Characterization of Amorphous-Particle Haze[1]

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ABSTRACT

A number of hazes in worts and in beers before final filtration were examined with nephelometry, electronic particle counting, optical microscopy, scanning electron microscopy, chemical analysis, and enzyme treatment. The relative merits of the various techniques in avoiding artifact formation, estimating the quantity of haze present, and characterizing the haze material to determine its origin were assessed. The hazes were almost entirely protein and carbohydrate. The carbohydrate contained a high percentage of reducing sugars, indicating an average degree of polymerization of 4. A large portion of the carbohydrate was nonglucan. Substantial amounts of mannose were found in one of the wort samples and in two of the beer samples.

Key words: *Analysis, Enzyme treatment, Measurement, Microscopy, Particle counting, Staining*

We have periodically experienced difficulties in clairification of beer during lagering. This has caused short filtration runs that result in greater processing expense. At various times different techniques have been used to try to characterize the "problem haze" and "normal haze" material. Some of the techniques have been much more helpful than others when dealing with amorphous haze particles. A study was conducted to compare results obtained with different analytical methods used on prefilter hazes of different origins—some naturally produced and others caused by the addition of tannic acid or β-glucan or by the alteration of pH or ethanol content.

EXPERIMENTAL

Sample Collection

Nineteen liters of wort or beer were collected in 5-gal Firestone tanks. Ethanol (5% v/v) was added to one of two plant-filtered wort samples and the other was adjusted to pH 3.6 with hydrochloric acid. Four of the six beer samples were ordinary production beers. Three of these, Process Beers 1, 3, and 4, were collected when they were ready to enter storage. Another (Process Beer 2) was taken at the end of storage. Process Beer 4 was a particularly hazy sample. The two final hazes were produced by adding, respectively, 105.3 mg/L of tannic acid (Mallinckrodt No. 1750) and 210.6 mg/L of barley β-glucan (Biocon U.S., Lexington, KY) to Firestone tanks of beer drawn just before storage. The beer used for this was the same as Process Beer 1, which served as a control for the two additions. All the samples were stored at 0° C for one week before sampling.

Nephelometry

Beer was placed in a cuvette, which was then inserted in a Coleman model 9 Nephelocolorimeter. The measurement was made in Coleman nephelos units. Coleman standards (N = 78 and N = 10) were used for calibration.

Chemical Analysis

Dry Weight. Beer from the Firestone tanks was collected in 1-L flasks. The samples were distributed equally among four centrifuge bottles and centrifuged at 13,200 × *g* for 40 min. The beer was carefully decanted, leaving the residue behind. The bottles were then inverted on paper toweling and allowed to drain for 30 min. The residues from the four bottles were washed with distilled water into a 25-ml volumetric flask and then diluted to volume with distilled water. The suspension was transferred to a 30-ml Corex centrifuge tube and agitated with a Vortex mixer until the suspension was finely divided. The tube was then centrifuged at 1,470 × *g* for 15 min. The supernatant was decanted into a 25-ml Erlenmeyer flask. The insoluble residue was transferred to a 25-ml volumetric flask and was diluted to volume with distilled water. The dry solids for these samples were determined by placing a 10-ml aliquot in a tared-aluminum moisture dish. The dish was placed in a 100° C oven for 24 hr and was then removed, cooled, and weighed.

Protein. Protein was determined by the Kjeldahl method.

Hydrolysis of Sugars. Aliquots (5 ml) of the soluble fraction and the suspended insoluble residue were placed in sealed hydrolysis tubes with 0.3 ml of concentrated H_2SO_4 (which made the solutions 2*N* with respect to sulfuric acid). The tubes were placed in a boiling water bath for 4 hr.

Reducing Sugars. The analysis of reducing sugars was conducted on both the hydrolyzed solutions (to determine total carbohydrate) and the nonhydrolyzed solutions, using the following method (4). Dinitrosalicylic acid (DNS) reagent (1.0 g) was added to 16.0 ml of 10% sodium hydroxide (w/v), 30 g of Rochelle salt (sodium potassium tartrate tetrahydrate), and 50 ml of distilled water. The mixture was warmed to dissolve the solids, then cooled and diluted to 100 ml. This reagent can be stored for up to five days at 5° C.

One milliliter of the solution to be analyzed was placed in each of several test tubes. DNS reagent (2.0 ml) was added to the tubes, which were then placed in a boiling water bath for 5 min. The tubes were cooled, and 10 ml of distilled water was added. The samples were vortexed, and the absorbance at 540 nm was measured with a Gilford 250 spectrophotometer. All of the analyses were carried out in triplicate.

Glucose. Glucose was determined with the Glucose Auto-Stat® kit (Pierce Chemical), which uses the glucose oxidase procedure. This method is specific for glucose even in the presence of other sugars. Glucose is converted to gluconic acid by the glucose oxidase. The gluconic acid is then reacted with a dye, and the color produced is measured with a spectrophotometer.

High Performance Liquid Chromatography. Samples were prepared essentially by the procedure described by the ASBC Subcommittee on Fermentable Carbohydrates in Wort (2,3). Approximately 1 g of mixed-bed ion-exchange resin was added to approximately 100 ml of wort or beer and shaken for 10 min. Samples were then filtered through a 0.45-μm Millipore filter (HAWP 01300) in a Swinny filter holder (Millipore XX30 01200) attached to a 10-ml syringe. The samples were further treated by passing the liquid through bonded nonpolar material (Waters Sep-Pak™ C_{18} Cartridge S1910). Samples approximately 2.5 ml in volume were prepared in this manner and were either chromatographed soon afterwards or frozen and held at −5° C until used.

High performance liquid chromatography (HPLC) was done using a DuPont 830 liquid chromatograph with a heated column oven and Haskell pneumatic piston amplifier pump. Detection was by means of a differential refractive index detector (DuPont). Separation by degree of polymerization was done with a 4.6-mm ID × 25-cm DuPont ZORBAX™ NH_2 column. Both column and detector were maintained at a constant 50° C; solvent was pumped at a pressure of 800 psig, resulting in a flow of approximately 1.5 ml/min.

Individual sugars were separated on a 25-cm × 4.6-mm ID column of Li-Chroma™ stainless steel tubing packed with Aminex® A-28 converted to the sulfate form. This column was prepared by pressure slurry packing at 6,000 psi. The mobile phase was 85% ethanol, and the flow rate of 0.3 ml/min was obtained at 800 psi. The column temperature was 75° C, and the eluting peaks were

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detected with the refractive index detector. This is essentially the procedure described by Havlicek and Samuelson (16). Peak areas were measured by hand calculation (triangulation), and the concentrations of mannose and glucose were calculated from the response of standards.

Enzyme Treatment

Enzyme Preparations. The α-amylase (Sigma A-6880, Sigma Chemical Co., St. Louis, MO), β-amylase (Sigma A-7130), and β-glucanase (CIBA) were all dry powders. In each case, 125 mg of the enzyme powder was dissolved in 50 ml of distilled water. The α-mannosidase (Sigma M-7257) was provided in solution. A portion of this solution, 115 μl, was dissolved in 25 ml of water.

Enzyme Digestions. One milliliter of the centrifuged and resuspended haze material, prepared as described above, was mixed with 1.0 ml of an enzyme stock solution and 0.5 ml of 1*M* sodium acetate buffer, pH 4.5. The mixture was incubated at 30° C for 1 hr. DNS reagent (2 ml) was then added and the reducing sugars were determined as described previously.

β-Glucanase Assay. Barley β-glucan substrate was prepared by adding 1.0 g of pure barley β-glucan (BIOCON) to approximately 80 ml of distilled water and stirring vigorously (only part of the barley β-glucan dissolved). The mixture was boiled for 5–10 min and cooled, and 10 ml of 1*M* sodium acetate buffer, pH 5.00, was added. The solution was made up to 100 ml.

For each determination, 1.0 ml of barley β-glucan substrate was added to a test tube, which was then placed in a 50° C water bath for 5 min. The tube was removed and 100 μl of enzyme solution was added. A control tube was also prepared in which 100 μl of water was substituted for the enzyme solution. The samples were mixed by vortexing and allowed to incubate 10 min at room temperature. DNS reagent (2.0 ml) was added to the tubes, which were then placed in a boiling water bath for 5 min. The tubes were cooled, and 10 ml of distilled water was added. The samples were vortexed, and the absorbance at 540 nm was measured. Each of the enzyme solutions described above was assayed in triplicate.

Amylase Assay. The substrate solution was 0.967 g of soluble starch (ASBC) dissolved and made to 100 ml as described for the β-glucan substrate solution.

The determination was performed in the same manner as was the β-glucanase assay, with the substitution of the starch substrate solution for the β-glucanase solution.

Mannosidase Assay. The substrate solution was prepared by combining 30 mg of *p*-nitrophenyl-α-D-mannoside (Sigma) and 10 ml of 1.0*M* sodium acetate buffer, pH 5.0, and diluting to 100 ml with distilled water.

An aliquot (0.6 ml) of the substrate solution was placed in a test tube. The enzyme preparation (10 μl) was added, and the tubes were mixed by vortexing. The samples were incubated at ambient temperature for 40 min. Two milliliters of 0.2*N* sodium carbonate (aqueous) was then added; the tubes were vortexed; and the absorbance at 400 nm was measured. Water blanks were included (10 μl of water in place of the enzyme solution). All of the enzymes were determined in triplicate.

Arabinase Assay. For the substrate solution, 30 mg of *p*-nitrophenyl-α-L-arabinofuranoside (Sigma) and 10 ml of 1.0*M* sodium acetate buffer, pH 5.0, were combined and diluted to 100 ml with distilled water.

The incubation and determination were performed as described for the mannosidase assay.

Xylanase Assay. For the substrate solution, 30 mg of *o*-nitrophenyl-β-D-xylopyranoside (Sigma) and 10 ml of 1.0*M* sodium acetate buffer, pH 5.0, were combined and diluted to 100 ml with distilled water.

The incubation and determination were performed as described for the mannosidase assay.

Optical Microscopy

Beer or wort samples (250 ml) were centrifuged at 13,200 × *g* for 40 min. The supernatant liquid was decanted and transferred to a flask. The residue in the centrifuge bottle was resuspended (Vortex mixer) with 3 ml of the supernatant liquid. For direct examination, one drop of this suspension was placed on a microscope slide, covered with a cover slip, and examined with an American Optical 110 series microscope under the following conditions: 1) brightfield at × 600 magnification, and 2) darkfield (phase contrast), green filter, at × 600 magnification. Photographs were made through the trinocular head of the microscope with a Polaroid MP4 camera.

Optical Microscopy With Staining.

Samples were centrifuged and resuspended as described for optical microscopy. A 25-μl aliquot of the suspension was placed in a test tube with 25 μl of prepared staining solution. After mixing with a Vortex mixer, one drop was transferred to a microscope slide and a cover slip was applied. The samples were viewed under the following conditions: 1) lactophenol blue stain,[2] brightfield illumination at × 600 magnification; 2) eosin yellow stain (13), brightfield illumination at × 600 magnification; 3) rhodamine B stain, fluorescence with AO 2074 Fluorocluster and mercury lamp, × 600 magnification; 4) 8-anilino-1-naphthalene-sulfonic acid (1,8-ANS), magnesium salt stain (13), fluorescence with AO 2073 Fluorocluster and mercury lamp, × 600 magnification; 5) fluorescein isothiocyanate-bound Concanavalin A (FITC-Con A) (Sigma), containing 10 mg of NaCl and 1.5 mg of potassium phosphate per milligram of protein, made to a concentration of 1 mg in 1 ml of distilled water, or 80 mg of protein per liter, fluorescence with AO 2072 Fluorocluster and mercury lamp, × 600 magnification.

Photography was performed as described under optical microscopy.

Scanning Electron Microscopy

Haze was collected from beer by centrifugation at 13,200 × *g* and resuspended in a small amount of distilled water. The samples were sent to a consulting laboratory, where they were filtered through 0.2-μm diameter Nuclepore filteres suspended on a wire mesh grid. The filters were dried and examined by scanning electron microscopy.

Electronic Particle Counting

A Coulter Counter model ZB_i was used for counting. A Coulter Channelyzer showed the particle size distribution and was used to adjust the settings that select the size range of the particles counted (although not the size range observed). The Channelyzer was also employed to plot the particle size distributions.

Samples (200 ml) were removed from each of the Firestone tanks and made 6% with respect to sodium chloride. One half of each sample was passed through a 0.8-μm membrane filter and then a 0.2-μm membrane filter. The filtered samples were used as blanks for the matching unfiltered samples. Particle size distributions were determined in two different size ranges with the Coulter Counter, using a 15-μm diameter aperture tube and the following settings: amplification, 1 or 4; aperture current, 5.65; lower threshold, 5; upper threshold, 100; channelyzer base channel threshold, 5; and window width, 100.

RESULTS AND DISCUSSION

Haze can arise from many different sources, some of which are more readily recognized than others (5,6). Oxalate haze, for instance, can be distinguished by the presence of regular, characteristic crystals that are readily apparent with light microscopy. Haze caused by microorganisms is similarly readily identifiable. Many forms of haze, however, are not comprised of particles with regular size or shape. We have investigated several haze problems in which the haze material was amorphous. At various times we employed light scattering, chemical analysis, enzyme treatment,

[2] Paul Glenister, personal communication.

electronic particle counting, optical microscopy with and without staining, and scanning electron microscopy. We found that some of these techniques were much more helpful in characterizing the haze and identifying its source than others. Because our impressions were based on a few investigations of haze at the end of lager storage, we were interested in comparing results of tests on a number of hazes of different origin. We selected different process points and drew beer samples of varying clarity; the haze in these samples (Process Beers 1–4) could be considered "natural" to our process. We also artificially induced haze in two samples of beer and two of wort. The additions to wort approximated two of the changes occurring during fermentation—the drop in pH and the production of ethanol.

The Problem of Artifact Formation

One of the principal problems in characterizing haze is its tendency to change on handling. Therefore, the methods used must be tested to see if they produce artifacts. Essentially the only method for examining haze in beer without any change is light scattering. Even here, artifacts may arise if significant warming of the samples occurs during handling and measurement.

Most of the analytical techniques that we applied to haze require the collection of haze material from the beer or wort in which it is suspended. Because the material is generally of colloidal size, this is not a simple matter. Some hazes are dissolved on warming, and therefore the samples must be held cold during the collection period. Prolonged (40 min) centrifugation in a high speed (13,200 × *g*) refrigerated centrifuge is capable of clarifying hazy beers. In one experiment, a large number of beers at the end of lager storage (and presumably with hazes of similar particle distributions) were examined by nephelometry and their hazes were collected by centrifugation as described above. The dry weight of the collected solids correlated well with the nephelometer results (Fig. 1). This suggests that the haze material was collected quantitatively by the centrifugation.

For the Coulter Counter work, the sample particles must be suspended in an electrolyte solution. Frequently this is done by pipetting a measured volume of sample into a measured volume of membrane-filtered electrolyte. When brewing samples containing haze are added to the aqueous sodium chloride electrolyte used for most biological samples, some of the haze dissolves (with some samples, as much as 90% of the haze is soluble). Beer itself, of course, contains salts and has been used as an electrolyte for measurements of beer haze (19). However, this work (19) used a 30-μm diameter aperture and covered a range of particle diameters from 0.5–12 μm. When observations of smaller particles are desired, a smaller aperture diameter and a better electrolyte are needed. An experiment was tried in which sodium chloride was added to beer to make it an improved electrolyte that would not dissolve haze. Nephelometer measurements were made at various salt concentrations to test for artifact formation. If no dissolution or salting out occurred, a straight line should result when haze is plotted against added salt. If the line curves up, salting out has occurred. If it curves down, dissolution of haze is indicated. When this test was performed (Fig. 2), the haze increased with increasing salt addition at 0° C. At 20° C, the haze remained constant with increasing salt concentration until about 6% NaCl was reached; then evidence of salting out was seen. In the current study, the Coulter Counter work was done at room temperature with 6% added NaCl.

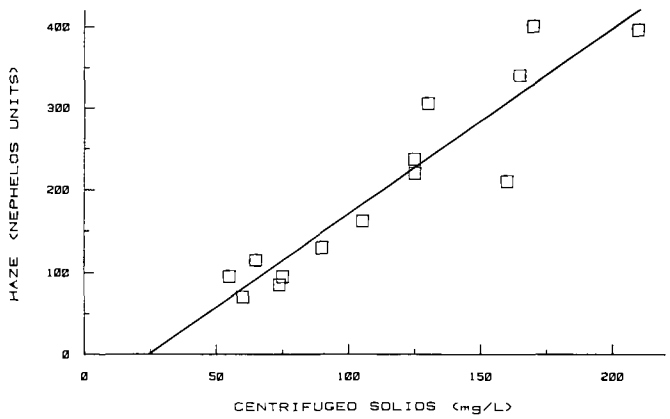

Fig. 1. Comparison of the weight of material collected by centrifugation at 13,200 × *g* and 0° C with the results of nephelometric measurement. All samples were drawn just before final filtration.

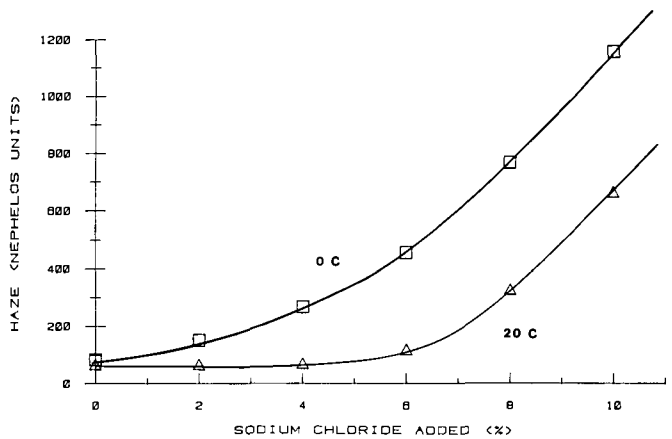

Fig. 2. Effect on light scattering of NaCl addition to beer at 0 and 20° C.

TABLE I
Various Assessments of the Amount of Haze in the Samples

Sample	Haze (nephelos)	Number of Particles[a] per Microliter Having Diameter (μm) in Range 0.305–0.842	1.00–2.76	Centrifuged Solids Concentration[b] (mg/L)
Wort + ethanol	282	395	914	261.0
Wort, pH 3.6	276	481	740	114.7
Beer 1 + tannic acid	⩾900	938	1,560	521.7
Beer 1 + β-glucan	618	595	1,096	340.5
Process beer				
1[c]	582	390	1,130	137.3
2[d]	60	721	848	62.0
3[c]	540	557	1,062	249.5
4[c]	>900	770	979	337.4

[a] Measured by electronic particle counting.
[b] Collected by centrifugation at 13,200 × *g* for 40 min at 0° C.
[c] Collected just before storage.
[d] Collected at the end of storage.

Quantitation of Haze

Virtually any investigation of haze must start with a measurement of the quantity of haze present. The results of several determinations of the amount of haze in the test samples are shown in Table I. All the techniques indicate that a considerable range of haze material is covered by the eight samples. In fact, two of the samples had so much haze that they overwhelmed the nephelometer (> 900 nephelos). A comparison of these results with one another is shown in Table II. The two high nephelometer results could not be used, and this made achievement of statistical significance more difficult. The centrifuged solids did not correlate significantly with the nephelometer results for the test samples (Table II). This could occur if the particle size distributions were dissimilar in the different samples. Examination of the Coulter Counter plots (Figs. 3 and 4) indicates that this is the case. The particles in the smaller size range did not correlate significantly with either the centrifuged solids or the nephelometer results. In contrast to this, the larger particles correlated significantly with both centrifuged solids and nephelometry. If the typical particle diameters for the two size ranges are taken as 0.6 and 1.5 μm and the equivalent volumes are calculated, an average larger particle would have 15 times the size of an average smaller particle. If the particles have similar densities, then a typical larger particle would have 15 times the weight of a smaller particle. This explains the lack of correlation between the number of small particles and the weight of collected solids. The lack of correlation with nephelometry comes about because 90° light scattering in this particle size range (where particle radius is comparable to the wavelength of the light used) is related to the radius raised to the fourth power (23). This relationship was described by Thorne for coherent light and spherical particles, neither of which is the case here. However, it does permit estimation of the relative amount of scattering of the two particle

TABLE II
Correlation Coefficients of Various Assessments of Haze

x	y	Number of Samples	Correlation Coefficient	Significance Level (%)
Centrifuged solids	Haze (nephelos)	6	0.651	ns[a]
Centrifuged solids	Particles,[b] 0.3–0.8 μm diameter	8	0.601	ns
Centrifuged solids	Particles,[b] 1.0–2.7 μm diameter	8	0.797	98
Particles,[b] 0.3–0.8 μm diameter	Haze (nephelos)	6	−0.360	ns
Particles,[b] 1.0–2.7 μm diameter	Haze (nephelos)	6	0.848	95

[a] Not significant.
[b] Per microliter.

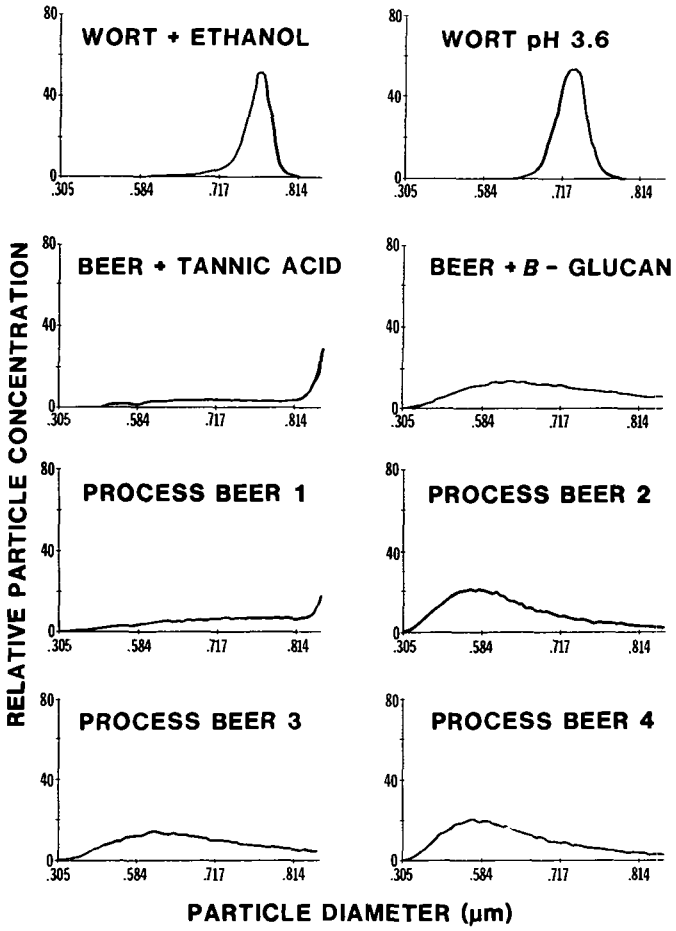

Fig. 3. Size distribution patterns for haze particles at 20° C, small particle size range.

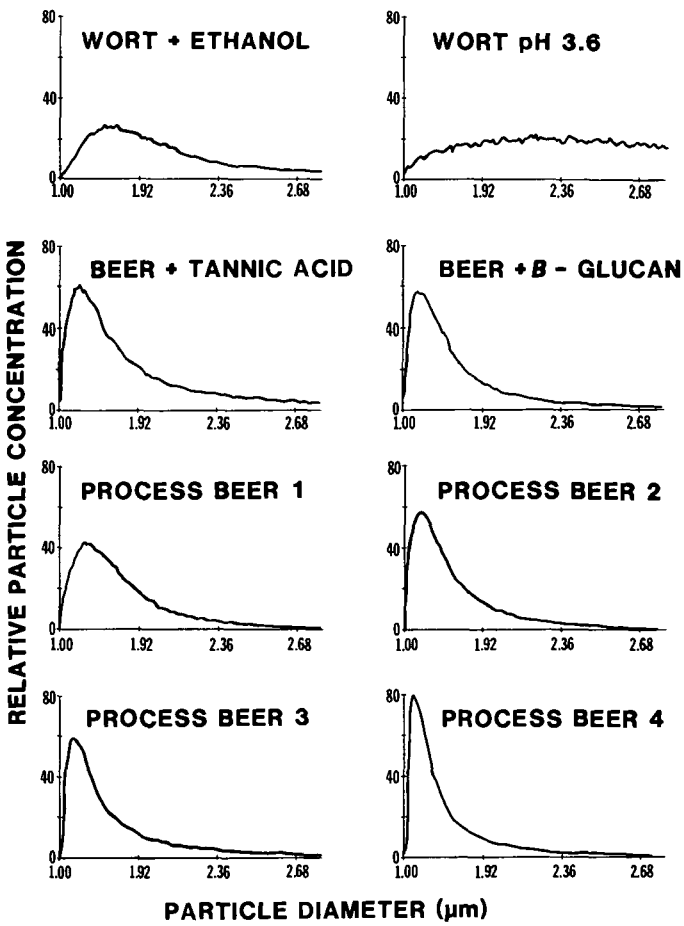

Fig. 4. Size distribution patterns for haze particles at 20° C, large particle size range.

sizes. A 1.5-μm diameter particle would scatter light 39 times as strongly as a 0.6-μm particle.

Characterization of Haze

The goal of most haze investigations is to characterize the haze so that its source can be identified. The first and most obvious approach is to examine some of the collected haze with an optical microscope. In the case of the eight haze samples studied here, this was of limited help. All eight were examined both with brightfield illumination and by using phase contrast optics (Table III). Most of the samples had irregularly shaped particles with no discernible structure (Fig. 5). In the wort sample to which ethanol was added, many small regular crystals could be seen (Fig. 6). These appeared square with rounded sides (barrel shaped); not much else was visible in this sample. The crystals were more readily apparent with phase contrast optics and looked very much like a somewhat unusual form of the oxalate crystals shown by Glenister (13). Figure 6 points out one of the difficulties of microscopic examination. Visually this sample appeared to contain little other than oxalate. Chemical analysis indicated, however, that the sum of the protein and carbohydrate accounted for nearly all of the weight with no room left for oxalate. At first we thought the crystals might not be oxalate, but they dissolved in 0.5*N* HCl, as did calcium oxalate. The sample was then reexamined microscopically. Initially it appeared as in Fig. 6. When the focus was shifted slightly and the intensity of light was reduced, large amorphous particles were seen (Fig. 7). The crystals had made a larger initial impression, and the microscopist had focused on these. A few crystals were apparent in the wort adjusted to pH 3.6, but these were very minor in comparison to the large amount of amorphous material.

In a previous haze study, a material very similar to Process Beer 4 was sent to a consulting laboratory for scanning electron microscopy. Magnifications as high as 10,000 times were employed. The resulting photographs (Fig. 8) were very clear and sharp, but the closeups of the material still showed no discernible structure. Greatly increased magnification was therefore not helpful for examining amorphous haze particles.

Staining followed by optical microscopy gives more information than microscopy alone (13). Two stains were employed with brightfield illumination (Table IV). Eosin yellow mainly stains

TABLE III
Examination of Collected Hazes by Optical Microscopy

Sample	Brightfield	Phase Contrast
Wort + ethanol	Primarily crystals, some irregular particles	Primarily barrel-shaped crystals, some irregular particles
Wort, pH 3.6	Mostly irregular particles, a few oxalate-type crystals	Irregular particles
Beer 1 + tannic acid	Irregular particles	Many irregular particles
Beer 1 + β-glucan	Many irregular particles	Many irregular particles
Process beer		
1[a]	Irregular particles	Irregular particles
2[b]	Few particles, irregular	Few particles, irregular
3[a]	Irregular particles	Irregular particles, some beer skins
4[a]	Many irregular particles, a few beer skins	Many irregular particles, a few beer skins

[a] Collected just before storage.
[b] Collected at the end of storage.

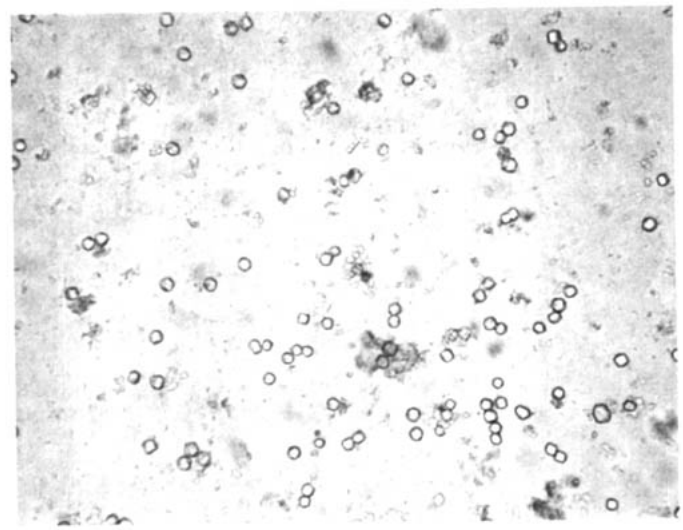

Fig. 6. Photomicrograph of haze from wort plus ethanol. Brightfield illumination at ×600 magnification.

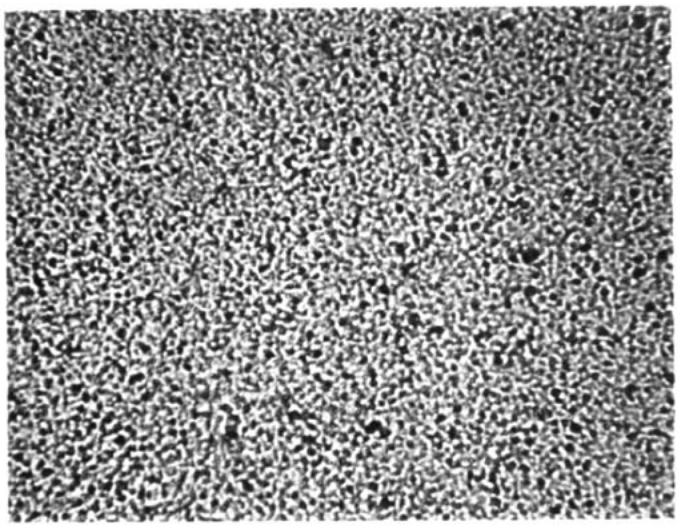

Fig. 5. Photomicrograph of haze from Process Beer 4. Phase contrast illumination at ×600 magnification.

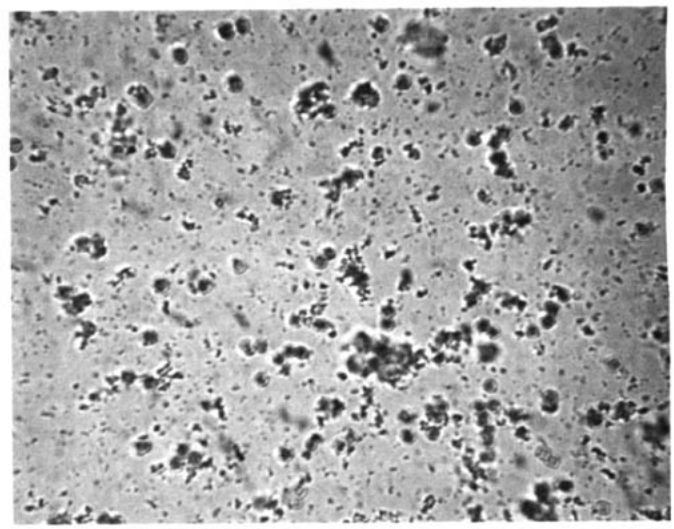

Fig. 7. Photomicrograph of haze from wort plus ethanol. Same sample and field of view as in Fig. 6 with light intensity reduced and focus shifted.

dextrin and proteinaceous matter (13). All eight hazes collected here accepted the Eosin yellow stain, indicating that all contained dextrinlike and/or proteinaceous material. Lactophenol blue is a stain that is fairly specific for the cell walls of fungi and yeast. Almost no staining was observed in the wort sample to which ethanol was added. A few round particles accepted the stain in the other wort sample, but most particles did not. This indicates, as would be expected, that little if any fungal or yeast material is present in the wort. All of the beer samples showed some degree of staining with lactophenol blue. Many of these particles were small and clumped together in spots, whereas a few larger particles appeared more like beer skins. These skins may be yeast cell "ghosts" where the cell membrane has been ruptured and the contents have been lost.

Fluorescent staining was also tried (Table V). The magnesium salt 1,8-ANS is thought to be quite specific for proteinaceous material (13). All the samples showed some degree of 1,8-ANS staining, indicating that all contained some protein. The wort adjusted to pH 3.6 and the beer with added tannic acid gave by far the strongest responses. The wort with added ethanol and Process Beer 3 gave somewhat weaker responses than did the other samples.

Rhodamine B stains many macromolecules and has been used as a yeast viability stain. All the hazes stained with Rhodamine. The crystalline material in the wort plus ethanol sample did not accept this stain.

Concanavalin A is a lectin (a carbohydrate-binding protein); it binds particularly strongly to biopolymers containing multiple α-D-mannopyranosyl residues and to a smaller degree to those with multiple α-D-glucopyranosyl residues (1). FITC-Con A acts as a fluroscent stain with a high degree of specificity. It readily stained yeast but gave little reaction with haze particles in a sample drawn from a fermenter. Viewing must be done promptly after staining because the fluorescent intensity fades quickly. FITC-Con A showed little staining with most of the haze samples. It gave a strong reaction with the wort with added ethanol, the beer plus tannic acid, and Process Beer 4.

TABLE IV
Examination of Collected Haze by Brightfield Microscopy of Stained Samples

Sample	Eosin Yellow	Lactophenol Blue
Wort + ethanol	Mainly globular and irregularly shaped particles of varying size, stain accepted to varying degrees; a few crystals that didn't stain	Virtually no staining
Wort, pH 3.6	Most particles irregular, stained; very few crystals that did not stain	A few round particles stained, mostly weakly; most particles not stained
Beer 1 + tannic acid	Many small irregular particles, stained	Some particles stained, varied from weak to strong
Beer 1 + β-glucan	Many small irregular particles, strongly stained; some large globules weakly stained	Some particles stained, with variable intensity
Process beer 1[a]	Many irregular small particles, stained	Most particles strongly stained
2[b]	A few irregularly shaped particles of varying size, strongly stained	A few particles stained, variable intensity
3[a]	Irregularly shaped particles of varying size, strongly stained	A few particles weakly stained
4[a]	Many irregular small particles, stained	Some particles stained, variable intensity

[a] Collected just before storage.
[b] Collected at the end of storage.

Chemical Analysis

The haze material collected by centrifugation was subjected to chemical analysis. The material was weighed and suspended in water at room temperature. This resulted in the dissolution of some of the haze in each case (Table VI). This may have been due either to warming from 0 to 20° C or to greater solubility of the haze material in water than in beer. Figure 9 demonstrates that in most cases well over half of the haze material was water soluble (WS). Only with the tannic acid and β-glucan additions was the larger proportion of the haze water insoluble (WI). The weight of collected solids varied over a wide range. Correlation coefficients of the WS and WI solids against the various assessments of total haze are shown in Table VII. Probably the most significant finding here is the strong correlation between the WI solids and the number of larger particles. This suggests that the WS fraction was of lesser importance in causing haze. The composition of the haze is shown in Fig. 10. Nearly all of the haze in each case appeared to be accounted for by the sum of the protein and carbohydrate results. The only significant exception to this was the beer with tannic acid added. In this case 50 mg/L of solid was present in addition to the carbohydrate and protein. This must be due to the tannic acid (105 mg/L) added. Although nearly all of the tannic acid would be expected to enter the haze, some of it would be detected as carbohydrate in the analysis; this is because tannic acid consists of glucose and gallic acid moieties. The tannic acid haze also had the highest percentage of protein (52%), which is not surprising in view of the well-known interactions of proteins with phenolic compounds. The other hazes had protein contents ranging from 16 to 32%, which is a fairly narrow range; the greater portion of these hazes, 68–87%, was carbohydrate. They thus differed more in quantity than in quality, and therefore identification of the source of the haze from the protein and carbohydrate results alone was not readily possible. Further characterization of protein by source (barley, yeast, adjunct, bacteria, etc.) would require sophisticated methods not available in the present study; antigenic methods, for instance, have been successfully applied to haze protein (22). The carbohydrate fraction can be further characterized, however. A number of approaches were tried, and the results are shown in Table VI. The

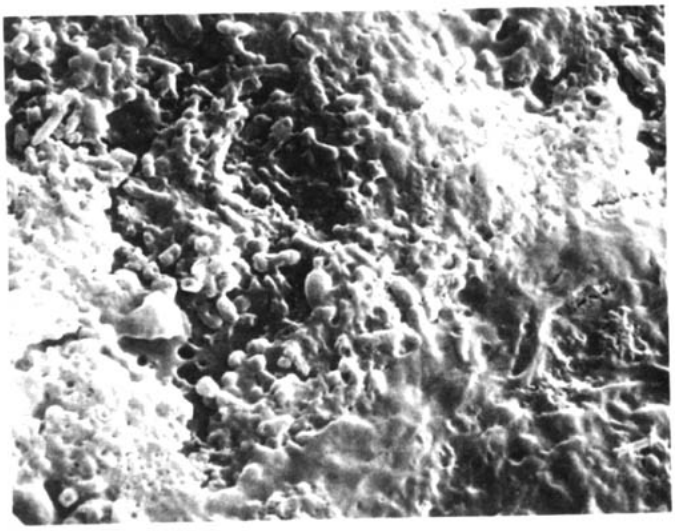

Fig. 8. Scanning electron micrograph of a haze similar to Process Beer 4 at ×5,000 magnification.

results for reducing sugar were quite surprising (Fig. 11). Except for the worts, in which a high degree of reducing sugar is expected and, in fact, hard to avoid when collecting the haze, the other hazes ranged from 19 to 34% of the carbohydrate. The average incidence of reducing sugar for the beer samples was 28% of the carbohydrate or roughly one sugar out of four. When polymeric saccharides are formed, the reducing group is used to connect each sugar to the next one. This means that each polymerc unit has only one reducing group. One reducing group for each four sugars indicates that the average carbohydrate molecule in the haze is a tetrasaccharide. This is surprisingly short; most haze carbohydrate has been presumed to be of quite large molecular weight. Although the reducing sugar reagent was alkaline, a test showed that hydrolysis of carbohydrate did not occur during the assay.

The suggestion has been made that polyphenols can cause a high result in reducing sugar tests (15). In polyphenol determinations made on hazes similar to some examined here, the amount of polyphenol detected was less than 1% of the weight of the haze material (data not shown). This is quite reasonable in view of several recent studies that have demonstrated that the dimeric polyphenols are active in haze formation, that monomeric polyphenols are essentially inactive but are entrained in haze by adsorption, and that trimeric and higher polyphenols are virtually insoluble in beer and so are present in very small quantities (9,12,14,18). The amounts of dimeric polyphenols in boiled worts (6–9.5 mg/L) and in beer 5–8 mg/L) have been determined by an isotope dilution method (18). These results indicate that approximately 1–2 mg/L of dimeric polyphenol may be lost

TABLE V
Examination of Collected Haze by Fluorescence Microscopy of Stained Samples

Sample	Stain		
	1,8-ANS[a]	Rhodamine B	FITC-Con A[b]
Wort + ethanol	Little staining	Coating on some crystals fluoresced; small irregularly shaped particles fluoresced and the entire background fluoresced	No crystals evident; very large irregular masses, strongly stained
Wort, pH 3.6	Many irregular large particles	Many large particles, strongly fluoresced	Irregular particles, weakly stained
Beer 1 + tannic acid	Some very large particles, strongly fluoresced; entire background weakly fluoresced	Many small irregular particles, some aggregated into very large clumps intensely fluoresced; background weakly fluoresced	Very large irregular mass
Beer 1 + β-glucan	Some medium-sized particles, fluoresced	Many large and medium-sized particles, strongly stained	Very weak staining
Process beer			
1[c]	A few medium-sized particles, stained	Many particles, weakly stained	No staining
2[d]	Many small particles	Moderate number of medium-sized particles, fluoresced	No staining
3[c]	A few medium-sized particles	Many medium-sized particles	Very weak staining
4[c]	Some small, a few medium-sized particles; entire background weakly fluoresced	Very many small particles, weakly stained; background fluoresced	Large irregular masses, well-stained

[a] 8-Anilino-1-naphthaline-sulfonic acid.
[b] Concanavaline A-fluorescein isothiocyanate.
[c] Collected just before storage.
[d] Collected at the end of storage.

TABLE VI
Chemical Analysis of Collected Haze Fractions

	Wort + Ethanol	Wort, pH 3.6	Beer 1 +		Process Beer			
			Tannic Acid	β-Glucan	1[a]	2[b]	3[a]	4[a]
Haze, nephelos	282	276	≫900	618	582	60	540	>900
Solids								
Total, mg/L	261	114.7	521.7	340.5	137.3	62.0	249.5	337.4
Water-soluble, mg/L (%[c])	159.4(61.1)	102.8(89.6)	125.7(24.0)	130.0(38.2)	130.1(94.8)	56.9(91.8)	205.4(82.4)	280.4(83.1)
Water-insoluble, mg/L (%[c])	108.5(41.6)	14.1(12.3)	401.0(76.9)	207.6(61.0)	8.2(6.0)	5.2(8.4)	39.8(16.0)	56.8(16.8)
Protein, mg/L (%[c])	54 (21)	30 (26)	271 (52)	55 (16)	33 (24)	11 (17)	73 (29)	107 (32)
Carbohydrate, mg/L (%[c])	208 (80)	82 (72)	201 (39)	295 (87)	99 (72)	51 (82)	174 (70)	228 (68)
Reducing sugars, mg/L (%[d])	120 (58)	47 (58)	54 (27)	76 (26)	31 (32)	9 (19)	59 (34)	61 (29)
Nonreducing sugars, mg/L (%[d])	88 (42)	35 (42)	147 (73)	219 (74)	68 (69)	41 (81)	115 (66)	152 (71)
Glucan, mg/L (%[d])	63 (31)	40 (49)	86 (43)	175 (59)	51 (51)	16 (32)	73 (42)	92 (43)
Nonglucan, mg/L (%[d])	145 (70)	42 (51)	115 (57)	121 (41)	48 (49)	34 (68)	101 (58)	122 (57)

[a] Collected just before storage.
[b] Collected at the end of storage.
[c] Of total solids.
[d] Of carbohydrate.

between wort and filtered beer (due to consumption by yeast, adsorption on yeast or vessel walls, deposition in the storage tank, and incorporation into the haze suspended in beer). Even if all the dimeric polyphenol entered the hazes determined here, it would amount at most (with Process Beer 2 at 62 mg/L) to 3.2% of the haze weight. The amount of monomeric polyphenol in WS haze formed after filtration has recently been shown to range from 0.1–0.33% (8). If the prefilter haze contains a similar amount of monomeric polyphenol, then the total polyphenol cannot exceed 3.5% of the haze. A test was made to assess the response of the DNS reducing sugar method to two phenolic compounds (Table VIII). The DNS procedure did give some response to the polyphenolic test compounds. However, if the maximum response (27%) is applied to the maximum estimate of polyphenolic material present (3.5% of the haze), the result is an overestimate of reducing sugar content of less than 1% of the weight of haze collected. The reducing sugar response therefore appears to be caused by actual reducing sugars.

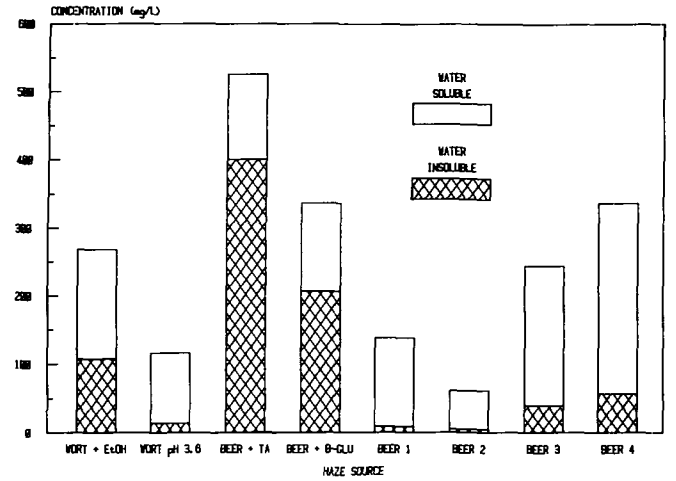

Fig. 9. Comparison of water-soluble and water-insoluble content of haze samples. EtOH = ethanol, TA = tannic acid, B-GLU = β-glucan.

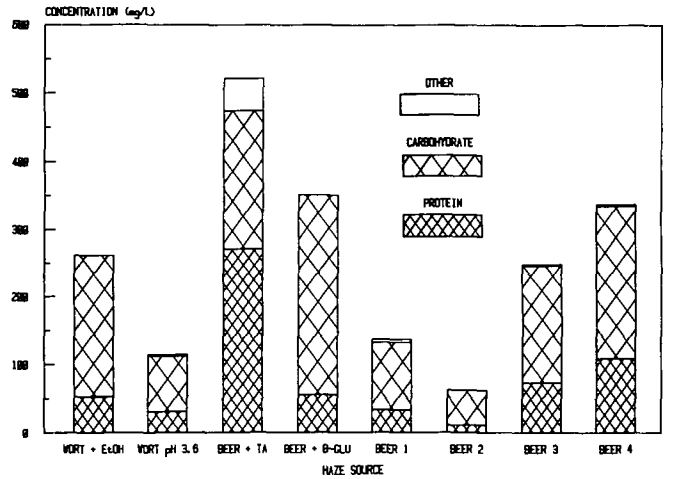

Fig. 10. Comparison of protein and carbohydrate content of haze samples. EtOH = ethanol, TA = tannic acid, B-GLU = β-glucan.

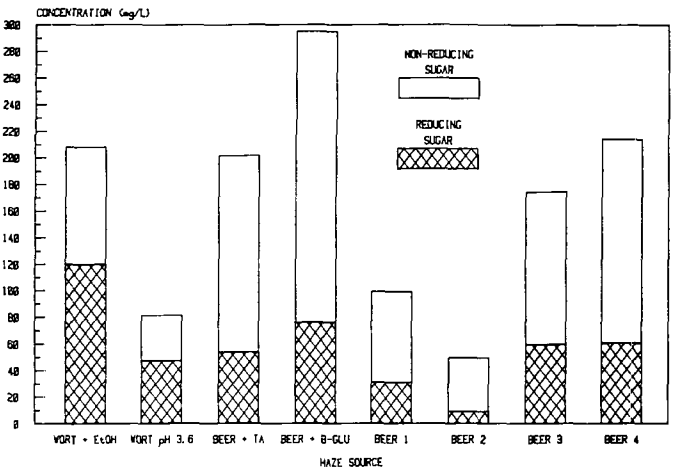

Fig. 11. Comparison of reducing and nonreducing sugar content of haze samples. EtOH = ethanol, TA = tannic acid, B-GLU = β-glucan.

The possibility was considered that if the removal of beer or wort from the centrifuge bottle after collection was incomplete, a figure might result that was partly due to soluble sugars in the unremoved liquid. Centrifuge bottles were filled with wort or beer and drained as in the normal collection procedure. The bottle was then washed with water and the reducing sugar content was determined. The result obtained with wort (27.6 mg/L) was larger and would cause a significant overestimation of the reducing sugar content of the haze. The result for beer was much smaller (2.22 mg/L) and would have an almost insignificant effect. This is supported by the finding that the percentage of reducing sugar in the carbohydrate is very nearly the same in the WI as in the WS hazes (Tables IX and X). If the results for reducing sugar were mainly caused by poor drainage of beer from the centrifuge bottles, the material should dissolve preferentially in water and the WI haze should have a lower percentage of reducing sugar. Some of the WS fractions were examined by HPLC and found to contain mainly DP4 carbohydrate with smaller amounts of DP3 and DP2. This confirms the above analysis and suggests that the carbohydrate fraction of the haze is largely of medium molecular weight with only a small amount of polysaccharide. It appears to be similar in composition to the most

TABLE VII
Correlations of Water-Soluble (WS) and Water-Insoluble (WI) Haze Solids Fractions with Assessments of Total Haze

Solids Fraction x	Total Haze y	Number of Samples	r	Significance
WS	Total solids	8	0.901	99.5%
WI	Total solids	8	0.888	99.5%
WS	Haze (nephelos)	6	0.636	ns[a]
WI	Haze (nephelos)	6	0.435	ns
WS	Particles,[b] 0.3–0.8 μm diam.	8	0.719	95%
WI	Particles,[b] 0.3–0.8 μm diam.	8	0.607	ns
WS	Particles,[b] 1.0–2.7 μm diam.	8	0.073	ns
WI	Particles,[b] 1.0–2.7 μm diam.	8	0.833	98%

[a] Not significant.
[b] Per microliter.

TABLE III
Polyphenol Response by the Dinitrosalicylic Acid Reducing Sugar Test

Test Compound	Molecular Weight	Weight Used (mg)	Reducing Sugar[a]	Percent Response
Rutin	610.5	96	26	27.1
Quercetin	302.2	96.6	6	6.2

[a] Milligrams of glucose equivalent.

prominent carbohydrates in beer.

The glucan fraction of the carbohydrate was estimated by hydrolysis followed by the glucose oxidase test, which permits the determination of glucose in the presence of other sugars. The results should include free glucose in the haze as well as glucans or glucose polymers of any length and linkage (α-1-4, α-1-6, β-1-3, β-1-4, etc.). The glucan content of the carbohydrate ranged from 31 to 59% (Fig. 12). It was highest (59%) in the sample to which β-glucan had been added. In the other samples the range was 31–51%. The wort to which ethanol was added had the largest proportion (70%) of nonglucan carbohydrate. With beer samples, HPLC was used to examine hydrolysates of haze; nearly all the sugar present was found to be either mannose or glucose.

The WS fractions of the haze (Table IX) tended to be lower in protein than the total haze was and correspondingly higher in carbohydrate. The WS nonglucans were more prominent than the glucans.

The WI fractions of haze (Table X) were particularly rich in protein (average 53%). Neither glucans nor nonglucans were dominant in the carbohydrate.

Enzymatic Digestion

Another approach to the characterization of haze is the use of enzymes. The specificity inherent in the enzyme can serve as a probe to test for the presence of a particular material that is attacked by that enzyme. Because commercially available enzymes are isolated from natural sources, testing enzyme preparations for activities of other than the stated enzyme is necessary before conclusions can be made. Enzymes can be used in several ways. They can be added to beer containing haze, which is then stored and periodically examined. A change in light scattering indicates that the enzyme in question caused a change in the haze. Alternatively, the Coulter Counter, rather than the nephelometer, can be used as the monitoring device for observing enzyme-caused changes in haze (19). Perhaps the best way to use enzymes, however, is to add them to isolated haze material and monitor their effect by measuring a change in the level of a reaction product.

None of the many proteolytic enzymes available can discriminate between proteins according to their source because nearly all proteins contain a mixture of the 20 or so common amino acids. The fact that a proteolytic enzyme attacks a haze therefore shows nothing that a protein (Kjeldahl nitrogen) determination has not already shown quantitatively.

Carbohydrase enzymes, however, offer some interesting possibilities. The activities of these can be assessed by measuring the reducing sugars liberated. β-Glucanases and amylases can indicate

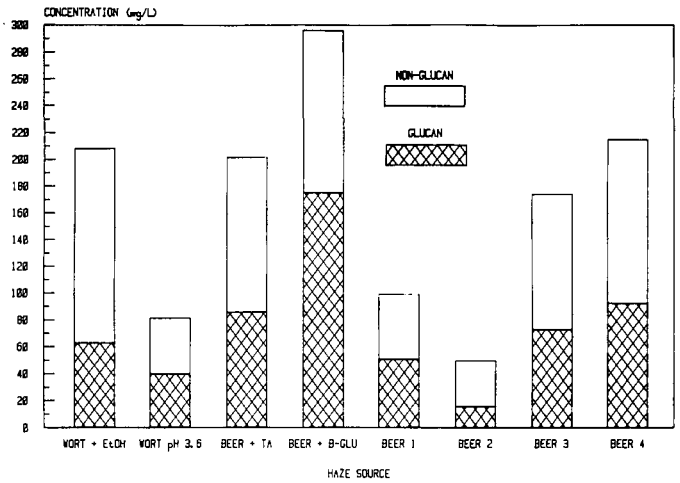

Fig. 12. Comparison of glucan and nonglucan content of haze carbohydrate. EtOH = ethanol, TA = tannic acid, B-GLU = β-glucan.

TABLE IX
Chemical Analysis of Water-Soluble (WS) Haze Fractions

	Wort + Ethanol	Wort, pH 3.6	Beer 1 + Tannic Acid	Beer 1 + β-Glucan	Process Beer 1[a]	Process Beer 2[b]	Process Beer 3[a]	Process Beer 4[a]
Solids, mg/L (%[c])	159.4(61.1)	102.8(89.6)	125.7(24.0)	130.0(38.2)	130.1(94.8)	56.9(91.8)	205.4(82.4)	280.4(83.1)
Protein, mg/L (%[d])	25 (15)	21 (20)	17 (14)	15 (11)	28 (21)	6.5(12)	50 (25)	79 (28)
Carbohydrate, mg/L (%[d])	133 (83)	79 (77)	107 (85)	125 (96)	98 (75)	49 (87)	154 (75)	199 (71)
Reducing sugars, mg/L (%[e])	85 (64)	46 (59)	20 (19)	27 (22)	31 (31)	8.9(18)	54 (35)	50 (25)
Nonreducing sugars, mg/L (%[e])	48 (36)	33 (41)	87 (81)	98 (78)	67 (69)	40 (82)	100 (65)	149 (75)
Glucan, mg/L (%[e])	40 (30)	40 (50)	52 (49)	27 (22)	50 (51)	16 (32)	59 (39)	32 (16)
Nonglucan, mg/L (%[e])	93 (70)	39 (50)	55 (51)	98 (79)	48 (49)	33 (68)	95 (62)	167 (84)

[a] Collected just before storage.
[b] Collected at the end of storage.
[c] Of total.
[d] Of WS solids.
[e] Of WS carbohydrate.

TABLE X
Chemical Analysis of Water-Insoluble (WI) Haze Fractions

	Wort + Ethanol	Wort, pH 3.6	Beer 1 + Tannic Acid	Beer 1 + β-Glucan	Process Beer 1[a]	Process Beer 2[b]	Process Beer 3[a]	Process Beer 4[a]
Solids, mg/L (%[c])	108.5(41.6)	14.1(12.3)	401.0(76.9)	207.6(61.0)	8.2(6.0)	5.2(8.4)	39.8(16.0)	56.8(16.8)
Protein, mg/L (%[d])	29 (27)	9 (64)	240 (60)	42 (20)	6 (73)	4 (76)	22 (56)	28 (49)
Carbohydrate, mg/L (%[d])	75 (70)	5.6(40)	94 (23)	170 (82)	1.4(18)	1.3(25)	20 (50)	30 (52)
Reducing sugars, mg/L (%[e])	36 (48)	0.6(43)	35 (37)	49 (29)	0.5(36)	0.4(31)	6.0(30)	11 (36)
Nonreducing sugars, mg/L (%[e])	39 (52)	0.8(57)	59 (63)	122 (71)	0.9(64)	0.9(69)	14 (70)	19 (64)
Glucan, mg/L (%[e])	46 (61)	0.8(53)	32 (34)	148 (87)	0.6(43)	0.2(15)	16 (80)	10 (34)
Nonglucan, mg/L (%[e])	30 (39)	0.7(47)	62 (66)	22 (13)	0.8(57)	1.1(85)	4.0(20)	20 (66)

[a] Collected just before storage.
[b] Collected at the end of storage.
[c] Of total.
[d] Of WI solids.
[e] Of WI carbohydrate.

if glucose is present in specific chemical linkages such as β-glucan or α-1-4 glucan (starch). Mannosidase can detect the presence of mannan without the lengthy preparations needed for HPLC or paper chromatography (hydrolysis and clean up).

Four different enzymes were used to digest the haze material. These preparations were characterized for their activities in attacking the linkages found in α-glucans (starch), β-glucans, α-mannans, α-arabinans, and β-xylans (Table XI). The latter two tests were included because pentose hazes have previously been reported in beer (7). For the starch and β-glucan tests, the enzymes were incubated with the indicated substrate and the amount of liberated reducing sugars was measured. In the other three cases, artificial substrates were used. The α-amylase preparation was quite pure, exhibiting only a trace of β-glucanase activity besides the amylase activity. The β-amylase, however, had small amounts of the four other enzyme activities tested for and was thus somewhat impure. The α-mannosidase was essentially pure. The β-glucanase preparation exhibited more amylolytic activity than β-glucanase activity. This points out the need for testing enzymes when conclusions are to be drawn regarding their substrates.

The four enzyme preparations were used to digest the haze material. The liberated reducing sugars were determined and the results are shown in Table XII. The α-amylase and β-amylase had fairly similar levels of activity in the process beer samples. These results are consistent with the reducing sugar results, which suggested that some of the normal α-glucan material present in wort and beer in large amounts is incorporated into the haze material. A portion of this carbohydrate can be degraded by amylases if they are given enough time (at least one domestic light beer is made by making use of this phenomenon[3]). The higher results for β-amylase appears to be due to the other activities present in this preparation. The α-amylase results can be taken as an indication of the amount of α-glucan in the hazes.

The β-glucanase preparation had some activity in all of the samples. Because of the starch-hydrolyzing ability of this preparation, however, the results should be compared with those obtained with α-amylase. When the two enzymes produced nearly equal values, the change was probably caused by α-glucan in the haze. When the β-glucanase figure exceeded the α-amylase result, the haze in question probably contained β-glucan. All four of the process beers appeared to have some β-glucan, although this was most pronounced in the case of beer 4. The beers to which tannic acid and β-glucan were added had very large amounts of β-glucan in the haze. The two wort samples had some β-glucan, particularly the one to which ethanol was added. β-Glucan is mainly considered to arise in barley, where many glucans with β-1-3 and β-1-4 linkages are present (11,20). We have found that nearly all β-glucanase preparations will also attack the glucans present in yeast cell walls;[4] these reportedly contain β-1-3 and β-1-6 linkages (10,17,21). The action of β-glucanase on yeast cell walls is frequently aided by the presence of protease, which presumably helps to remove cell wall protein and render the β-glucan more accessible to the β-glucanase.

Mannosidase had no effect on three of the beers and one of the worts. It had substantial activity on the beer treated with tannic acid, on Process Beer 4, and on wort plus ethanol. A smaller amount of mannosidase activity was found on the beer with added β-glucan. The results indicate the presence of mannan in these samples. Interestingly enough, the same samples had substantial amounts of nonglucan material (Table VI) and also stained well with FITC-Con A. Mannan comprises a substantial part of the carbohydrate in yeast cell walls (10,17,21). The mannose results and the lactophenol blue staining patterns suggest that the mannan found in two of the beer samples is of yeast origin. The mannose found in the wort plus ethanol sample presumably originated in malt. Barley has been shown to contain mannose, and one particular fraction of cell wall had as much as 20–26% mannan (11,20). Mannan in the malt may survive mashing preferentially due to a lower level of enzymatic attack than is the case for β-glucans or starch. If so, the wort would contain mannan of high molecular weight, which would be readily precipitated by ethanol.

[3] Unpublished data.

TABLE XI
Characterization of Enzyme Preparations

Enzyme Preparation	Activity[a]				
	α-Amylase	β-Glucanase	α-Mannosidase	α-Arabinase	α-Xylanase
α-Amylase	1.81	0.011	0	0.005	0
β-Amylase	1.62	0.144	0.130	0.052	0.079
α-Mannosidase	0	0.007	0.962	0.016	0
β-Glucanase	1.85	1.04	0.004	0.016	0

[a] In μmol of hexose or pentose liberated per liter per minute.

TABLE XII
Increase in Reducing Sugars[a] Caused by Enzyme Digestion of Haze

Sample	α-Amylase	β-Amylase	β-Glucanase	α-Mannosidase
Wort + EtOH	578	1,111	640	431
Wort, pH 3.6	267	978	328	0
Beer 1 + tannic acid	178	889	1,367	653
Beer 1 + β-glucan	178	311	1,822	87
Process beer				
1[b]	311	345	444	0
2[c]	87	87	108	0
3[b]	267	311	328	0
4[b]	311	311	499	501

[a] In μg of glucose equivalent per liter per minute.
[b] Collected just before storage.
[c] Collected at the end of storage.

CONCLUSIONS

A number of different methods were compared on a series of hazes. Nephelometry is very simple operationally and has the least risk of artifact formation. It cannot, however, be considered quantitative unless the particle size distribution of the haze particles remains constant. Collecting haze material in a refrigerated, high speed centrifuge appeared to be satisfactory; it clarified the samples, and the weight of material obtained correlated well with nephelometry in a situation where the haze particles were known to be similar. Electronic particle counting must be done very carefully because it is prone to artifact formation. Under proper conditions it gave better agreement with the amount of solids collected by centrifugation than did nephelometry, and it can also show the particle size distribution of a haze. The concentration of larger particles, 1.0–2.9 μm diameter, had a significant correlation with nephelometer and centrifuged haze results, but the level of smaller particles, 0.3–0.8 μm diameter, did not.

Optical microscopy was of little help for amorphous samples. Scanning electron microscopy had the same difficulty; it showed highly magnified pictures of irregular material without discernible structure. Optical microscopy of stained samples was more informative because, even if particles are of varying shapes, they may accept or reject characteristic stains. Some stains are absorbed by proteins, others by carbohydrates, and so on. This is often helpful but is not always definitive, as many stains lack specificity. Of the stains used in this study, lactophenol blue and, particularly, FITC-Con A were most helpful. Qualitative microscopy such as was done here must be interpreted cautiously because the conditions selected may emphasize a minor constituent of the haze and give a misleading impression. Much of the haze tends to be water

[4] Unpublished data.

soluble and presumably dissolves to some degree on mixing with the staining reagent.

Chemical analysis is clearly the most quantitative of the techniques employed and can give a more realistic impression of the relative amounts of various kinds of material present than does microscopy. Chemical analysis is very laborious, and the results obtained may not be conclusive. All the hazes examined in this study were almost entirely protein and carbohydrate. Determination of the relative amounts of glucan and nonglucan carbohydrates was helpful in giving some indication of the origin of the haze.

Enzymatic digestion of the haze gives a good idea of the carbohydrate composition of haze with much less work than is needed for chemical analysis and can provide information about the nature of the carbohydrate linkages.

All of the hazes were basically similar. Only the haze caused by tannic acid addition had a significant amount of material other than protein and carbohydrate. The hazes from beers with added tannic acid and barley β-glucan were mainly WI. With all the other samples, well over half of the haze material was WS. A surprisingly large percentage (typically 25%) of the haze carbohydrate was reducing sugar, which indicated that it had, on the average, a degree of polymerization of 4. All of the hazes had substantial amounts of nonglucan as well as glucan carbohydrate, including the beer with added barley β-glucan. The hazes from ethanol-treated wort, beer plus tannic acid, and one of the process beer samples contained a substantial amount of mannose, as indicated by enzyme treatment and FITC-Con A staining. This apparently can arise either through yeast lysis or from barley cell walls. Lactophenol blue stained yeast cell wall material but did not stain haze material from wort.

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