CHLOROETHANOL AS A CEREAL PROTEIN DISPERSANT[1]

N. W. TSCHOEGL[2]

ABSTRACT

Wheat gluten may be dispersed virtually completely in a 0.01*N* solution of hydrochloric acid in 70% aqueous 2-chloroethanol, from which it may be recovered by precipitation with diethyl ether with its properties apparently unaltered. A 0.1*N* solution of hydrogen chloride in anhydrous chloroethanol also disperses wheat gluten completely, but there is some loss of amide nitrogen and some of the free carboxyl groups become esterified. After precipitation with diethyl ether the cohesive properties of the gluten are lost. Anhydrous acid chloroethanol may be used also in the direct extraction of the proteins from wheat and rye flour, but is less effective in the extraction of barley and oat proteins. The extraction of the protein from the germ flour of the carob bean *(Ceratonia siliqua)* is still less complete. Acid-free anhydrous chloroethanol disperses about 60% of wheat gluten. Aqueous acid chloroethanol is about as efficient as anhydrous acid chloroethanol for wheat but extracts more oat flour protein.

The intrinsic (bulk) viscosity of gluten dispersed in aqueous acid chloroethanol is much higher than that of gluten dispersed in anhydrous acid chloroethanol, but the reverse is true in regard to the limiting surface viscosities of gluten spread from these dispersions at an oil/water interface.

The investigation reported in this paper arose from a study of spread monolayers of wheat gluten and other cereal proteins (15,16), in particular the choice of a suitable dispersion medium for spreading purposes. Dispersions in dilute acids and in sodium salicylate were the obvious first choice. From the former, however, gluten could not be spread without the aid of a spreading agent. Furthermore, dispersions of gluten in dilute acids are known (14) to be very polydisperse. Gluten was found to spread readily from dispersions in 10% w/v sodium salicylate, the salicylate itself apparently acting as the spreading agent, but the films gave rather low surface viscosities.

Attention was therefore turned to the chance observation by Hess (9) that native wheat flour protein could be dispersed completely in 2-chloroethanol and recovered by precipitation with diethyl ether with its properties apparently unaltered. When this observation was checked, virtually complete dispersion of vacuum-dried gluten was obtained in a clear but not specially purified laboratory sample of chloroethanol. The gluten particles first swelled, then disintegrated, and were completely dispersed on shaking after about 3 days. Centrifugation removed some starch and also some

[1]Manuscript received August 31, 1959.
[2]Senior Research Officer, Bread Research Institute of Australia, North Ryde, N.S.W.

protein, but most of the latter could be redispersed by shaking up the residue with a few ml. of the dispersant. The supernatant was much less turbid than a dispersion of the same concentration in sodium salicylate and gave no iodine reaction. Gluten films could be spread readily from a suitable dilution and showed much higher surface viscosities than films spread from dispersion in sodium salicylate. However, when the experiment was repeated with a carefully purified sample of chloroethanol, only about 60% of the gluten would disperse.

As chloroethanol appeared to be an attractive dispersion medium for surface chemical studies in particular and physicochemical investigations in general, some of the factors influencing its gluten-dispersing power were investigated. These investigations were then extended to the direct extraction of the proteins from wheat flour and from the flours of rye, barley, and oat, and the germ flour of the carob bean *(Ceratonia siliqua)*. Carob germ flour was included since it (and perhaps that of some related species (3)) is apparently the only known nonwheat-plant protein to possess gluten-forming ability (12,13).

Materials and Methods

Reagents were of A.R. quality unless otherwise stated. Water was first deionized and then distilled from an all-glass still containing a trace of sulfuric acid.

Flours. Wheat flours were milled on a Buhler experimental mill from sound whole grain of known variety. Rye, barley, and oat flours were milled on an Allis-Chalmers mill from whole rye, pearled barley, and dehulled oats.

Carob bean seeds were softened by boiling in water for 30 minutes and soaking in a fresh portion of water overnight. They were then opened by hand, and the germ was picked out and dried over anhydrous calcium chloride. This procedure was thought to be preferable to that of Rice and Ramstad (12) in which the seed coats are carbonized in concentrated sulfuric acid. The germ was ground in a laboratory hammer mill and bolted through 56-mesh nylon gauze. The samples were analyzed by the official methods of the AOAC (2) for protein, moisture, ash, and fat. Doughs made from the wheat flours were tested on the Chopin Alveograph. The analytical and physical test data are shown in Table I.

Preparation of Gluten. Gluten was washed from the medium wheat flour following the standard procedure detailed in *Cereal Laboratory Methods* (1), except that Dill and Alsberg's (6) phosphate

TABLE I

ANALYTICAL DATA OF FLOURS

ANALYTICAL MEASUREMENT	FLOUR						
	Strong Wheat	Medium Wheat	Weak Wheat	Rye	Barley	Oat	Carob Germ
	%	%	%	%	%	%	%
Protein (dry basis)	11.71	12.50	9.92	6.52	6.15	8.20	62.2
Moisture	15.0	14.4	12.8	13.4	12.2	9.2	9.3
Ash (dry basis)	0.55	0.48	0.53	0.38	0.59	0.74	...
Fat (dry basis)	1.91	1.80	2.06	1.56	1.70	8.68	9.20
Alveogram "strength"	84	48	13	..	..	..	...

buffer was substituted for tapwater. The gluten was dried *in vacuo,* powdered, bolted through 56-mesh nylon gauze, and stored over phosphorus pentoxide in a vacuum desiccator. The protein content of the dried gluten was about 80%.

Dispersion Media. 2-Chloroethanol was purified from laboratory-grade material. After a preliminary distillation under reduced pressure, the sample was carefully dried and freed from acid over desiccated potassium carbonate and twice redistilled under a vacuum of about 18–22 mm. Hg. The purified chloroethanol had an acidity of less than 0.005% w/v expressed as hydrochloric acid and a water content of less than 0.5% w/v. Solutions of hydrogen chloride in purified chloroethanol were prepared by passing in dry hydrogen chloride gas (19) until a concentration of 0.365% (0.1*N*) was attained. Solutions of lower acidity were obtained by dilution of the 0.1*N* sample with purified chloroethanol. Aqueous acid chloroethanol solutions were prepared by diluting anhydrous acid chloroethanol of the required acidity with hydrochloric acid of the same strength.

Solutions of other acids in purified chloroethanol were obtained by adding the required amounts of concentrated sulfuric, anhydrous (90%) formic, glacial acetic, or trichloroacetic acid.

Solutions of acids in alcohols were similarly prepared from redistilled n-butanol, isopropanol, and from absolute ethanol freshly distilled from calcium oxide.

Preparation of Dispersions. Dispersions were prepared by extracting about 100 mg. of dried gluten or an equivalent amount of flour with 10 ml. of the dispersing agent in glass-stoppered test tubes. In most of the experiments the tubes were kept in a thermostat at 25°C. for 3 days and were shaken at regular intervals. Some experiments were carried out in which the tubes were placed in boiling water for 15 minutes.

The dispersions were separated from the residues by centrifuging at about 1,000 g. for 20 minutes and decanting the supernatants. The residues were then shaken up with 3–5 ml. of the dispersant, the tubes recentrifuged, and the supernatant decanted. This process was repeated and the three supernatants combined.

Degree of Dispersion. This was obtained from the amount of protein in dispersion and the amount of undispersed protein remaining in the residue. Suitable aliquots of the supernatants and the residues were analyzed by a semimicro-Kjeldahl procedure. Water was added to the supernatant aliquots to distill off the chloroethanol or alcohol and prevent the formation of volatile sulfates. Gluten residues were usually dissolved in 50% sulfuric acid. Very small gluten residues were digested *in toto*. All determinations were carried out in duplicate.

Precipitated Gluten. About 2 g. of gluten dispersed in 200 ml. of dispersant were precipitated by the addition of 200–400 ml. of ether. After standing for about 5 days, the clear supernatant was centrifuged off and a fresh portion of ether was added to the precipitate. The ether was removed after another 3–5 days and the precipitated acid gluten was dispersed in 200 ml. of water in a Waring Blendor. After the foam had settled, the gluten was reprecipitated by addition of 0.1*N* sodium hydroxide to pH 6.8. The precipitated gluten was then dried *in vacuo*.

Amide Content. This was determined by the 2*N* hydrochloric acid extrapolation method as described by Leach and Parkhill (11). No correction was made for free ammonia.

Esterification. The number of alkoxy groups per 10^5 g. of protein were determined by the Zeisel method. These determinations were carried out by the CSIRO Microanalytical Service, Melbourne, in sixfold replication for each sample, and the results were averaged. Calculation of the degree of esterification was based on Fraenkel-Conrat and Olcott's (7) figure of 36 carboxyl groups per 10^5 g. of original gluten protein. The blank due to the methionine content of the original gluten protein was subtracted from the number of alkoxy groups found in the precipitated gluten proteins (4,7).

Viscosity. Bulk viscosities were determined in B.S. No. 1 capillary viscometers. No kinetic energy correction was made.

Surface Viscosity. Surface viscosity measurements were carried out at the oil/water interface with an oscillating-needle, surface-torsion pendulum as described by Tschoegl and Alexander (16).

Results

Influence of Water and Hydrochloric Acid Content. To find an explanation for the difference in gluten-dispersing power of unpurified and distilled chloroethanol, the influence of the two most likely impurities, namely water and hydrochloric acid, was investigated. Analysis of several laboratory-grade samples showed them to contain about 3–5% water, with acidities from 0.006 to 0.06*N*, or from 0.02 to 0.23% w/v expressed as hydrochloric acid.

Gluten was therefore dispersed in 0.01 and 0.001*N* solutions of hydrochloric acid in chloroethanol containing from 0 to 40% water. Figure 1 shows a plot of the degree of dispersion obtained as a function of the hydrochloric acid and water content of the choloroethanol.

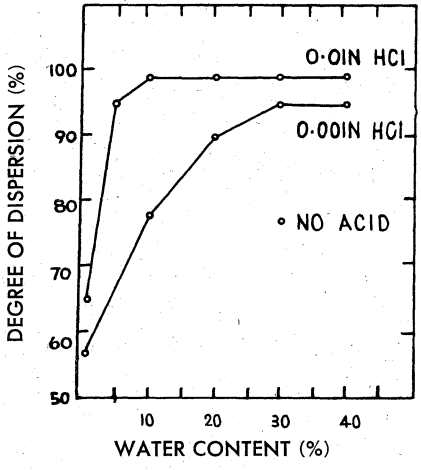

Fig. 1. Influence of water and hydrochloric acid content on degree of dispersion of gluten in chloroethanol.

In the 0.01*N* solution 95% dispersion was obtained with only 5% water and dispersion was virtually complete with 10% water; further increases in the water content had no significant effect. In the 0.001*N* solution, on the other hand, dispersion was only 95% with 40% water, although the same degree of dispersion was already reached with 30%. In carefully neutralized chloroethanol of 30% water content the degree of dispersion was only 77%.

In another experiment the influence of increasing amounts of hydrogen chloride in anhydrous chloroethanol was investigated. Table II shows that the degree of dispersion of gluten increased with the hydrogen chloride content, and complete dispersion was obtained

TABLE II

INFLUENCE OF HYDROGEN CHLORIDE CONTENT ON DEGREE OF DISPERSION OF GLUTEN IN ANHYDROUS CHLOROETHANOL

NORMALITY OF ACID	DEGREE OF DISPERSION	NORMALITY OF ACID	DEGREE OF DISPERSION
0.001	56.1	0.04	89.6
0.005	64.3	0.06	97.6
0.01	61.2	0.08	98.8
0.02	77.6	0.10	99.2

with about 0.08–0.10*N* hydrogen chloride.

Effect of Time on Degree of Dispersion. Dispersion was most rapid in the 0.01*N* aqueous acid chloroethanol solutions. It was usually complete within 24 hours and could be speeded up by more frequent shaking. Dispersion in 0.001*N* aqueous solutions took a little longer, and was slowest in 0.1*N* anhydrous solutions. The effect of time on the degree of dispersion in the latter was therefore studied in more detail. One hundred-milligram portions of gluten were shaken at regular intervals with 10 ml. of anhydrous 0.1*N* acid chloroethanol. From time to time a tube was withdrawn and centrifuged, and the degree of dispersion was determined. Table III shows that the greatest increase in the degree of dispersion occurred during the first 24 hours (overnight) and that dispersion was virtually complete in 3 days.

TABLE III

EFFECT OF TIME OF EXTRACTION ON DEGREE OF DISPERSION OF GLUTEN IN ANHYDROUS ACID (0.1*N*) CHLOROETHANOL

HOURS	DEGREE OF DISPERSION	HOURS	DEGREE OF DISPERSION
7	1.5	47	95.0
15	58.8	66	98.0
24	88.4	72	98.0
31	91.0		

Other Anhydrous Acid-Alcohol Systems. To see whether the combination of chloroethanol and hydrochloric acid was unique, another experiment was carried out using 0.1*N* solutions of various acids in chloroethanol on the one hand, and in unchlorinated alcohols, on the other. The results are shown in Table IV.

No swelling at all occurred and very little protein was dispersed in the unchlorinated alcohols. In chloroethanol, sulfuric acid was about as effective as hydrochloric acid, although the gluten particles seemed to disintegrate somewhat more rapidly. Formic acid was not as effective as the mineral acids. Trichloroacetic acid, a protein pre-

TABLE IV

DEGREE OF DISPERSION OF GLUTEN IN VARIOUS ANHYDROUS ACID-ALCOHOL SYSTEMS

Dispersant		Protein Dispersed	
Acid	Alcohol	at 25° C.	In Boiling Water Bath
		%	%
Hydrochloric, 0.1*N*	Ethanol (abs.)	0.6	...
Hydrochloric, 0.1*N*	Isopropanol	2.8	...
Sulfuric, 0.1*N*	n-Butanol	7.3	...
Hydrochloric, 0.001*N*	Chloroethanol (anhyd.)	56.1	70.6
Hydrochloric, 0.1*N*	Chloroethanol (anhyd.)	99.2	99.5[a]
Sulfuric, 0.1*N*	Chloroethanol (anhyd.)	99.4	99.6[a]
Formic, 0.1*N*	Chloroethanol (anhyd.)	81.6	87.0
Trichloracetic, 0.1*N*	Chloroethanol (anhyd.)	82.1	...

[a] Dissolved.

cipitant in aqueous solution, increased the dispersion in chloroethanol slightly more than formic acid. Precipitation occurred only after the addition of water.

Effect of Temperature. The effect of temperature was not investigated in detail. However, some observations were made in experiments in which the tubes were placed in a boiling-water bath for 15 minutes. The degrees of dispersion obtained in this way with gluten in anhydrous chloroethanol containing different acids are shown in the last column of Table IV. Heating of the dispersions caused a significant increase in the degree of dispersion in case of the 0.001*N* hydrochloric acid (control) and the 0.1*N* formic acid solutions. With the mineral acids, 0.1*N* hydrochloric and 0.1*N* sulfuric, the increase may not have been significant but the gluten dispersed spontaneously without shaking. With the other two dispersions, the undispersed gluten particles did not disintegrate on shaking. No precipitation occurred on cooling. The dispersions remained clear, although of a slightly yellowish-brownish color. When gluten was heated with 10% sodium salicylate or with 0.01*N* hydrochloric acid, it dispersed more easily on shaking but did not disperse spontaneously.

Particle Size and Agitation. Particle size had a considerable effect in the case of the less effective dispersants, the larger particles usually taking longer to disperse. Comparable results could only be obtained by using dry gluten bolted through gauze of the same mesh size.

The effect of agitation was more noticeable with the more effective dispersants, such as 0.01*N* hydrochloric acid in aqueous chloroethanol, where an increase in the frequency of agitation shortened the time required for complete dispersion. Simple mixing instead of shaking, in the case of 0.1*N* hydrogen chloride in chloroethanol, lowered the degree of dispersion by a few percent.

Precipitation. Gluten dispersed in anhydrous 0.1*N* acid chloroethanol could be precipitated with diethyl ether, acetone, isopropanol, and n-butanol. Ether caused an immediate precipitation of about 97% of gluten dispersed at room temperature and about 95% of gluten dispersed at the temperature of boiling water. Observations made on these precipitations largely paralleled those made by Jaffe and De Coene (10) on the precipitation of zein dispersed in 70% aqueous alcohol. When the supernatant was decanted from the centrifuged precipitate immediately after precipitation, the precipitate darkened and formed a smeary, sticky gel. When left standing for several days under fresh portions of ether before centrifugation, the precipitate could be obtained in the form of a grayish powder. The material precipitated from aqueous redispersion, as described in the section on methods, showed no cohesion and resembled oat protein as obtained by the method of Cunningham *et al.* (5). A vacuum-dried sample of this material could not be redispersed in a 0.01*N* solution of hydrochloric acid in aqueous chloroethanol.

Addition of water to anhydrous acid chloroethanol dispersions produced very fine colloidal suspensions which were perfectly stable, no precipitation occurring after standing for several weeks. Dilution (tenfold) with virtually acid-free chloroethanol likewise produced no precipitation.

From dispersion in a 0.01*N* solution of hydrochloric acid in chloroethanol containing 30% of water, ether precipitated about 98% of the gluten in the form of a fine suspension which settled out on several (usually 5) days' standing to a clear supernatant. The material precipitated from aqueous redispersion resembled in every respect the wet gluten obtained by dispersion of dry gluten in dilute acid and precipitation by neutralization with dilute sodium hydroxide as described by Udy (17).

Deamidation and Esterification. Column 2 of the table below shows the percent loss in amide nitrogen relative to the original gluten, and column 3 the extent of esterification (percent of free

Sample	*Loss in Amide N*	*Esterification*
	%	%
A	0.4	..
B	4.0	19
C	16.4	41

carboxyl groups esterified), for gluten recovered by precipitation with ether, redispersion in water, and reprecipitation by neutralization.

Sample A represents gluten recovered from dispersion in 0.01*N* hydrochloric acid in aqueous (30%) chloroethanol. Samples B and C were recovered from dispersion in 0.1*N* hydrogen chloride in anhydrous chloroethanol. Sample B was dispersed at room temperature and sample C at the temperature of boiling water.

No esterification and only an insignificant loss of amide nitrogen occurred in the aqueous chloroethanol. Some esterification and loss of amide nitrogen occurred in the anhydrous chloroethanol at room temperature. Temperature had a marked effect on both esterification and loss of amide nitrogen.

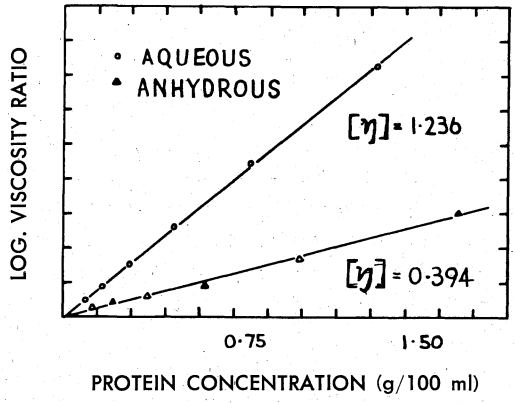

Fig. 2. Dependence of the logarithm of the viscosity ratio on gluten concentration. Disperson in aqueous (30%) acid (0.01*N* HCl) and anhydrous acid (0.1*N* HCl) chloroethanol.

Bulk Viscosity Measurements. Figure 2 shows the results of bulk viscosity measurements carried out on anhydrous (0.1*N* HCl) and aqueous (30% water, 0.01*N* HCl) acid chloroethanol dispersions of gluten. The logarithm of the viscosity ratio (η/η_0) is plotted against concentration. Since the regression lines pass through the origin, the limiting viscosity number (intrinsic viscosity) may be obtained simply from $[\eta] = 2.303b$ where b is the slope of the regression line. The limiting viscosity number was 0.394 in anhydrous acid chloroethanol and 1.236 in aqueous acid chloroethanol. Dispersions containing more than about 17–18 mg. per ml. were not obtainable.

Surface Viscosity Measurements. Figure 3 shows plots of the surface viscosity against time for films spread from anhydrous and aqueous acid chloroethanol. The curve obtained with gluten dispersed in 10% w/v sodium salicylate is included for comparison. The limiting surface viscosity (16) was 4.0 surface poises for films

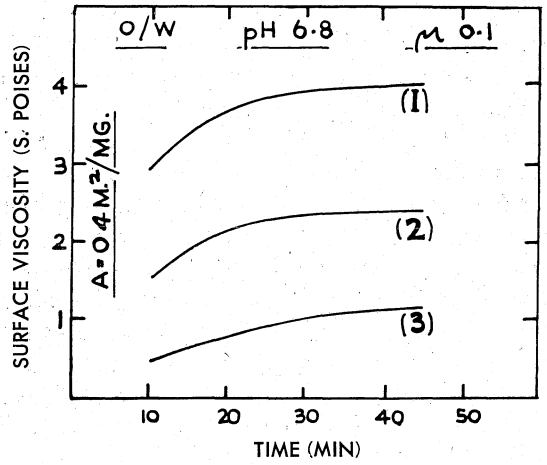

Fig. 3. Surface viscosity of gluten films spread from dispersion in anhydrous acid (0.1*N* HCl) chloroethanol (1), aqueous (30%) acid (0.01*N* HCl) chloroethanol (2), and 10% w/v sodium salicylate (3).

spread from dispersion in anhydrous acid chloroethanol, 2.4 surface poises for films from aqueous acid chloroethanol, and 1.4 surface poises for films from salicylate dipersions.

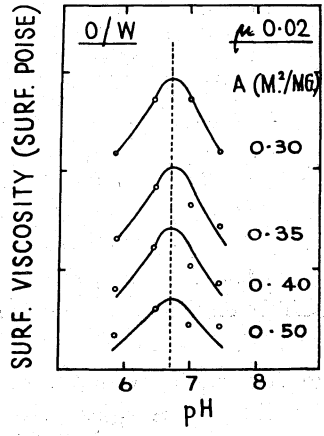

Fig. 4. Surface isoelectric point of gluten from films spread from dispersion in aqueous (30%) acid (0.01*N* HCl) chloroethanol.

Figure 4 shows a plot of the surface viscosity against the pH of the substrate at various areas per mg. protein for films spread from dispersion in aqueous acid chloroethanol. These plots show maxima

corresponding to a surface isoelectric point of about 6.7.

Direct Extraction of Flours with Anhydrous Acid Chloroethanol. Some experiments were carried out to investigate the possibility of extracting the protein free from the influence of added water directly from wheat flour and from other cereal flours from which glutens cannot be washed out.

Portions containing 100 mg. of protein of strong, medium, and weak wheat flour, and of rye, barley, oat, and carob-germ flour were therefore repeatedly extracted with 10 ml. of anhydrous 0.1*N* acid chloroethanol. Complete extraction could not be obtained in a single operation. The percentage of protein extracted increased, however, with the number of successive extractions, as shown in Fig. 5, which gives the cumulative percentages achieved in four extractions.

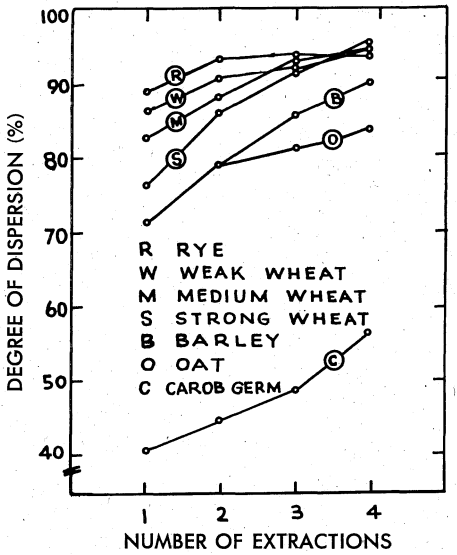

Fig. 5. Degree of dispersion of cereal flour proteins in anhydrous acid (0.1*N* HCl) chloroethanol.

Extraction of about 95% of the wheat and rye flour proteins was attained in four extractions. Of the wheat flours the weak flour was most and the strong flour least rapidly extracted. This was confirmed in a repeat experiment. Rye flour behaved like a rather weak wheat flour. Extraction of barley (90%) and oat (84%) flours was less satisfactory, and only about 56% of the carob-germ flour could be extracted under the same conditions.

Direct Extraction of Wheat and Oat Flour with Aqueous Acid

Chloroethanol. By contrast, aqueous 0.01*N* acid chloroethanol extracted about 80% of wheat protein from the medium wheat flour and about 81% of oat protein in a single extraction, and about 91% of wheat protein and about 86% of oat protein in two extractions.

Discussion

From the results presented here it appears that the chloroethanol used by Hess (9) could not have been free from acid and/or water. Only about 60% of gluten disperses in acid-free anhydrous chloroethanol. More gluten can be dispersed in the presence of hydrochloric acid, but this involves some deamidation and the esterification of part of the free carboxyl groups. These changes result in a shift of the isoelectric point, which was found to be about 7.5 by surface viscosity and surface pressure measurements (15,16). Whether the loss of cohesion in the reprecipitated gluten is due to esterification is difficult to say at this stage, since other possibilities such as the removal of lipids would have to be excluded.

When the chemical changes are either of little consequence or may be taken into account (e.g. in molecular weight determinations), anhydrous acid chloroethanol allows the examination of wheat and other cereal proteins free from the influence of added water. This dispersant may also facilitate examination by infrared spectroscopy which is difficult in the presence of water.

The esterification of gluten in 0.1*N* solutions of hydrogen chloride in various alcohols was investigated by Fraenkel-Conrat and Olcott (7), who found that esterification was complete only in methanolic solutions. Ethanol, propanol and n-butanol reacted more sluggishly, and hardly any esterification occurred in isopropanolic solutions. The esterification of gluten in chloroethanolic solution appears to be intermediate between that in propanolic and isopropanolic solution. It is interesting to note that the partly esterified gluten remains soluble in anhydrous acid chloroethanol, whereas it is apparently insoluble in the unchlorinated acid alcohols (7).

From a dispersion in aqueous acid chloroethanol, gluten may be recovered with its chemical and physical properties essentially unaltered. The isoelectric point was found to be about 6.7 by surface viscosity measurements (Fig. 4). It is worth noting that gluten dispersed virtually completely in a 0.01*N* solution of hydrochloric acid in 70% aqueous chloroethanol, whereas a similar ethanolic solution dispersed only about 72% of the gluten under identical conditions. The limiting viscosity number of gluten was 1.236 in aqueous acid chloroethanol and 0.394 in anhydrous acid chloroethanol. In 8%

sodium salicylate Udy (18) found the limiting viscosity number of gluten to be 0.309, while from the data of Geerdes and Harris (8) it appears to be about 0.200 to 0.250 in dilute acetic acid.

Gluten dispersed in either anhydrous or aqueous acid chloroethanol spreads readily at an interface. The limiting surface viscosities (16) from the aqueous dispersion are higher than those obtained from dispersion in sodium salicylate (cf. Fig. 3). Still higher limiting surface viscosities are obtained from the anhydrous dispersion. While the aqueous dispersion therefore yields higher limiting bulk viscosity numbers, the anhydrous dispersion gives higher surface viscosities. At the surface concentrations used, the surface viscosities are more of the nature of solid viscosities than solution viscosities. Further work is required to elucidate the difference in the limiting surface viscosities obtained from the two different dispersions.

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