constant regardless of mash temperature. Although $Ca_3(PO_4)_2$ is quite soluble at mash pH (Mortimer Brenner, *personal communication*), a reaction between dissolved phosphate and calcium to form insoluble $Ca_3(PO_4)_2$ is widely assumed to account for the effect of Ca^{++} on mash pH (4). Ca^{++} decreased in concentration at 70° C in wort, but only in the presence of mash particles with which we suppose it reacts to affect final mash pH at this temperature. Although a direct reaction between added Ca^{++} and dissolved phosphate does not establish final mash pH, such a reaction may explain the effect of Ca^{++} on initial mash pH. But even here the evidence is not clear because, contrary to expectation, Ca^{++} and phosphate concentration in wort and mash pH all rise during the protein rest. The two-stage effect of Ca^{++} on mash pH requires additional clarification.

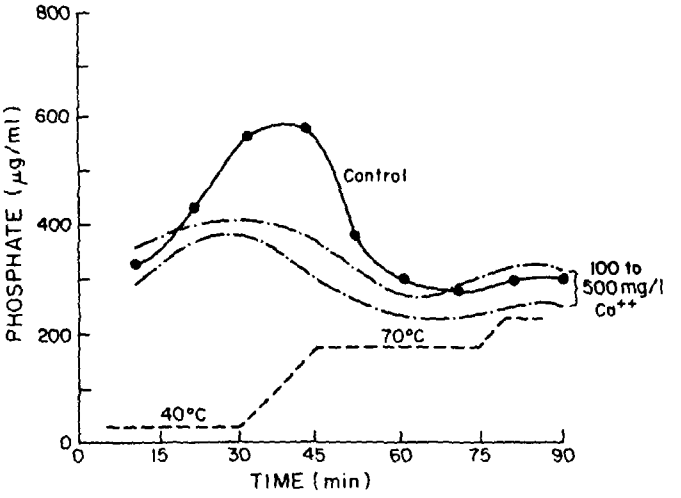

Fig. 10. Effect of added Ca^{++} in the range of concentration shown on the concentration of dissolved total orthophosphate (as phosphorus) in wort during mashing. Control = no added calcium.

In this work we have demonstrated conclusively that protein dissolves substantially, then partly precipitates in mashing, and that the changes in dissolved protein concentration during mashing can be entirely described by precipitation without proteolysis. Ca^{++} and phosphate concentration in solution during mashing and mash pH follow a similar pattern to protein solution and precipitation but are not related chemically to that phenomenon. Although added Ca^{++} significantly lowers mash pH, it appears to do so, at least at 70° C, by reacting with mash particles, not with phosphate in solution. The elements we have chosen to measure here, therefore, appear to react in the mash independently, although all are under the influence of mash temperature and the presence of mash particles. This work is continuing.

LITERATURE CITED

1. American Society of Brewing Chemists. Methods of Analysis, 7th ed. Malt-4. The Society: St. Paul, MN, 1976.
2. Barratt, J., Bathgate, G. N., and Clapperton, J. F. *J. Inst. Brew.* 81:31, 1975.
3. Bradford, M. M., *Anal. Biochem.* 72:248, 1976.
4. Hough, J. S., Briggs, D. E., and Stevens, R. Pages 209-211 in: Malting & Brewing Science. Chapman Hall: London, 1971.
5. Lewis, M. J., Krumland, S. C., and Muhleman, D. J. *J. Am. Soc. Brew. Chem.* 38:37, 1980.
6. Lewis, M. J., and Serbia, J. W. *J. Am. Soc. Brew. Chem.* 42:40, 1984.
7. Moll, M., Flayeux, R., Lipus, G., and Marc, A. *Tech. Q. Master Brew. Assoc. Am.* 18:166, 1981.
8. Singleton, J. L., and Slinkard, K. *Am. J. Enol. Vitic.* 28:49, 1977.
9. Yadav, K., Weissler, H., Garza, A., and Gurley, J. *Am. Soc. Brew. Chem., Proc. 1969*, p. 59.

[Received February 24, 1984]