A STUDY ON THE CORN-GLUTEN OF STRIPPER-STARCH

by

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THESIS

submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

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Montreal, Quebec.

April, 1948.

ACKNOWLEDGEMENTS

Sincere thanks and appreciation are due to Dr. O.F. Denstedt for the guidance and consideration which he, as Director of Research, has given to this work.

The stripper-starch samples were made available through the efforts and kindness of Mr. George Graham, of the Canada Starch Co. Montreal.

Thanks are also due to Mr. Hugh MacLennan for technical assistance during the past summer, and to Miss R. Mayhew for the great care and patience which she has devoted to typing this manuscript.

I wish also to thank all the friends who have helped with many of the details of preparing and correcting the manuscript.

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A Study on The Corn Gluten Found in Stripper-Starch

The work presented in this study on the corn gluten of stripper-starch centres about two problems. The first problem was to determine the form in which the gluten is present in stripper-starch, and the nature of its association with the starch. The second problem was to find a neutral solvent capable of extracting the complete native corn gluten complex from the accompanying starch without dispersing the latter.

The first problem is quite important in industry. At the time when this study was begun, there was an acute shortage of corn-starch. As a consequence the market price of starch had risen greatly, and wheat and potatoes were exploited as raw materials for starch production. At periods such as this, an economical process for removing the gluten from stripper-starch would be of considerable value both to private industry and to the national economy. Stripper-starch contains 2 to 10 per cent gluten, and this small proportion of protein imparts to it objectionable tastes, odours, or colour when used in most of the commercial applications for corn-starch.

The second problem is of great importance for fundamental studies on corn gluten. At present, dilute alkali is the only known agent that will remove the gluten more or less quantitatively from the accompanying starch, and alkali or acid is known to denature wheat gluten. A neutral solvent, capable of extracting native

gluten quantitatively from starch, would make possible many fundamental studies, which at present are limited to wheat gluten, because the latter, alone among cereal glutens, can be separated by mechanical operations nearly quantitatively from the associated starch.

Introduction to Zea Mays

Maize appears to have originated in Central America (1) and to have been distributed, largely by human agency, throughout the world. Accumulative modification and specialization of the various tissues of the plant have produced many varieties differing greatly in size and appearance of the plant and seed.

The varieties of maize fall into six distinct classes which hybridize readily. These classes differ mainly in the shape of the grain or seed, and the nature of the endosperm. Dent corn is the principal type grown in the U.S. corn-belt, because of its enormous yield. Its 300 odd varieties provide most of the fodder, ensilage and commercial corn products. Sweet corn has most of its carbohydrate as sugar, both because of an inherent defect in its starch forming mechanism, and because it is used before ripening. It is the chief type used for canning in the cooler northern and eastern parts of the continent.

Maize is an annual grass and the tallest cereal, many varieties reaching a height of 15 feet. The staminate flowers form a tassel at the top of the plant, and the pistillate flowers on the cob or ear are down on the stalk, protected by the leaves. The ovaries, arranged in rows on the cob, become the kernels when mature.

The commercially marketed corn, cornstarch, and corn products in the United States come mainly from the mid-western "corn-belt" where they represent about one-fifth of the corn crop. By considerable research in breeding, to-day's popular corn varieties give a high yield of starch, and less oil and protein than did earlier varieties.

The following brief description of the morphological divisions of the corn seed, is condensed from Hector (2).

The Corn Grain

The mature grain varies in shape, the crowding of the kernels at the base being much greater than at the apex of the ear. The base of the kernel is drawn out into an apex of "tip" which fits into the pedicel on the husk. The outer side of the kernel is rounded in flint corns, pointed in sweet corns and grooved in dent corns. Colour varies from white through yellow to purple or black, and is a blend of all colouring layers of the pericarp and the aleurone.

The Pericarp

The pericarp is a protective and structural coating around the outside of the kernel. Anatomically it consists of concentric layers, an outer epidermis, a mesocarp, a spongy parenchyma and an inner layer of tube cells. The pericarp layer may contain a water-soluble pigment giving a purplish or cherry colour, and a fairly insoluble pigment giving orange or red colours. The pericarp constitutes about 6% by weight of the grain. Inside the pericarp a thin suberized testa layer, and inside this a thin transparent nucellus may be found, but are usually absent.

The Endosperm

The aleurone layer is the outer-most layer of the endosperm, and consists of a single layer of closely packed cells, ranging from cubical to short columnar. The aleurone layer is formed when the endosperm is nearly mature, by a series of periclinal divisions of the outermost endosperm cells. It has negligible storage function since it

contains little or no starch, although it does contain some fat and protein. It may be the resting meristematic region of the endosperm (3).

The endosperm originates as a number of free nuclei arranged about the outside of the embryo sac. Cell walls form, and rapid cell division outwards from the embryo follows (4). Gordon (5) considers that cell division occurs at the outer layer of endosperm cells, which function as a cambium layer and eventually reaches a resting state, then constituting the aleurone layer.

Starch deposition in the endosperm appears to begin in the older endosperm cells near the embryo, and spread from here outward into the newer cells. On the other hand, protein seems to be deposited first in the newer cells, and to extend toward the older inner cells. The deposition of the protein seems to control the formation of "crown" and "horny" starch. All mature endosperm cells contain starch granules in a colloidal matrix of protein and carbohydrates. An uncharacterized alcohol-soluble yellow pigment is associated with this matrix in yellow maize and in certain coloured corns. If the matrix does not fill the space between granules, the resulting granules are more or less round in shape, and tend to be soft and "fluffy". These granules are fragile and easily injured in milling. This is so-called "crown starch". If the matrix completely fills the interstices of the granules, the granules become close packed, irregular in shape, tend to be hard or "flinty". This constitutes the so called "horny" starch. The given matrix becomes hard corneous and translucent. The horny type of structure is always associated with interstices that are small relative to size of granule, and usually but not always has

a higher than average ratio of protein to carbohydrate.

The deposition of the gluten matrix apparently begins and reaches its greatest development in the outer cells, and usually the entire periphery of the endosperm is horny in nature. The inward extent of the gluten matrix varies in different species. Soft corn varieties have only a thin horny layer, in flint corns it is thicker, in dent corns it is fairly thick, but does not cover the apex. Sweet corns have lost the ability to form large granules, and an outer horny layer. Hence the seeds dry more thoroughly at the end of the season, and give shriveled seeds with a hard translucent gluten matrix containing small irregularly shaped granules and much sugar which has not been converted into starch.

There has been no general agreement concerning the relative importance of dehydration, granule growth, and heavy gluten deposition in producing the irregular shapes and hard structures of horny endosperm.

The Embryo

The embryo is a small complex structure lying in a shallow groove along the side of the grain nearest the tip of the ear. There are three major parts to it, the scutellum which acts as a food reserve for the developing embryo, the plumule which matures into the stalk and leaves, and the root system.

The embryo contains a high percentage of oil, some protein and carbohydrate. The oil contains over 1.5 per cent sterol and

1.5 per cent lecithin. The protein is mostly zein but contains some globulin. The carbohydrates are non-reducing; sucrose is evenly distributed, and starch is present in most cells except the root tip.

The germ is removed by a flotation process after steeping in aqueous medium. The softened corn grains are ground in coarse mills. This grinding action tears apart the hulls, germs, and endosperms very completely. The heavy hulls are separated by settling, and then the germs are floated off in large V-bottom settlers. The endosperms and some of the looser crown starch granules suspended in the water raise its specific gravity to the point where the light, oil-containing germs all float. Only very minute amounts of germ are ever found in the endosperm starch which settles out at the bottom of the tank. Lipid, extractable from processed starch, is mainly intra-granular, i.e., not ether-extractable. The separated germ is pressed mechanically, and the very impure oil, which is expressed, is clarified in centrifuges. The germ residue is dried and sold as cattle feed.

Chemical Composition and Uses of the Parts of the Corn Grain

The chemical composition of the various parts of the corn grain determines the uses of the fractions produced from the wet-milling process.

The endosperm constitutes about 82 per cent of the dry weight of the corn grain. It contains about 84-89 per cent of starch and about 6 - 13 per cent of protein, and these are the materials with which this study will be particularly concerned. The endosperm also contains

relatively small amounts of lipid, sugar, and ash, but the last two components because of their solubility are largely removed during milling. The milling process recovers most of the starch of the endosperm in a state nearly free of protein.

The "pure" starch has a great variety of uses. In the granular form it is used in food products such as cakes, ice-cream and soups. By carefully-reproduced processes of gelatinization and dextrinization, the granular starch may be converted into various adhesives, sizings for cloth or paper, inks, etc. For all these uses it is important that the structure of the starch granule should not have been weakened by swelling or hydrolytic changes. A large part of the starch produced nowadays is hydrolyzed to corn syrup, from which dextrose and corn molasses are obtained.

The protein part of the endosperm is called corn gluten and is sold chiefly in cattle feeds. A small part of it is used for the commercial extraction of zein. Stripper-starch, a starch fraction containing 5-10 per cent protein is unsuitable for most of the uses of pure starch because it imparts an objectionable odour, taste, or colour. It is sold chiefly in cattle feeds or as core-binder for foundries.

The germ fraction constitutes about 12 per cent of the corn grain and is remarkable for its high content of all components except starch. The germ is made up of about 10 per cent ash, 19 per cent protein, 35 per cent oil, 10 per cent sugar, and only 5-10 per cent starch. The pericarp, or bran is the smallest fraction, about 5 per cent of the dry weight of the corn, and is composed mainly of cellulose and hemicellulose. It contains about 4 per cent of protein and 3-10 per cent starch, but very little ash, lipid or sugar.

Physiology of Gluten

The greater part of the protein that occurs in plants is stored as a reserve in seeds. It apparently is formed from the amino acids, amides, nitrogenous bases, nitrates and carbohydrates in the cells. The disappearance of most of the free asparagine, arginine, histidine, tryptophane, and mono amino acids during maturation of the seeds has been amply demonstrated (7-11). The other nitrogen bases, nitrates and amides also decrease during protein formation in seeds (12-14). Since the presence of carbohydrate is essential for protein formation in all parts of the plant, (15,16) it is probable that amination of α-keto acids formed from carbohydrate metabolites occurs in reactions similar to those postulated in animal tissues (17). It is interesting to note, however, that asparagine instead of glutamine apparently plays a major role in the amidations and transaminations. Thus wheat grains just before ripening contain large amounts of asparagine but no glutamine (18-20). In addition, Wassilieff (7) showed that during a period of increasing amide nitrogen content of the seeds, amino nitrogen decreased.

The actual storage of gluten proteins in the endosperm does not occur until desiccation of the mature seed begins. This is to be contrasted with starch storage, which begins very soon after cell walls form in the endosperm and continues until desiccation begins, or as long as the leaves are green and form an excess of sugar. The full-grown, still-green wheat kernel contains about 90 per cent moisture (18) and barley, at flowering time, about 80 per cent

moisture in the seed (21). Drying is very regular, 1 or 2 per cent per day (21,22). During this desiccation process, the gluten protein is laid down. Drying of immature wheat kernels also will change their plentiful amino acid content into protein (18). The enzymes concerned with protein formation are apparently damaged by freezing, so that the ripe seeds contain substantial portions of their nitrogen still as non-protein nitrogen (23,11). Loew (24) was unable to obtain any intermediates when asparagine and other amino acids are being converted into protein, although polypeptides have been demonstrated in ungerminated maize, wheat and rye kernels (25-27).

The changes in the protein fractions of the gluten during growth have also been studied. Teller (13) showed in developing wheat kernels, all protein fractions at first increased, with rapid decrease in non-protein nitrogen. The albumin plus globulin fraction soon became relatively constant. Initially the glutelin fraction was about 60 per cent of the total endosperm protein, but after a short increase, and at an early stage, it began to decline abruptly in terms of per cent total protein to reach a fairly constant value in the more mature stages of about 30 per cent total protein. On the other hand gliadin showed a steady rise, calculated as per cent total protein, to a final equilibrium value of about 52 per cent. Teller suggests that the rise in proportion of gliadin is due to the increase in relative amount of endosperm gluten, which is high in gliadin compared to bran. He does not discuss the possibility of gliadin formation by amidation of globulins or glutelins.

The possible enzymic role of gluten has been suggested. The very presence of storage starch and gluten makes it probable that there would be some amylolytic and some proteolytic enzymes associated. That these enzymes should be destroyed during ripening, and then relatively suddenly regenerated upon germination, in order to make the stored material available to the growing seed, seems unlikely. A more plausible theory would be that the starch and gluten deposition are halted by desiccation, or by some change in concentration of a diffusible enzyme activator. Germination then occurs in moist conditions, and either this or the formation of activator renews the enzyme activity, but in the catabolic direction.

If the enzymes postulated were soluble, they should be demonstrable in wheat flour, corn meal, etc., but largely absent from such preparations as corn starch, wheat starch, or wheat gluten where extensive washing has occurred. Furthermore, the commercial drying procedures involving temperatures of 190^{o}F would almost certainly destroy enzyme activity.

There is a controversy concerning the quantitative importance of proteolytic enzymes in washed wheat gluten. Landis and Frey (28) have demonstrated a very weak proteinase activity of wheat gluten on gelatin. Others consider that the action of oxidizing and reducing agents on gluten stickiness, extensibility, etc., indicates an inhibition or activation of a papain-like proteinase with gluten as its substrate (29-33). However several objections to this theory exist. Oxidants such as potassium bromate have little inhibiting action on papain at concentrations that are used commercially (34).

Furthermore, Sandstedt and Fortmann (35) showed that flour which had been 'softened' by the action of reducing agents could be hardened again by adding oxidizing agents. Other observations, for example that glutathione softens dough more rapidly than papain (36), that gluten dispersions in dilute acetic acid, after heating to destroy enzyme activity, could still be softened by added glutathione (37) are not so clear-cut or decisive. The total of all work however, heavily supports the conclusion of Sullivan et al (38) that the action of oxidizing and reducing agents on gluten consistency is primarily on the sulfur linkages of the gluten.

Formation and Growth of the Starch Granule

The details of the formation and structure of the type of starch granule found as the storage form in the seeds of cereal plants are only partly known. Starch granules are formed only in plastids (39). In the chloroplasts, numerous granules are formed, but they remain small and are usually without a definite structure. Zirkle believes them to be situated in the central vacuole of the chloroplast, although they sometimes stretch the chloroplast into an irregular outline. It is generally conceded that the chloroplast granules represent a temporary storage form, and this starch is withdrawn for use or storage elsewhere as soon as the plant's physiology permits.

The leucoplasts are the site of formation of the commercially important starch granules, which form the main and more-or-less permanent food storage depots of plants. Only one starch

granule is thought to form in each leucoplast (39) although true compound granules are occasionally found (41). The commonly observed compound granules are really two or more granules fitting tightly together, and separable with a little manipulation.

The position of the "hilum" or growth center of the granule is determined by the position of initial starch formation in the granule. If the first starch is deposited in the center of the leucoplast, growth occurs evenly in all directions. The finished granule will have striations arranged symmetrically about the hilum. But if granule formation does not begin in the center of the leucoplast, then growth is greater in the direction of the greater amount of leucoplast material. Then the finished granule has an off-center hilum and eccentric striations. Whether the leucoplast bursts as the starch grain grows, or whether it stretches to form an unbroken film around the granule, is at present unknown. If the leucoplast is postulated to break, then the role of the leucoplast must simply be the control of initial starch deposition and initial granule organization. It is interesting to note in this connection that Lindet and Nottin (42) observed a sugar content of 0.5 to 1.5 per cent in the cell sap of the young potato, but were unable to detect any soluble starch in homogenized potato slices.

There are two theories concerning the method of granule growth. Continual deposition of starch at the hilum, pushing the granule outwards as it forms is called growth by intussusception. This theory was supported mainly by the difficulty of explaining the softer nature of the granule center. According to the other theory

General Structure of the Starch Granule

The size of starch granules is usually given as the length in microns of the longest axis. Maize granules vary greatly in size; both horny and floury granule types have an average size of 15 microns. With modern manufacturing methods, commercial corn starch has a minimum granule size of about 5 microns and a maximum of about 25 microns. On the other hand, wheat granules are usually either small (2-10 microns) or large (20-35 microns), with few intermediate sizes.

The hilum in some types of starch granule is easily observable under the microscope, appearing as a more or less circular dark area or cavity. In the growing granule the centre is always the softest, has the highest moisture content, and upon drying cracks may develop radially from the hilum. Arranged concentrically about the hilum are markings, called striations, usually seen only with oblique lighting and careful focussing of the microscope. Very faint iodine or dilute chromic acid stains make the striations more visible. Sande-Bakhuyzen (48) grew wheat under constant humidity and illumination and found no striations in the granules. When these granules were heated to swelling, refractive radial needles could be detected, projecting from the centre radially for 17-20 microns to a base 2-3 microns wide. Additional support for this radial or "trichite" structure of starch granules lies in the radial markings seen in rye granules, or acid-modified potato starch under controlled swelling pressures, with Sjostrom's technique (43). In addition, when a granule is crushed under a cover glass it breaks along radial lines.

The granule also has a concentric organization. The striations apparently arise from variations in plastid activity during growth of the granule and are visual evidence of differences in refractive index between layers. Observation of swelling of normal granules, and the fracture lines which are usually formed, emphasize concentric structure much more than radial. This weakening of the granule primarily along concentric lines occurs with both dry and moist heating, as contrasted with acid steeping which weakens the granule along radial lines (43,49).

Evidence for subgranule units has been offered. Hanson and Katz (50) stained granules after "lintnerizing" for 10 days followed by swelling in 2M calcium nitrate solution, and claimed to show remarkably cubical units, about 1 micron across and arranged in concentric layers. Sjostrom's (43) photographs of the swelling of acid-modified potato starch also suggest subgranule units in the swollen debris.

Moisture is normally an element of granule structure. Granules observed under polarised light are brilliantly illuminated except for a dark unlighted cross intersecting at the hilum. If the hilum is very eccentric the cross may appear more like a "V". When the granules are dried very thoroughly they lose this refraction, but moistening restores it. Similarly, the normal X-ray powder-type of spectrum given by dry granules is reported to be sharpened by addition of small amounts of water (51). Both these optical effects indicate greater organization of the granule when an optimum amount of water is present, and both effects are destroyed by gelatinization.

The water content of a starch granule when in equilibrium with atmospheres of varying water vapour pressure follows a modified adsorption type curve. The function has been carefully investigated by Sair and Fetzer (52,53) for representative starches including corn starch and by Rakowski (54) and Katz (55). The hygroscopicity curves all show a hysterisis-like divergence of water content values depending on whether vapour pressure is increasing or decreasing. One important practical result of this fact is that the water content of a starch sample in equilibrium with laboratory air will change slightly from day to day with changing humidity. In this study, with laboratory air varying in water vapour pressure from about 10 to 18 mm.Hg. in winter and 13 to 24 mm.Hg. in summer, exposed starch granules might be expected to vary in moisture content from 16 to 24 per cent (dry basis). Sair and Fetzer found that desiccation, especially at high temperatures, permanently reduced the sorptive capacity of starch. They attribute this effect to elimination of water from the molecular structure followed by mutual bonding of hydroxyls. Both the birefringence and X-ray spectrum indicate a radial arrangement of chains in the granule.

An important fact about the birefringence effect was observed long ago by Meyer. Granules, soaked in dilute sulfuric acid, lose 50 per cent of their weight without losing any of their birefringence. The deduction is that a relatively resistant, crystalline, radial framework is interlaced with approximately the same amount of amorphous non-crystalline, less resistant material.

The possibility of the existence of an external membrane on the starch granule was debated for many years. At present it is generally considered that no membrane, differing significantly in structure from the rest of the starch granule, exists. Evidence for such an exterior membrane is discussed by Badenhuizen (45) and Alsberg (44). The protoplast which generates the granule would be expected to form an outer membrane. It has been suggested that there is a delicate membrane, ordinarily lost in preparation (50,56, 57), and Alsberg (44) cites evidence that very mild conditions of preparation (58) give a starch granule which differs in its swelling and gelatinizing properties from commercial starches. A recent paper by Haller (59) claims that an iodine-water hydrosol does not penetrate the starch granule but is adsorbed on its surface. On the other hand, iodine in potassium iodide solution penetrates the granule. He suggests that this is analogous to the behaviour of cellulose with substantive and basic dyes respectively, and that it indicates the presence of a cellulose compound in the wall of the starch granule. In addition, the outer membrane which is left intact after digestion by diastatic enzymes, gives typical cellulose staining reactions with iodine sol or with iodine-zinc chloride preparation. Frequently cited as support for the theory of a peripheral membrane are the well known observations concerning granules that have been chipped or cut. The cut faces swell in cold water, stain with dyes, etc., whereas the undamaged portions behave in a normally resistant manner. None of these interesting observations has apparently been studied systematically.

A very considerable amount of literature has accumulated relative to the theory that the high amylopectin content of the outer layers of the granule is responsible for its membrane-like properties. Partly gelatinized starch suspensions show swollen membranes which exhibit elasticity when micromanipulated. Microscopically, the least soluble portion of the granules appears to be these membranes. Chemically, the least soluble portion of the starch is the amylopectin fraction, which is high in phosphate. Samec assumed therefore that the membrane was amylopectin and later demonstrated that amylophosphoric acid gave solutions more viscous than amylose, even approaching the viscosity of amylopectin solutions (60,61). Hence there arose the idea of a resistant outer layer in the granule, owing much of its properties to its high phosphate content.

Evidence was advanced against this hypothesis. Koets (62) claimed the nitrogen-free amylose could be converted to amylophosphuric acid that gave solutions only slightly raised in viscosity. Alsberg (63) has shown that there is no correlation of phosphorous content and granule resistance, and concludes that amylopectin is probably widely distributed through the molecule. The very observations about chipped granules can be used to argue against a distinct membrane, since swelling is limited to the cut surface, and dyes do not penetrate to the relatively non-resistant centre of the granule (44). What appears to be conclusive evidence against the role of amylopectin in the membrane rests in work done by Schoch (64). He demonstrated that the phosphate could be almost totally removed from wheat and

corn granules by Soxhlet extraction with 80 per cent aqueous dioxane or aqueous methanol, without apparently producing significant changes in the granule structure or resistance (65). This is decisive evidence in favour of Badenhuizen's conclusion (45) that the membrane properties merely arise from the more stable nature of the outer layers of the granule, and not from any chemical difference.

Since there has been no explanation of the mechanism of this resistance, and since observations such as those of Haller (59) and Hanson and Katz (57) have not been extended or discredited the existence of an external membrane must be considered unsettled.

Swelling and Gelatinization of Starch Granules

Starch granules suspended in cold water swell to a slight extent, and the degree of swelling increases gradually as the temperature is raised to about 50°C. The swelling is reversible. There is no apparent change in the properties of the granule and no significant increase in viscosity. If the liquid phase is starch-free initially, no starch can be detected in solution after the heating. There is no evidence of chemical degradation in the form of rupture of chemical bonds, or other irreversible changes.

The second stage of swelling takes place within a small temperature range below the gelatinization temperature. Hanson and Katz (56) have estimated this range as 50°C to 65°C for wheat starch. In this range the granule suddenly swells to many times its original size, loses its birefringence, and the viscosity of the suspension increases greatly. The loss of birefringence is

gradual and is difficult to correlate with the other changes. Viscosity may not begin to increase until 10°C above the temperature at which birefringence disappeared (66). Some starch can be detected dissolved in the supernatant after centrifuging. Moreover, the changes are irreversible; when cooled the granules do not regain their initial compact shape or birefringence.

During the third phase of swelling, at and above the gelatinization temperature the granule sacs become increasingly swollen, shapeless, and indistinct, and more and more of the starch goes into solution. The granule sacs apparently persist to some extent even after they become invisible, their interlocking forms creating a high viscosity that can be reduced considerably by vigorous boiling, autoclaving, stirring, or homogenizing (44,67).

A strictly analogous swelling and gelatinization process may be induced in starch by suspending it in solutions of such reagents as sodium hydroxide, sodium salicylate, thiocyanates, iodine, urea and others. Samec (68) has given a concise account of the effect of crystalloids in low concentration on the gelatinization of starch suspensions. He points out that organic crystalloids have essentially the same action. The experiments of Schoch (69) show that granule lipids also shift the gelatinization temperature. A general statement of the effect is that the anion determines primarily the direction of the shift of gelatinization temperature, while the nature of the cation affects primarily the relative extent of shift. Both anions and cations can be arranged in a Hofmeister series with respect to type and extent of influence on

swelling and gelatinization temperature. Salts which upon hydrolysis produce an increase in hydroxyl ions usually increase swelling and thereby lower the gelatinization temperature. It is interesting to notice that phosphate ion, an important constituent of many native starch granules, tends to raise the gelatinization temperature.

Samec (70) observed that increasing concentrations of urea caused increasing swelling of the starch granule with increasing depression of the gelatinization temperature. The similar action of potassium thiocyanate and salicylate was studied in detail by Ostwald and Frankel (71). Studies by Lejeune (72) showed that, at room temperature in 10 per cent sodium salicylate, barley starch approximately tripled its volume, wheat starch swelled slightly, cornstarch and potato starch did not swell more than in pure water. But even the latter more resistant starches gelatinized after about 1 hour in 17 per cent sodium salicylate, and almost instantaneously in 20 per cent salicylate. Experiments at pH 4.7 and at pH 7.0 indicate that the peptization is not sensitive to pH variations in this range. The swelling could be checked almost completely by organic solvents, e.g. 10 per cent ethanol or 5 per cent acetone, and appeared to be decreased by the small amounts of calcium associated with the granules.

Factors important in swelling and eventual gelatinization have been indicated by physical studies. Films of oriented starch molecules, when treated with swelling agents, only increase about 50 per cent in length parallel to the molecular axis, but increase

several times in thickness normal to the molecular axis (41). Since data from the studies on X-ray spectra and birefringence indicate that a great proportion of starch molecules normally are oriented radially in the granule, we should expect tangential swelling to occur. This is confirmed by the appearance of a space in the center of the granule (73) during swelling, indicating that forces are pulling from the periphery rather than acting outward from the center of the granule. Another important factor may be the decreased association of water molecules caused by heat or by lyotropic ions (74). Caesar (75) suggests that swelling occurs due to increased penetrating power of the smaller-sized water aggregates so formed.

Hydrolytic changes may occur in the starch molecules more-or-less independently of gelatinization changes in the granule structure. In our study, where the primary interest has been to prevent changes in the starch granule, only initial hydrolytic changes, occurring under mild treatments such as steeping are of interest. The action of cold dilute acid in weakening granule structure radially, and in solubilizing the less crystalline components of the granule have already been mentioned. Haworth (76) claims that the initial rate of acid hydrolysis of starch is faster than the later stages, and that the "disaggregation", or initial hydrolytic stage has a slightly lower activation energy than that for the normal 1-4-α glucosidic bond. There have been no definite conclusions as to the nature of the bonds which are hydrolyzed initially. Acid steeping treatments are

commonly used in present day milling of starch. Kerr (41) points out that the steep waters, containing 0.15 to 0.20 per cent SO_2 at a pH of 3.5 to 4.2 and about 125°F, almost certainly cause some hydrolysis, with loss of potential hot paste viscosity. Schoch (67) claims that certain laboratory preparations of starch have much higher potential viscosity than commercially steeped starches.

Extensive studies have been carried out on the mineral constituents of starches and flours of corn and wheat, chiefly to determine their nutritional value. It has been known for over two decades, that the mineral elements are not an integral part of the structure of the starch molecule. Thus Samec (77-79) showed that prolonged washing with dilute hydrochloric acid reduced gradually the amount of metal ions and acidic ions in the starch granule to negligible levels. The first washings gave an equal removal of acid and base. The potassium, magnesium, sodium and sulphate were readily extracted, whereas the calcium and silica were extracted relatively slowly. The phosphate of wheat starch was only about 40 per cent extractable; even with ten extractions there was considerable residual nitrogen. Extraction with dilute KOH gave similar results except that nitrogen and phosphorus were more completely removed.

Cereal starches had been shown (80,81) to be especially rich in a component that was resistant to the action of diastase from ungerminated barley. Ling and Nanji (82) concluded that this fraction, which contained consistenly from 0.83 to 0.98 per cent of silica was a silicic acid ester of amylose. Defatted corn starch yielded only about 0.15 per cent silica in this resistant fraction (83) and the

silica could be largely eliminated by dispersion in alkali followed by reprecipitation in acid. Even earlier Samec showed that a dilute hydrofluoric acid solution removed the silica without altering the properties of the fraction. Samec (79) discusses the mineral elements and considers that they are all impurities with the possible exception of phosphorous and nitrogen. The structural role of nitrogen and phosphate is discussed elsewhere in detail.

Lipid Constituents of the Starch Granule

The importance of lipids in starch structure was a controversial topic for many years. Early work suggested that the lipids might be incorporated in the granule structure, but recent work seems to have shown conclusively that they are merely adsorbed or held in physical mixture. The normal milling procedures remove the germ very completely, and the endosperm is left with a very small amount of fatty acids. Granular corn starch, thoroughly extracted with ether, still contains about 0.6 per cent lipid (84-86). Fatty acids were found to constitute about 94 per cent of the lipid; of these oleic acid represented 37.7 per cent and linoleic acid 31.1 per cent with 30 per cent saturated fatty acids. Extraction which removed 95 per cent of the fatty acid removed only 25 per cent of the phosphorous, suggesting that the major portion of these fatty acids are not present in phosphatides. Fatty acids from wheat starch (87) were similar in composition to those of corn starch and averaged 41 per cent oleic, 24 per cent linoleic and 35 per cent palmitic. Schoch (88) who used 80 per cent aqueous dioxane to extract granule lipids, found that in the case

of wheat starch, most of the phosphorous was also extracted, indicating that a phospholipid was being extracted. Taylor (89) attempted to show that the fatty acids of corn starch were esterified with the amylopectin fraction, and contributed to its properties of anodic migration, solubility in alkaline solutions and insolubility in acid solutions. Removal of virtually all lipid material from the intact granules by Soxhlet extraction with neutral 80 per cent dioxane or 85 per cent methanol by Schoch (64,88) proves conclusively that the fatty acids are not bound chemically in the polysaccharide. Schoch's explanation, for the successful extraction by such lyophilic fat solvents, is that solvents such as ether are too non-polar to penetrate the granule. Studies on the lipids led Lehrman (90) to conclude that the fatty acids were adsorbed. Strong support for this conclusion was the demonstration by Schoch and Jensen (65) that fatty acids in methanol or "Methyl Cellosolve" were absorbed by defatted starch, and could only be extracted by hydrophilic fat solvents.

Linkage of Starch and Gluten

Corn and wheat starch contain a nitrogen fraction that is very difficult to remove. In this respect they differ from some other starches, such as potato starch. Thus corn and wheat starch, purified by commercial methods, commonly contain 0.3 to 0.8 per cent protein, and even repeated washing in dilute alkali will not completely remove this protein. There have been two main theories of the relation of this nitrogenous fraction to starch; the older

theory assumes some type of chemical linkage through the phosphoric acid component, the more recent theory involves linkage by co-acervation.

The earlier theory was based mainly on conclusions derived from inadequate methods for separating starch constituents (91-94). By mild alkaline gelatinization of the granules of wheat starch followed by fractional precipitation with alcohol, Ling and Nanji (93) concluded that wheat amylopectin was a hexa-amylose containing two β-linkages and with two phosphoric acid groups, whereas wheat amylose was a hexa-amylose containing α-linkages and a negligible content of phosphate. With the application of electrophoretic techniques to starch fractionation, the phosphorous was shown to be overwhelmingly in the branched amylopectin fraction (95-97). The electrical conductivity of an amylopectin suspension was less than would be expected from its phosphate content, and Samec concluded that part of the phosphate formed an ester linkage with the nitrogenous constituent.

In more recent years observations accumulated which did not agree with the above-mentioned theory. Posternak (98) isolated glucose-6-phosphate from potato starch, but was unable to isolate any from wheat starch. He also showed that alcoholic extraction removed most of the phosphate and nitrogen from alkali-gelatinized wheat starch, whereas similar treatment did not significantly reduce the potato phosphate. Kerr and Severson (94) have recently shown for potato starch that extraction with aqueous alcohol leaves a residue rich in phosphate, but that such treatment gives no significant

fractionation of the phosphate in wheat starch. Also quite recently Schoch (88) has shown that Soxhlet extraction of wheat starch for 48 hours with 80 per cent aqueous dioxane removes the phosphorous and nitrogen almost quantitatively, although the same treatment of potato starch removes only about 10 per cent of its phosphate.

Such differences in behaviour of potato starch and the cereal starches had already caused Samec (99) to admit the inadequacy of the theory of salt linkage of protein and amylophosphoric acid in the case of cereal starches. It would appear to be still quite possible in the case of potato starch, and some other root starches where the phosphate is in chemical union with the starch. Schoch's extraction of phosphate and nitrogen supports the conclusion of Posternak (98) that the phosphorous is present as phospholipid impurity in simple physical mixture inside the granule. However, Kerr (41) quotes Samec as having removed the nitrogen from wheat starch by digestion with pepsin. This and the variability of N:P ratio in all papers indicates that the nitrogen is not only phospholipid, but is probably largely protein.

At present the coacervation theory of starch-gluten linkage is generally accepted. Von Przylecki and co-workers investigated the coacervation of many nitrogenous compounds with starch and starch components. Coacervation of egg albumen with amylose occurred, almost independently of pH, in the pH range 1.7 to 7.5 (100), whereas with amylopectin coacervation was greater and showed a minimum at pH 5 (101). Simple ampholytes such as asparagine and creatinine

showed a minimum at pH 5 also, but coacervates of amylopectin with creatin or peptone were completely dissociated above pH 7. The protein-binding capacity of starch is a maximum when it is just gelatinized, and further heating or alkali apparently removes some of the ionizable phosphate, silicate, and fatty acids leaving a decreased binding capacity (104). Koets (62) prepared amylophosphoric acid from amylose and demonstrated coacervate formation with gelatin, wheat leucosin, and purified potato albumen. Both systems showed coacervate droplets with minimum hydration in the pH range 2.5 to 4.0.

Koets (62) reviews considerable evidence in favour of coacervation between starch and the residual gluten that is difficult to remove. Thus Koets points out that Samec (102) had shown that the nitrogen content of potato starch is small compared to its phosphoric acid content. Hence coacervates would be expected to exhibit a negative charge, and in fact, potato amylopectin containing the phosphorous and nitrogen, does migrate toward the anode. On the other hand, some wheat amylopectin fractions contain nitrogen in amounts equal to, or slightly in excess of, their phosphorous content (78,96). Hence it is an important observation that, during electrodialysis of gelatinized wheat starch, some fractions have been observed to deposit on the cathode, indicating a positive charge (103).

In all studies of coacervation, the addition of electrolytes, dilute acids or alkalis either decreased or abolished the linkage, and this effect is considered diagnostic of a coacervate system. Thus removal of the nitrogenous constituent of wheat amylopectin with dilute

acid or alkali would be expected to break up the coacervate and leave an amylopectin with properties similar to potato amylopectin. Samec (104) had demonstrated this. The reversal of this process, the addition of protein to potato amylopectin to give a coacervate with properties more nearly similar to wheat amylopectin, has also been effected (99). From the data presented by Koets (62) it is apparent that the coacervates with amylophosphoric acid are stable in the pH range 2.0 to 4.0, but rapidly dissociate as the pH is raised to 5.0. It would appear doubtful whether any coacervation of starch with protein could exist in media more alkaline than this.

In the removal of nitrogen from cereal starches, the final traces of gluten are not appreciably diminished by washing with solutions of electrolytes, and are only partially removed by successive washings with dilute alkali. This would seem to be decisive evidence against a coacervation linkage of the last traces of nitrogen. However Koets (62) has made the important observation that, whereas fresh coacervates exhibited typical qualities of dispersion upon adding alkali, acid, or neutral electolytes, after some time the viscous or liquid coacervates dehydrated further to form rather resistant floccula. Strong peptizing agents such as potassium thiocyanate and alkali were required to redisperse these floccula. The comparative inertness of the residual gluten toward solutions of electrolytes, dilute acids, and dilute alkalis does not indicate coacervation with starch and has not been sufficiently considered. Moreover, all artificial coacervate systems of starch with gluten have involved gelatinized starch rather than granules, and have used solutions of

proteins which, in their molecular structure and solubilities, do not approximate gluten. There has been no demonstration that intact granules will bind an appreciable amount of gluten, or that the gluten would not be easily removed. All electrical properties and solubility characteristics of the system simply indicate an association of starch with a gluten fraction that is very difficult to hydrate, and cannot be considered as adequate proof that the association is due to any particular force or forces.

The Solubility Properties of Gluten

Definition of "Gluten"

There is no exact definition of gluten, and by this term different workers imply different amounts of the total cereal proteins. The word originally designated the coherent, slightly yellowish mass of protein that could be 'washed-out' of wheat flour. The washing-out process has been investigated and modified many times, usually with the object of recovering more completely the total flour proteins (105-107). Some workers speak of the total flour protein as gluten. Others argue that the most soluble portion of the flour protein, approximating the fraction soluble in dilute salt solutions, is not part of gluten (108,109). Up to the present time no decisive studies have been reported by which to define what fraction of the seed proteins are gluten on a basis of physiological activity, physico-chemical properties, or chemical composition.

The terminology commonly used in the cereal trades and in their technical publications will be used in this discussion. The complete protein system of the endosperm will be called 'corn-meal proteins', or 'wheat-flour proteins', as the case may be. 'Corn gluten' will mean the proteins of the main waste fraction obtained from the overflow in tabling or from the centrifuges. The term 'wheat gluten' will designate the coherent mass of protein that can be washed out of wheat flour. The empirical and inexact nature of these terms is apparent, but unavoidable in the present state of our knowledge.

Separation of the Corn Endosperm

The quantitative separation of the corn kernel into bran, germ, and endosperm is simple but tedious. Commercial milling methods adapted to a laboratory scale do not give a sufficiently complete separation. The bran coat may be loosened sufficiently by steeping in water to allow hand separation of the components of the kernel. A steeping treatment of 5 to 30 minutes in cold water is the minimum (110), but an easier separation may be effected by steeping 2 to 3 hours at 50°C (111). The germ is then cut away from the endosperm with a scalpel. The tissues are dried if necessary, and ground. The total protein content is estimated usually from a Kjeldahl analysis.

The relative amounts of these seed tissues and the protein distribution in the tissues have been tabulated by Osborne and Mendel (112) as follows.

	Per cent of Whole Seed	Protein Content in per cent	Protein Content as % of Total Seed Protein
Endosperm	83.5	11.1	73.3
Bran	15.0	18.7	23.3
Embryo	1.5	36.7	4.4

Some controversy has occurred concerning the conversion factor to be used in deducing protein content from the nitrogen content. The common conversion factor of 6.25 was changed, on the basis of Osborne's early work, to 5.69. This was a weighted average

for the different proteins in wheat flour. Jones (113) has emphasized that the bran and embryo proteins differ in composition. He lists conversion factors of 6.31 for bran, 5.70 for endosperm, 5.80 for embryo, and 5.83 as a weighted over-all factor. In view of the variability in nitrogen content of the fractions reported by different workers, ths absolute accuracy of such figures is probably not more than $\pm$ 2 per cent. Workers commonly specify which factor they employ. The present official assay of total protein, as listed in the "Methods of Analysis" of the A.O.A.C. (6th edition), involves nitrogen determination by either Kjeldahl, Kjeldahl-Gunning, or Kjeldahl-Gunning-Arnold methods. In any case, the conversion factor of N x5.7 is used for both wheat and corn, and for both whole kernel and endosperm proteins. This method represents a simplification for industrial usefulness, and may be satisfactory in certain comparative studies, but obviously is undesirable in accurate studies.

One of the early methods of fractionating corn protein was that of Osborne (114), who extracted the globulins with dilute salt solution, the prolamines with strong aqueous alcohol, and the residue, - called the glutelins, - with dilute acidic or alkaline solutions. Later workers frequently ignored the fact that the early studies had shown that almost 10 per cent of the total protein in corn is present as albumens and globulins. Thus Osborne and Clapp (115) fractionated the ground-up, whole seeds by exhaustive extraction with cold alcohol (85 per cent by volume), followed by extraction with 0.20 per cent sodium hydroxide. Similarly Hoffman and Gortner (116) prepared zein by direct and repeated extraction of gluten waste with 70 per

cent alcohol. Other early studies do not describe completely how the gluten was fractionated. The unsatisfactory nature of such variable and inexact fractionation procedures became evident from the numerous investigations of the solubility properties of the gluten fractions of wheat. Very few investigations of corn gluten fractions other than zein have been reported; it is assumed usually that the other fractions of corn proteins have properties analogous to the properties of the corresponding fractions of wheat.

Solubility of Gluten in Water

Because corn, corn meal, wheat flour, and even the gluten mass "washed out" of wheat flour, are known to contain minerals, the simple suspension of any of these substances in water must be considered as a treatment with a dilute salt solution. Indeed the amounts of mineral present in meal are sufficently large to produce variability in extractions with added amounts of known dilute salt concentrations. Hence only those gluten proteins which remain in solution after extraction with water or aqueous solutions of neutral compounds, followed by dialysis against distilled water, may properly be called water-soluble. However, so few studies meet this requirement, that protein soluble after prolonged dialysis against tap water will also be considered as albumen.

Osborne (114) did not directly determine the 'albumen' fraction of corn proteins, since he was convinced that there was no sharp distinction in composition between the protein fraction still in solution after dialysis and the precipitate. He made a thorough

study of the more plentiful albumen fraction of wheat, which he named "leucosin" (120). The wheat kernels, after grinding, were extracted with dilute saline, and the extract was dialyzed to precipitate the globulin. The albumen was recovered by heating to coagulation, and he concluded that it constituted 3 to 6 per cent of the total protein.

Herzner (121) electrodialyzed an aqueous extract of wheat until the soluble protein contained no ash. The resulting preparation contained 16.11 per cent nitrogen, and had an isoelectric point, as determined by electrophoresis, of pH 5.10. The maximum coagulation by heat, in the presence of buffer salts, occurred at pH 4.97. He does not state what per cent of the flour proteins were in this albumen fraction. After the work of Staker and Gortner (122) in which albumen fractions from several varieties of wheat were determined, interest in differentiating the water-soluble protein from the salt-soluble protein practically disappeared.

Recent studies have discovered water-soluble proteinases in gluten (30,32) and non-proteolytic dissociating enzymes (123,124). These cause extractions to be unreproducible (108), and may explain some experiments in which up to 60 per cent of gluten was extracted by water (125,126). It is probable that any extraction and purification in neutral aqueous media of albumens (or globulins) must be relatively rapid in order to prevent the production of soluble fractions by breakdown of the more insoluble components of gluten and in order to secure reproducible results.

Solubility of Gluten in Electrolyte Solutions

The variability of the peptizing effect of salts was slowly established. Blish (127) showed that extraction with 1 per cent sodium chloride gave a fraction with amide nitrogen unusually high for albumens and globulins. In support of this observation, Olson (128) decided that 1 per cent sodium chloride extracted a fraction containing about 30 per cent gliadin, but that 10 per cent sodium chloride gave much less extraction of gliadin. Later, Jones and Gersdorf (129) showed that even the small amount of mineral salt in the bran was sufficient to cause peptization of significant amounts of globulin, when the bran was extracted with distilled water. The protein fractions extracted by Hoffman and Gortner (130) with 5 per cent potassium sulphate and 10 per cent sodium chloride each gave different ratios of albumento globulin.

The most thorough investigation of the peptizing action of salts on gluten was performed by Gortner, Hoffman and Sinclair (131,132) and certain parts confirmed and extended by others (122,126,133-135). These researches showed that, compared with extraction by distilled water, many salts tended to decrease the peptization of gluten protein, others tended to increase peptization in varying degrees, and with others the peptization exhibited a maximum at one of the salt concentrations investigated. As could have been predicted from the work of Jones and Gersdorf (129), peptization by sodium chloride decreased slightly but steadily as the concentration was increased from 0.5 N to 2.0 N. The observation that 5 per cent potassium sulphate (0.3 N) extracted only very slightly more protein than did 10 per cent sodium chloride is important since most studies have used one or other of these solutions.

From all the studies on peptization of gluten, Hofmeister series for anions and for cations were constructed. It was apparent, however, that the albumen:globulin ratio varied widely with the different solutions used for extraction. Also, many of the salts showed a maximum peptization at quite low concentrations, and even in the usual dilute solutions exerted a salting-out effect.

The two salt solutions, 5 per cent potassium sulphate and 10 per cent NaCl, which are most commonly used for extraction of albumens and globulins, differ considerably in their actions on cereal flours (130). Suspensions of flour in 5 per cent potassium sulphate have a low viscosity and the particles settle rapidly, the moderately clear supernatant comprising about 70 per cent of the salt solution added. By contrast a suspension in 10 per cent sodium chloride gives a very high viscosity, the particles sediment slowly, and the sediment formed does not pack solidly, so that only about 50 per cent of the original salt solution can be recovered as supernatant. Obviously much greater hydration of the particles occurs in the 10 per cent sodium chloride, and it seems probable that this holds true for the protein constituent at least as much as for the starch.

Another variable discovered in the fractionation technique was the influence of protein concentration on solubility. The data of Gortner, Hoffman and Sinclair (132), of Geddes and Goulden (136) and of Harris (134) all showed that increased total protein per unit of extractant solution gave a decreased percentage extracted as albumen and globulin. Rich (137) recalculated much of this work and

showed a high positive correlation between ash content of the flours and the proportion of protein peptized. He concluded that the quantity of protein peptized by saline solvents was independent of total protein content. A later study, however, showed that the amount of protein peptized was not proportional to ash content, when water was the extractant (126). It seems probable that ash content affects peptization only by altering the types and concentrations of ions in the extractant, and it is highly probable that the total protein: extractant ratio is very important.

The effect of extraction time and temperature also was investigated. Harris (134) found that extraction at 20°, 38° and 58°C respectively, made little difference in the amount of protein extracted. This temperature range can probably be extended to 6°C in view of the extractions obtained by Csonka (138). Harris observed also that about 70 per cent of the extractable protein was peptized almost instantaneously, the rate of extraction decreasing thereafter, although dispersed nitrogen was still increasing slightly after one hour. If the suspension is not continually agitated mechanically, the extraction period must be greatly prolonged. Thus a shaking time of one hour is considered equivalent to three hours extraction with shaking only at intervals of thirty minutes.

The number of extractions required to remove completely salt-soluble proteins was investigated by Geddes and Goulden (136). Their data indicate that three successive 30 minute extractions of 6 gm. of flour with 50 c.c. of 0.5 N magnesium sulphate removed over 99 per cent of the salt-soluble protein. This held for flours varying

in total protein content from 8.6 to 13.7 per cent. Later workers seem to have accepted three extractions as sufficient (138) for quantitative work, and one extraction is considered sufficient for comparative work (139) although a lower total protein-extractant ratio is usually used. The work of Rich (126) indicates, however, that one extraction, even with 150 ml of solvent:16 gm. flour removes only about 45 per cent of the total protein removable by successive extractions, and that up to five extractions may be necessary to remove all dispersable protein. If the total protein peptized by many extractions with any given salt solution is termed "peptizable protein", then the amount extracted by any one extraction appeared to be nearly proportional to the amount of "peptizable protein" still undispersed.

Solubility of Gluten in Organic Solvents

In 1821, J. Gorham applied the name "zein" to the fraction of gluten which was soluble in strong alcohol. In one of the pioneering papers on this protein fraction, Osborne (114) reported that zein was readily dispersed by 85 to 95 per cent aqueous ethanol, 95 per cent aqueous methanol, aqueous isopropanol, glacial acetic acid, and phenol, and in glycerol at 150°C. Osborne observed that when dispersed in glycerol, it could be heated to 200°C without appearing to denature. Later workers (119, 140-143) used the extraction procedures of Osborne with little modification. The next phase of study on the peptization of prolamine concerned gliadin almost exclusively.

The composition and concentration of the extracting medium

were thoroughly studied. Up to very recent years, 70-80 per cent (by volume) ethanol concentrations have been popular as extractants. Nevertheless, as early as 1898 it had been shown that 40-60 per cent ethanol extracted maximum nitrogen from wheat flour, and this was confirmed by later workers (143-145). Similar studies were carried out with other solvents. Thus Schleimer (145) obtained optimum extraction of gliadin with 55-60 per cent methanol 55 per cent ethanol and 40 per cent propanol, and the total protein extracted increased in that order. Dill and Alsberg (146) found that isopropyl alcohol, chloral hydrate and methyl-ethyl-ketone in aqueous solutions of the proper strength could peptize gliadin. In addition glycerol, propylene glycols, and ethylene glycol, alone or in aqueous solutions of the proper strength gave clear solutions of gliadin at room temperature.

Dill extended these studies on the critical peptization temperature (CPT) of gliadin to investigate zein in aqueous alcohols (147). He observed that the C.P.T. was independent of the zein concentration if the latter was greater than 3 to 5 per cent, but that below 3 per cent zein, the C.P.T. tended to be lower. Above the C.P.T. zein appeared to be miscible in all proportions, and the upper limit of zein concentration seems to be determined by the extreme viscosity of the dispersions. Below the C.P.T. the zein is only 2 to 3 per cent soluble.

The most complete published study on zein solvent systems is that of Evans and Manley on primary (148) binary (149) and ternary (150) solvent systems. Numerous compounds were listed as primary

solvents for zein, i.e., capable by themselves of dispersing zein into a clear "solution". The C.P.T.s varied from below -40°C to above 173°C, indicating considerable differences in dispersing power. All of the solvents contained one or more hydroxyl, amine, amide, or carboxyl groups or some combination of these. Carbon chains attached to these groups were mostly two or three carbon atoms in length. Longer chains appear to decrease the solvent power and raise the C.P.T. In aniline, phenol, pyridine, benzyl alcohol and furfuryl alcohol, the carbon atoms are in the form of a ring. Some mono-phenyl ethers and mono-benzyl esters of glycerol, ethylene or propylene glycol, or ethanolamine also are solvents, but the disubstituted compounds have no solvent power. Swallen (151) shows curves for the per cent total protein of a dried sieved gluten meal peptized by aqueous ethanol and by aqueous isopropanol of 40 to 90 per cent concentration at different temperatures. The curves show a broad maximum, an increase of solubility with temperature increase up to 60 or 70°C, and greater solubility in isopropyl alcohol than ethanol at any given concentration and temperature. In these respects zein is similar to gliadin.

Swallen (151) describes the addition of secondary solvents to improve the solubility of zein in a "primary solvent" in which it does not disperse at room temperature. For example, zein was found to form a gel with aniline and did not disperse even when warmed, but when a small amount of alcohol was added a clear dispersion formed. Manley and Evans (149) give a much more complete treatment of such secondary solvents. They observed that in the case of poor primary solvents, which must be heated to effect dispersion, the addition of secondary

solvents, even in relatively large proportions, could greatly aid dispersion. On the other hand the same secondary solvents did not greatly increase dispersing power of good primary solvents such as glycerol, glycol, etc. Addition of relatively large amounts of the secondary solvents to strong primary solvents tended to cause precipitation, i.e., raised the C.P.T.

Among the secondary solvents are several partially chlorinated hydrocarbons and some nitroparaffins. Certain studies had shown that their heats of solution in certain donor solvents (152), and the composition of their azeotropic solutions in certain donor solvents, were related to their capacity to form hydrogen bonds. Manley and Evans observed that the effectiveness of these substituted paraffins in aiding zein dispersion followed the same sequence as the measure of their active hydrogen.

In a third paper these authors discuss ternary solvents containing primary and secondary solvents plus a diluent. The diluent does not increase the solvent effectiveness of either primary or secondary components, and it can be added to a dispersed system in relatively large amounts without causing precipitation or formation of two phases.

The authors emphasize the gradation of solvent properties in the three classes and between the three classes. The dispersing capacities of the primary solvents are indicated by their C.P.T.'s, and the secondary solvents may be graded according to their capacity to lower the C.P.T. when mixed with a primary solvent. The poorest primary solvents, which disperse zein only at comparatively high

temperatures, are also excellent secondary solvents. Thus absolute ethanol is a very weak primary solvent with C.P.T. about 120°C (153). Water would probably disperse zein if the system could be heated enough without denaturation, but must be classed as a secondary solvent, along with acetone. Yet mixtures of aqueous ethanol or aqueous acetone, or alcohol-acetone mixtures of the proper concentrations can all readily disperse zein. Similarly, the structural differences between secondary solvents and diluents are not great. Thus hexane is a diluent, but the slightly greater activity of the hydrogens of benzene makes the latter a weak secondary solvent.

Temperature has a very marked effect on the extraction of prolamines. More gliadin was extracted at 65°C than at room temperature (143,154) and Sharp and Herrington (144) estimated about 20 per cent increase in extraction with boiling 50-70 per cent ethanol. The use of higher extraction temperatures was considered unwise, because the extra protein did not have a specific rotation characteristic of gliadin, and Blish and Sandstedt (155) showed that successive extractions with hot alcohol could peptize about 25 per cent of the fraction normally regarded as glutenin.

The extraction of gliadin is considered to be slower than the extraction of globulins. Prolonged extraction periods with occasional shaking were used in the earlier work; a typical example is found in the 'Methods of Analysis' (6th Ed.) of the A.O.A.C. which specifies thorough shaking 10 to 12 times at intervals of 30 minutes, and then allowing the mixture to stand overnight. The suspension is then shaken thoroughly again and filtered. However, Hoagland (143) had

shown in 1911 that 60 to 90 minutes of continuous shaking is just as effective. The latest A.O.A.C. method uses 1 hour of continuous shaking followed by standing overnight, and filtering.

In the extraction of gliadin, successive extractions do not appear to be of value. Successive extraction of 120 gm. of "globulin-free", washed gluten with two-litre volumes of boiling 70 per cent alcohol for periods of three hours (155) gave a first extract containing approximately 98 per cent of the total peptizable nitrogen, a second extract containing about 1 per cent and a third extract containing about 0.7 per cent. The amide nitrogen as per cent of total nitrogen in the extract decreased slightly with each successive refluxing.

A very early study had distinguished three zein fractions (156). The α-zein was soluble in amyl and ethyl alcohol, β-zein was soluble in ethyl alcohol only, and γ-zein was insoluble in both solvents. These fractions had different specific optical rotations (157). Gliadin dispersions in alcohol, which were prepared at room temperature, also exhibited decreasing solubility as the temperature is lowered (158). By cooling such a dispersion to successive temperatures of $0^{\circ}C$ and $-11^{\circ}C$, three gliadin fractions were produced. The data indicate that of the original 10 per cent gliadin dispersion, about 47 per cent was insoluble at $0^{\circ}C$, another 47 per cent insoluble at $-11^{\circ}C$ and only 2.5 per cent still in solution at $-11^{\circ}C$. Analysis showed no definite differences in chemical composition. More recently, zein has been fractionated by successive dilution with water of a 70 per cent ethanol dispersion of zein (159), by successive extractions with methanol and 70 per cent ethanol (160), and by successive additions

of water to a dispersion of zein in either methyl or ethyl cellosolve (161).

Gluten Solubility in Acids and Alkalis

One of the distinctive features of the zein fraction of corn gluten, as purified by Osborne (114), was its insolubility in dilute acid. In contrast, the prolamine of wheat is readily soluble in dilute weak acids such as acetic, lactic and propionic (125). More recent studies have shown that this difference is the result of denaturation caused by drastic purification procedures, and that the undenatured zein is soluble in organic acids, although less so than gliadin (148, 149). With respect to dispersion and solubility characteristics, corn gluten is usually assumed to be qualitatively similar to the better known wheat gluten system. There have been no comprehensive studies on the solubility of the complete corn protein system, or the corn glutelin fraction, in acids and alkalis.

In an early study on wheat gluten, Rich (126) showed that in aqueous medium a minimum of protein was dispersed near neutrality, with sharply increased extraction as either acid or alkali was added. He emphasized that, although alkali would solubilize the total flour protein, acids such as the commonly used acetic acid, did not remove approximately 25 - 35 per cent of flour protein and were suitable only for dispersing the washed, starch-free gluten. Viscosity measurements indicate that maximum hydration and peptization of glutenin, the least soluble fraction, should occur at about pH 2.5 to 3.0 or pH 10.5 to 11.5 (125) with a relatively slight and constant hydration between pH 4 and 10.

The solubility of the glutelin fraction of corn gluten in acids and alkalis is very important since this is the only method available at present for assaying, or isolating this fraction. Again little more is known than has been described by Osborne (6). Some workers have pretreated the corn meal or gluten with salt solutions followed by 80 per cent ethanol, after which the glutelins were extracted with 0.2 per cent aqueous sodium hydroxide (162). Jones and Csonka found the glutelin was insoluble in dilute alkali after zein had been removed with 85 per cent alcohol (163). They dispersed the corn proteins with 0.2 per cent sodium hydroxide in 60 per cent ethanol, and precipitated the glutelin by neutralizing the dispersion or by adding ammonium sulphate. In a later paper, Csonka (164) declared that corn glutelin could be dispersed in alkali only.

Wheat glutenin is more soluble than corn glutelin in both acid and alkali. An early method of isolation of glutenin involved dispersion of whole gluten by 0.08 per cent sodium hydroxide in 70 per cent methanol. The glutenin is then precipitated by adjusting the pH to 5.2 with dilute acid (165). The amount of glutenin recovered by this method is very critically affected by the concentration of sodium hydroxide. Modifications have been suggested involving dispersion in dilute ammonium hydroxide in 70 per cent methanol, with subsequent removal of ammonia by aeration at 50°C, or else dispersion in aqueous barium hydroxide, from which glutenin precipitates when the concentration of methanol is adjusted to 92 per cent (166).

Blish and Sandstedt (165) found that glutenin, purified by Osborne's method, could be precipitated from an aqueous alkaline

dispersion by adjusting the pH to 5.2, and was soluble on either side of this pH. At this pH gliadin also tended to form a milky suspension although it precipitated very slowly. In a recent paper, Olcott and Mecham (167) have fractionated a gluten dispersion in dilute acetic acid by adjusting the pH to 5.0 to precipitate 46 per cent of the protein, then removing 13 per cent of the total protein as a fraction soluble between pH 5.0 and 6.8, leaving 41 per cent soluble in neutral salt solution. It seems scarcely justifiable to call the latter fraction gliadin, and for such a large proportion of gluten to be soluble in neutral salt solution suggests that some hydrolysis had occurred. Tague (168) found that gliadin had a minimum solubility in phosphate buffer at about pH 6.8, and this minimum was 1/10 to 1/20 of its maximum solubility in acid or alkali.

The question of which medium is best for extracting glutenin was discussed by Blish and Sandstedt (169) who claimed that dispersion of gluten in dilute sodium hydroxide gave preparations with lower total nitrogen, lower amide nitrogen, and higher arginine content than when dispersion in dilute acetic acid was used. An excellent comparative study by Larmour and Sallans (170) showed that glutenin prepared by dispersion in a very dilute acetic acid solution (.007N) apparently was freer of carbohydrate, had lost less amide nitrogen, and was lower in arginine than preparations derived from dispersion in stronger acetic or in alkali.

Solubility in Neutral Dispersants

The ability of sodium salicylate to peptize gluten was demon-

strated by Mangels and Bailey (171). Later, Rich (126) found that a single extraction with 0.25N sodium salicylate peptized 85 per cent of the gluten, and a second extraction removed all gluten. At higher concentrations of sodium salicylate the starch dispersed also.

The treatment of wheat flours by increasing concentrations of sodium salicylate (172) demonstrated a virtually linear increase in per cent of total nitrogen dispersed from a level of between 5 and 20 per cent in water to 100 per cent at sodium salicylate concentrations of 7 to 10 per cent.

There is evidence that the ratio of total nitrogen:extraction-volume is important. At any given concentration of sodium salicylate, the proportion of the total protein that was dispersed was inversely related to the total protein content (172). Apparently this was not due to any qualitative differences in the proteins of the different flours, since a detailed fractionation of the gluten dispersion obtained from each flour showed no significant differences.

There has been no published work on the effect of pH on the dispersion of gluten in sodium salicylate. Spencer and McCalla have suggested that variations in pH of different salicylate preparations may explain certain variations in amount of dispersed gluten (108).

Time of extraction as a variable has not been well studied. Washed glutens from wheat flours of differing qualities had different rates of dispersion in 10 per cent sodium salicylate (173). With constant gentle shaking, the dispersion of the gluten had an initial "inductive" period of about 1/2 to 4 hours, followed by a period of 4 to 8 hours of rapid dispersion, then a period of transition to a

constant value. The glutens from 'soft' wheats were found to be dispersed most rapidly. Dispersion of the 'hardest' glutens was complete in 24 hours and most varieties had reached complete dispersion within 15 hours. Vigorous and frequent shaking during a dispersion period of 6 hours, followed by standing overnight in the refrigerator, has been used (172,174). Later it was reported (108) that stirring or shaking had a denaturing action on the gluten, and that stirring was useless until the salicylate solution had been allowed to penetrate the gluten. The gluten was allowed to soak in the salicylate for several hours in the icebox, then stirred at intervals in order to expose fresh surfaces.

Successive extractions with low concentrations of salicylate each peptized decreasing amounts of protein, although the later extractions were important only with 2 to 5 per cent salicylate concentrations. With 8 per cent sodium salicylate concentrations or stronger, a single extraction will ordinarily peptize nearly all the gluten, the later extractions removing only an additional one per cent of the nitrogen. In a later section of this paper, Spencer and McCalla concluded that successive extractions at the lower concentrations merely served to expose fresh surfaces, and that the same effect could be achieved by stirring at intervals of about one day. Since removal of dispersed nitrogen has no effect, an equilibrium between dispersed and non-dispersed protein cannot be involved.

Gluten dispersed in sodium salicylate is present in colloidal aggregates of varying size. McCalla and Gralen (109) considered that the gluten which did not precipitate in their high speed centrifuge was molecularly dispersed, and that the protein fractions removed by

centrifuging at speeds of 3,000 r.p.m. and 18,000 r.p.m. represented colloidal aggregates. Of the protein peptized in 2 per cent sodium salicylate only about 40 per cent was molecularly dispersed, in 12 per cent sodium salicylate about 85 per cent of the peptized gluten was molecularly dispersed. In higher concentrations of salicylate the degree of aggregation was increased, possibly an indication of denaturation.

Salting-Out Gluten Fractions

Salting-out has been one of the most useful procedures for the recovery of gluten fractions from their dispersions. Osborne (6,120) used half or complete saturation with ammonium sulphate to precipitate albumens and globulins from their dispersions in dilute saline media. Hoffman and Gortner (130) precipitated a wheat albumen fraction from aqueous medium by half-saturation with ammonium sulphate. An important study of gluten peptization by salts was performed by Gortner, Hoffman and Sinclair (131) but indicated salting-out effects only with certain salts. A later study by Rich (126) described nearly complete salting-in and salting-out curves for some salt solutions. In general, salt solutions below 0.2 - 0.5 N have a salting-in effect, and salting-out occurs in more concentrated solutions. Salt concentrations around 5N are the most dilute that will completely salt out the most soluble flour proteins. Recovery of albumen and globulin fractions by salting-out procedures has not been commonly used since the many studies concerning the effect of electrolyte solutions on gluten have disclosed that the fractions obtained have

no real chemical identity.

Electrolytes affect the solubility of gluten in alcoholic media. Dill and Alsberg (147) precipitated gliadin by pouring the syrup, obtained by concentrating the 70 per cent alcoholic extract, into 5 volumes of 1 per cent sodium chloride. They give no reasons for adding the salt, but it would appear that the salt caused the gliadin to separate much more quickly, and in a more dehydrated form than is obtained by mere dilution with water. They give no data from which one might estimate the effect of the salt. When dilute acid or alkali in 50-70 per cent aqueous alcohol was used to disperse gluten, the gluten was readily precipitated from the dispersion by adding electrolytes (175). There appeared to be no salting-in effect at very low concentrations. The salting-out effects depended on valency and ion species and, at low salt concentrations, the ions could be arranged in a Hofmeister series on the basis of their capacity to cause precipitation of gluten.

The salting-out process for gluten solutions in neutral dispersants has been studied more fully. The salting-out of gluten dispersed in 10 per cent sodium salicylate solution by magnesium sulphate increased continuously and assymptomatically to a value near 95 per cent as magnesium sulphate concentration was increased to half saturation (172). The data were admittedly qualitative, since the salting-out action was accompanied by precipitation due to increasing dilution of the salicylate as the aqueous magnesium sulphate

was added. The magnitude of this dilution effect became significant only after some 90 per cent of the protein had been salted-out.

Gluten dispersed in 30 per cent urea, buffered at pH 6.9, may be salted-out in a similar manner. Presumably because the urea concentration is well above the minimum required for dispersion, a considerable salt concentration is required to start precipitation. Cook and Alsberg (175) observed that no precipitation occurred below 0.15 saturation with magnesium sulphate. Between about 0.15 saturation and 0.27 saturation, with magnesium sulphate, Cook (175) separated five fractions, from which unfortunately, the urea could not be completely extracted. However, the arginine content was found to decrease steadily with increasing solubility, as in the case of salting-out from salicylate. Amide nitrogen content and total nitrogen content were quite irregular because of adsorbed urea. However, analyses of amide nitrogen and arginine in the various fractions suggested that the gradation of properties obtained was the same as with salting-out from sodium salicylate.

DENATURATION

Definition and Estimation

The meaning of the term denaturation is not as yet clearly definable. Two recent reviewers of the subject (178,179) differ in their opinions whether the changes following certain treatments constitute denaturation, whether denaturation is all-or-none or variable in extent and whether it is truly reversible. In view of this disagreement, denaturation will be considered as being any physical or chemical change detectable in the properties of gluten or of its fractions. Change in solubility is a basic criterion of denaturation, and much of the knowledge of the denaturation of gluten by heat, (drying) or solvents has been based on solubility studies. Other physico-chemical characteristics such as the specific rotation, iso-electric point, and viscosity have frequently been used. Changes in chemical groups, such as amide nitrogen, sulfhydryl and free-amino groups appear to require comparatively drastic treatments involving major changes in structure of the molecules. In no case has chemical analysis proven adequate to detect the more subtle but definite changes in coherence, elasticity or solubility of gluten or its fractions.

Loss of coherence and elasticity in wheat gluten appears to be a sensitive criterion of denaturation. These properties have frequently been used in a qualitative manner in studies on wheat gluten. Unfortunately, none of the other cereal glutens possesses a coherence and tenacity of the same order, so that these properties

are not generally applicable. Even in the case of wheat gluten, the mechanism of these changes as they affect molecular structure is not understood, and the accurate measurement is difficult.

It must be emphasized that many of the conclusions regarding susceptibility to denaturation which are based on work with wheat gluten, may not apply in the same degree, if at all, to other glutens. Thus, the denaturation of wheat gluten by vigorous stirring while it is being dispersed in sodium salicylate or urea solution, is almost certainly related to the property of coherence, and a relatively non-coherent gluten such as that of corn, probably is not affected to the same extent.

Effect of Drying

Drying procedures have been shown to denature gluten. Glutenin, salted-out of a 30 per cent urea dispersion, became insoluble in 30 per cent urea when dried at a low temperature (175). Likewise, quick drying in an air blast at room temperature made the glutenin insoluble in 30 per cent urea. Cook (177) observed that dried gliadin preparations were rendered insoluble less easily than most gliadin by heating in 60 per cent alcohol. Drying gluten, even at temperatures as low as 45-50°C, greatly decreased its hydration capacity in acid dispersion, and glutenin was the fraction most affected (180). Drying gluten in vacuo at 60-65°C rendered the glutenin insoluble in dilute acetic acid (181) and drying at 80°C for three days denatured gliadin so that when precipitated from the dilute acetic it had lost its normal coherence. Recent studies (149,151) have emphasized the

view that water is a powerful denaturing agent for zein, and that zein is stable in anhydrous solvents. Furthermore, zein that has been dried at a low temperature retains its solubility when heated to well over 100°C. The effect of drying on corn glutenin has not been studied thoroughly.

Effect of Heat

Few observations have been reported on the denaturation of corn gluten by heat. The extraction of zein does not seem to be decreased by heating if the corn meal or gluten has been thoroughly dried beforehand (151). Also, zein appears to be relatively stable to heat when dispersed in anhydrous solvents (148). Solubility and critical peptization temperature were usually unchanged after heating to temperatures over 100°C for several hours. Herd (133) attempted to judge the denaturation of wheat gluten by heat, using as criteria the solubility of the fractions, especially of the gliadin, coagulation in water, and the viscosity in dilute acid solutions. He concluded that heat decreased the solubility of all fractions, although drying to a moisture content of about 4 per cent greatly diminished the rate of denaturation. Associated with the decreased solubility, an increased imbibitional power of the gluten in the heat-treated flours was deduced from their improved "baking strength" (182). Cook (177) concluded that gliadin, when dispersed in 30 per cent urea, was less easily denatured by heating than was glutenin. It seems possible that the method of preparation of the gliadin, involving preheating to 70°C, and extraction with 60 per cent aqueous alcohol already may have denatured the more

sensitive structures of the gliadin. Heating a wheat flour at low pressure did not cause denaturation of its gliadin, as judged by constancy of the critical peptization temperature (C.P.T.). The same heat, applied to flour containing 14 per cent moisture, rendered about 40 to 80 per cent of the gliadin insoluble in 60 per cent alcohol. Almost all the denaturation occurred within the first 15 minutes of heating and the C.P.T. of the soluble gliadin fraction was found to be lower. The shift of C.P.T. was independent of time of heating. Cook concluded that those fractions of gliadin which are least soluble and have the highest C.P.T., are the most sensitive to heat denaturation.

When gluten, dispersed in urea or sodium salicylate, was heated to temperatures not exceeding 60°C, viscosity tests and salting-out tests indicated little change in the protein except a slightly improved degree of dispersion. Dispersions heated above 70°C on the contrary showed increased viscosity and greater susceptibility to salting-out, indicating some coagulation.

Heat did not appear to coagulate gluten when it was dispersed in dilute acid or alkali (183). Instead, an increased rate and intensity of all the hydrolytic changes associated with acid or alkali at room temperature was observed. Recent work indicates that the hydrolytic changes in dilute acetic acid (pH 3-4) are caused by enzymes associated with the gluten (37). If the dispersion is heated to about 95°C for 5 to 10 minutes and then cooled, the gluten does not coagulate, the viscosity stays constant, and no increase of free amino groups occurs. There has been no demonstration that prolonged heating of gluten

dispersed in dilute acetic acid will not cause slight, but measureable, hydrolysis. Likewise, it seems probable that the hydrolytic effects observed in alkali are caused by the alkali rather than by proteinases. Denaturing actions other than hydrolysis evidently occurs when gluten is heated in these media (184), since the viscosity of a gluten dispersion in 0.1 N sodium hydroxide at 25°C was found to remain nearly constant in spite of increasing and extensive hydrolysis. In contrast, relatively little increase in free amino groups occurred in dilute acetic acid dispersions, yet the viscosity decreased considerably.

Effect of Organic Solvents

Alcohol appears to have a denaturing effect on wheat gluten. In an early study, freshly prepared gliadin solutions in aqueous alcohol were observed to have a slightly higher specific rotation than after standing 12 to 48 hours (185). In a later study, gliadin in aqueous ethanol solutions ranging from 20 to 90 per cent ethanol, showed significant changes in critical peptization temperature over periods up to 15 days (186). Dill and Alsberg also observed some changes in the solubility of gliadin. When dry gliadin that had been initially completely soluble in 70 per cent alcohol was left in contact with strong aqueous ethanol for 2 to 3 days, a portion of the gliadin had become insoluble in any strength of alcohol (146). Also, gliadin was precipitated from an alcoholic solution by adding just enough alcohol to precipitate the gliadin in a viscous, quite hydrated form. Upon redissolving in 70 per cent alcohol, much of the gliadin was denatured.

These authors also observed that drying a precipitated gliadin with absolute alcohol and dry ether denatures part of it so that this part will not dissolve in any strength of alcohol. Aqueous alcohols also denature the glutenin fraction. Prolonged extraction of gluten with aqueous alcohol left a glutenin residue that was insoluble in 30 per cent urea (175). Similarly, glutenin which had been precipitated from a 30 per cent urea dispersion by adding methanol to 60 per cent (by vol.) final concentration, or ethanol to 70 per cent (by vol.) final concentration was only partly redispersible in 30 per cent urea. Chilling to 0°C while in contact with the alcohol decreased the proportion of glutenin that was insoluble in urea. In agreement with this is the observation that corn glutenin is fairly soluble in 0.2 per cent sodium hydroxide, but is relatively insoluble after treatment with warm 60 per cent ethanol (163).

Recent studies tend to discredit the theory that the above denaturations are a specific effect of the organic solvent. The relative stability of zein in alcohol solvents containing less than 10 per cent water has been mentioned above (151), and zein seems to be most stable in anhydrous organic solvents. An important observation is that the sedimentation diagrams of wheat gluten dispersed in sodium salicylate show no denaturation changes after extraction with ether.

An interesting problem is raised by these considerations. Water alone is not commonly regarded as a denaturant for gluten except when warmed. If alcohol, acetone, glycol and other organic molecules do not cause denaturation, why do their aqueous solutions promote denaturation? An early suggestion of Dill and Alsberg (146) concerns

this problem. They concluded that less denaturation occurs if the solvents are adjusted so that the prolamine changes rapidly from its dissolved state to a rather completely dehydrated form, or vice versa, and is not left in contact with strong aqueous alcohol while in the partly hydrated form. This view agrees with the theory that water is a denaturing agent for gluten only when the latter is strongly hydrated or dispersed. Swallen (151) observes that addition of highly acid resins to a zein dispersion in 85 per cent alcohol tends to stabilize the protein against denaturation. Dispersions of zein in a concentration of 5 per cent or above change to a gel when the zein denatures and the denatured zein is insoluble in mixtures of organic solvents. Agitation accelerates denaturation, even in dilute solutions. The formation of some denatured zein in a previously stable dispersion will cause the whole dispersion to gel within a few hours, thus indicating that denaturation is autocatalytic. Carotenoid pigments or other lipids appear to be catalysts for the denaturation, since zein that has been extracted with lipid solvents until nearly colourless forms very stable dispersions (187).

Effect of Acid and Alkali

Acid or alkali seems to cause a shift in the isoelectric point of wheat gluten proteins. Thus, while the fresh alkaline dispersion of flour proteins always precipitated when adjusted to pH 5.8-6.0 (165) the purified glutenin, after redispersion, usually had an isoelectric point near pH 5.2 (188,189). After a longer dispersion in 0.5 N sodium hydroxide, part of the glutenin was denatured further and this fraction precipitated fairly sharply at pH 4.4 (188,190). Two glutenin preparations

have been described which had been prepared by dispersion in alkali, and had isoelectric points in the pH range 6.5-7.0 (191,192).

One of the principal denaturing effects of acid or alkali is the loss of amide nitrogen from the gluten. Glutenin fractions prepared by dispersion in .007 N acetic acid were found to have a higher amide nitrogen content than preparations which had been dispersed in stronger acetic acid or in alkali (190). It has been observed that glutamine and asparagine are most stable at pH 5, and that loss of the amide group becomes appreciable outside the range pH 4-6.5 (193). The terminal amide group is removed from glutamine much more easily than from asparagine, and glutamine is appreciably hydrolyzed even in buffers at pH 6.5 if warmed to 45°C or above. On considerations of stability of the amide groups, dispersion of gluten in very dilute acetic acid at pH 4-5 should cause no more denaturation than salicylate does at pH 7.

It is very important to remember that the least soluble glutenin fractions apparently have the lowest content of amide nitrogen. Therefore a relatively low amide nitrogen content in the glutenin fraction may indicate deamidation or an especially complete extraction of glutenin. When comparing the losses of amide nitrogen in different strengths of dilute acetic acid or alkali, it is very important to show that there has been no appreciable difference in amount of undispersed protein. This precaution usually has not been observed.

Hydrolysis of peptide bonds probably occurs in acid and alkali. The rate of hydrolysis of wheat gluten dispersed in acetic acid and sodium hydroxide has been measured by Cook and Rose (184). Recent observations indicate that wheat gluten dispersed in dilute acetic acid is stable to hydrolysis of the peptide linkages if the proteinases

have first been destroyed. There has been no demonstration that the hydrolytic changes observed in alkali are enzymic. A further effect of alkali on glutenin is racemization. The specific rotation of fresh glutenin dispersed in 0.5 N sodium hydroxide decreased to half its initial magnitude after standing for 10 days (194). The racemization seems to be associated with the shift in isoelectric point, and both are quite possibly the result of hydrolysis.

Cysteine is destroyed partly by dispersion in dilute alkali. When an alkaline dispersion of flour proteins is neutralized a strong mercaptan-like smell was observed (165). This smell was not noticed when working with purified glutenins, probably because of the repeated alkali treatments they had already undergone. Other studies show that dispersion of glutenin in alkali results in a lower cystine content (195).

Effect of Reducing Substances

Several denaturants seem to affect the disulfide linkage of wheat gluten (31). When small amounts of cysteine, of the order of 5 mg. of cysteine hydrochloride per gram of gluten, were mixed well with fresh moist gluten and incubated at 37°C for 30 to 60 minutes, the gluten liquified and became water-soluble. Electrolytes in low concentration caused precipitation of this dispersed gluten. Thus 0.5 per cent sodium chloride precipitated approximately 60 per cent of the liquified gluten. The coagulum formed a sticky mass, resembling freshly precipitated gliadin but was not completely soluble in 70 per cent alcohol. The coagulum could be repeatedly redissolved and salted-

out. The dispersed cysteine-gluten was not coagulated by boiling, even when the sulphydryl groups had been oxidized with excess hydrogen peroxide. The well-washed coagulum gave a strong nitroprusside test. Even after the reduced cysteine-gluten was precipitated, dried and powdered, it was still soluble in water. Drying and powdering after oxidation with hydrogen peroxide, however, gave an insoluble product.

Glutathione, potassium cyanide and sodium sulphite in slightly acid solutions produced similar changes, except that an initial partial liquefaction was followed by formation of a rubbery coagulum, insoluble even after thorough washing with water. However, part of the gluten remained in solution and could be coagulated by salts or acids. This fraction gave a strong nitroprusside test, which was not from cysteine, since Sullivan's test was negative. Potassium cyanide seemed to have a more drastic action than glutathione. After the removal of all coagulable nitrogenous material, there remained a soluble fraction rich in sulphydryl groups, which could be precipitated by tungstic acid. Treatment with sodium sulphate also produced a soluble, sulfhydryl-containing fraction, but it appeared to consist of smaller molecules, since it could not be precipitated by tungstic acid.

There are certain other observations which are not normally connected with the above facts, but which seem quite possibly related. For example, repeated emulsification of hand-washed wheat gluten in water by means of the Waring Blendor lowered the sulfhydryl content of the gluten (196). The authors regarded this as purification of the gluten, and suggested that the soluble material removed had an effect on gluten similar to the effect of glutathione, since the gluten was

considerably less elastic when recovered. This denaturing effect is probably identical with the denaturation observed when agitating or stirring gluten to aid dispersion (109). It is possible that the lipoprotein extracted from wheat flour by petroleum ether (197) is related to these soluble sulfhydryl-containing fractions. Mild acid hydrolysis liberated a water-soluble polypeptide fraction rich in arginine and cysteine. The polypeptide fraction had a variable composition, and only one of its fractions "purothionin", has been crystallized (198).

There are apparently one or more non-proteolytic enzymes which dissociate wheat gluten. This dissociation without appreciable increase of free amino groups has been described by Blagoveschenski and Yurgenson (123,124). It has been suggested that this effect occurred during a prolonged aqueous extraction of gluten (108). The action of such an enzyme also seems to be indicated by the observation (184) that the viscosity of gluten stored in dispersion in dilute acetic acid decreases very greatly without a corresponding increase in free amino groups. Heating such a dispersion to 95°C for 5-10 minutes prevents the change.

This particular grouping of the above observations is novel, but it offers an attractive and plausible basis for considering many of the changes in denaturation, solubility and structure which do not seem to be correlated with loss of amide nitrogen, coacervation, or hydrolysis. Many observations have been reported which suggest that any chemical or physical manipulation of gluten will cause denaturation. Thus, the precipitation of wheat flour proteins from an 8 per cent

sodium salicylate dispersion, either by dilution or by salting-out, creates irregularities in the sedimentation diagram (109). The sedimentation diagram is initially very simple, indicating a very homogeneous dispersed phase. It has also been observed that prolonged washing of wheat gluten in 10 per cent sodium sulphate causes surface denaturation, although the authors did not specify their criterion. It must be emphasized that up to the present, chemical conditions have been adjusted to prevent structural degradation by hydrogenion activity and heat, but no thorough control of conditions affecting factors such as hydrogen bonding or reduction-oxidation potential has been applied in these studies.

COMPOSITION OF THE GLUTEN COMPLEX

The Relation of Fractions to the Gluten Complex

The most complete and simple concept of the gluten complex has been obtained from the behaviour of gluten towards fractionation in neutral dispersants. The fractionation of gluten by extraction with increasing concentrations of salicylate, or by increasing dilution of a dispersion in 10 per cent sodium salicylate, or by salting-out protein from a similar dispersion, give virtually the same results (108,172). Urea dispersions give fractions with very variable nitrogen content (175). However, the analyses for arginine on an incomplete fractionation by salting-out from a 30 per cent urea dispersion suggests that a similar fractionation is obtained with urea.

The least soluble fraction is characterized by an amide nitrogen content of 15-17 per cent of the total nitrogen and an arginine nitrogen equal to 11-13 per cent of total nitrogen. When the dispersion has been brought to the point of precipitation, this fraction may be precipitated by a very small increase in salt concentration. The least soluble fractions cohere as a tough, tenacious, smooth mass, which redisperses with difficulty to give an opaque solution. Increasing solubility in the various fractions is characterized by an increase in the content of amide nitrogen, a decrease in arginine, and increasingly large changes in salt concentration are required to precipitate a given amount of protein. These fractions become more gel-like in consistency, have reduced coherence and can-

not be collected into a tenacious mass.

After approximately 70 per cent of the gluten has been precipitated, the next fraction, containing 10-15 per cent of the gluten, has the highest amide-nitrogen content of all fractions, amounting to 25-27 per cent, and has the lowest arginine content, approximately 5 per cent. This fraction precipitates as small particles which have little coherence, and are readily redispersed in 10 per cent sodium salicylate to give an almost clear solution. The fractions comprising the most soluble portion (about 15 per cent) of the gluten has an amide-nitrogen content which sharply decreases, and an arginine content which correspondingly increases, with increasing solubility. The percentage of amide-nitrogen in the final fraction was about the same as the values for the least soluble glutenin fraction. Also, the arginine content of these two fractions was approximately equal. The most soluble fraction of gluten could not be salted-out; by calculation the amide content was found to be distinctly below, and the arginine content above, that of the least soluble fraction.

Spencer and McCalla (108) suggested that the most soluble fraction of gluten in flour is related to the flour proteins removed by water in preparing the gluten, and they did not regard it as being part of the gluten system. Their gluten fraction represented only about 82 per cent of the flour protein, and they observed a further 16 per cent of the flour protein was extracted from the gluten by a single extraction with distilled water. They concluded from the fractional analysis that a further 10 per cent of the

total flour protein, or some 40-45 per cent in all, is not part of the gluten system.

The critical importance of the above theory has not been sufficiently emphasized. Since the studies of peptization of gluten by different salts in 1929 (132) much attention has been given to the boundaries of the globulin fraction and the relation of this fraction to the gliadin and glutenin fractions. The gradation of properties between globulin and "gliadin" fractions is quite a controversial question and is just as basic to an understanding of the gluten system as is the gradation between the gliadin and glutenin fractions.

There are theoretical objections to the evidence given by Spencer and McCalla for a separate "water-extractable protein" constituting 10-15 per cent of the gluten and over 40 per cent of the flour protein. A discontinuity in the properties of the end fractions obtained in one medium should not be linked with the initial fractions obtained in another medium, without strong supporting evidence. When wheat gluten, initially dispersed in 10 per cent urea, was gradually salted-out with magnesium sulphate, a smooth curve was obtained showing no discontinuity up to the point where only 5 per cent of the protein remained in solution (172). Similarly, when flour samples were extracted with increasing concentrations of sodium salicylate, an essentially linear increase of peptized nitrogen with increased salicylate concentration was observed (172). There was no evidence of a discontinuity in these relationships, in the region of 40 per cent peptization, as suggested by Spencer and McCalla.

It is commonly assumed that essentially the same order of solubility holds for all aqueous solvents. A comparison of the Van Slyke analyses of several early studies of albumens and globulins (51) suggests that this may not be true. Although the values are very irregular, there is a tendency for globulins to contain much more arginine, and only slightly less ammonia nitrogen than the accompanying albumens. This is the reverse of what would be expected according to the fractionation series in salicylate, if the salt solutions used were aiding peptization. This assumption must be true, since the final operation used in all these separations was dialysis. Similarly, in an investigation of the peptizing action of potassium iodide on gliadin, unexpected results were obtained (199). Since some 74 per cent of gluten can be peptized by 0.5 N potassium iodide (132), this electrolyte solution presumably can peptize gliadin. However, only 45 per cent of an alcohol-extracted preparation of gliadin dispersed in 0.5 N potassium iodide. When this dispersible fraction was reprecipitated, it was found that a second extraction dispersed approximately the same proportion of both the originally dispersible gliadin and the originally undispersible material. It appears to be unjustified to assume that the solubility pattern obtained with such neutral dispersants as sodium salicylate and urea is obtained also with lyotropic salt solutions.

Most other fractionation studies contribute to the impression that the glutelin is the least soluble fraction of the gluten system. However, the other methods of fractionation all involve an aqueous-alcohol medium in which the gliadin fraction would be expected to be

more soluble. If gluten dispersed in dilute acetic acid or sodium hydroxide were fractionated by salting-out, it is probable that a different progression of properties would be obtained. This is supported by the observations of Csonka and Jones (199a), who fractionated glutenin, dispersed in 0.2 per cent sodium hydroxide, by salting-out with ammonium sulphate. The less soluble fraction had an amide-nitrogen content and a specific rotation that was closer to gliadin than was that of the more soluble fraction of glutenin.

It has been suggested (108) that the smooth progression of properties, which is obtained by serial fractionation in neutral dispersants, is proof that gluten is not composed of a small number of distinct but chemically related proteins. In this case, gluten might be an example of the type of "reversibly dissociating complex". suggested by Sørensen (199b,200). On the other hand, it has been suggested that gluten is a series of protein species differing slightly and smoothly in molecular properties. Some evidence has been reported which weakens the above argument. For example, Kunitz (201) mixed purified α-chymotrypsin with γ-chymotrypsin and observed that at pH 4.0 the mixture gave a smooth solubility curve typical of a solid solution. When the solubility curve was determined at pH 5.5 however, two distinct breaks in the slope were evident. Further examples of protein mixtures, which show a smooth variation in properties under certain conditions, have been discussed by Butler (202) and by Northrop and Kunitz (203).

Bungenberg de Jong has shown that coacervation between mixtures of glutenin and gliadin can be expected in the range pH 5-7 (204,205). Sodium salicylate solutions commonly have a pH between 5.5 and 7, and when urea dispersions have been used, the general practice has been to buffer the solution at pH 6.8. It is unfortunate that these studies on the fractionation of gluten have not been done with neutral dispersants at various pH levels.

Gortner and Macdonald (161) have fractionated zein into a hydrophobic type of protein and a hydrophilic type. They deduced that there were three components. Two components, with molecular weights of 45,000 and 30,000, comprised 65 per cent and 25 per cent respectively of the total zein. These components were precipitated as compact coagula when water was added to their dispersions in methyl cellosolve, and were designated as hydrophobic. The third component formed a stable suspension and was not precipitated by addition of water. It was called hydrophilic. It had a molecular weight of 23,350 and made up only 10 per cent of the zein. The solubility of zein is very dependent on the presence of the hydrophilic component. Dispersions of either of the purified hydrophobic components tended to separate relatively sharply and completely when water was added. When a little of the hydrophilic component was added, the dispersion showed a less complete separation with an optimum precipitation of protein at 40-45 per cent (by volume) water, and an increasing solubility as more water was added. Certain other studies (206,207) have indicated that prolamines are not homogeneous, but Gortner and Macdonald are the first to demonstrate convincingly the presence

of distinct proteins rather than fractions.

Lipoprotein in Gluten

Recent work indicates that lipids, especially phospholipids, can become associated with gluten, probably by adsorption, to form a lipoprotein complex. Gluten, washed out of wheat flour, retains about 75 per cent of the flour lipids (206). Only small amounts of the lipid could be extracted with ether (208,209) suggesting that a lipoprotein complex was formed during the mixing of the dough (210). Recently Olcott and Mecham (167) claim to have shown that the lipid complex involves the glutenin fraction primarily and that phospholipid is preferentially bound. Complex formation occurs in aqueous media, or flours containing above 30 per cent moisture, and is facilitated by agitation. The lipoprotein is stable in dilute acetic acid, and in dispersions in urea or sodium salicylate, but is broken up when alcohol is added to precipitate the glutenin. The observation that 30 per cent of total lipids of flour are not ether-extractable before wetting suggests that some "lipoglutenin" already is present. The lipoprotein however is readily dissociated by treatment with aqueous alcohols.

Amino Acid Content of Gluten Fractions

The amino acid analyses which have been reported for different gluten fractions by various workers are not consistent enough to warrant definite conclusions as to the structure of the different fractions. Since such great differences exist between the proportion

of amide nitrogen found in the gluten fractions, it would seem more logical to compare the distribution of nitrogen among the amino-acids on an amide-free basis, especially in view of the unavoidable hydrolysis of amide groups which Larmour and Sallans (170) have shown to occur in varying degrees with different methods of preparation. Also, it would be advisable to adjust the analyses for nitrogen distribution to eliminate the effect of the three terminal nitrogen atoms in arginine.

Bailey (51) has collected data of several workers on nitrogen distribution of gluten fractions by the Van Slyke method. These data are chiefly notable for their lack of consistency; using the basis of calculations suggested above gives only a partial correction of the irregularities. In data on the analyses of glutenin and gliadin quoted by Bailey, the total basic nitrogen content is relatively consistent at 19-25 per cent and 10-12 per cent respectively. The nitrogen distribution among the rest of the amino acids, however, is extremely irregular. The albumin and globulin fractions show great irregularity throughout, tending to have a high basic nitrogen amounting to 20-32 per cent of total nitrogen. The most consistent values for an individual amino acid are those for arginine which constitutes 4.5-6.2 per cent of gliadin, 8-12 per cent of glutenin and 10-18 per cent of albumens and globulins. Individual differences in using the Van Slyke analytical technique probably account for most of the irregularity. Therefore the comprehensive investigation carried out by Larmour and Sallans (170), on the glutenin and gliadin fractions obtained by various methods, is of especial importance. Their ranges of values

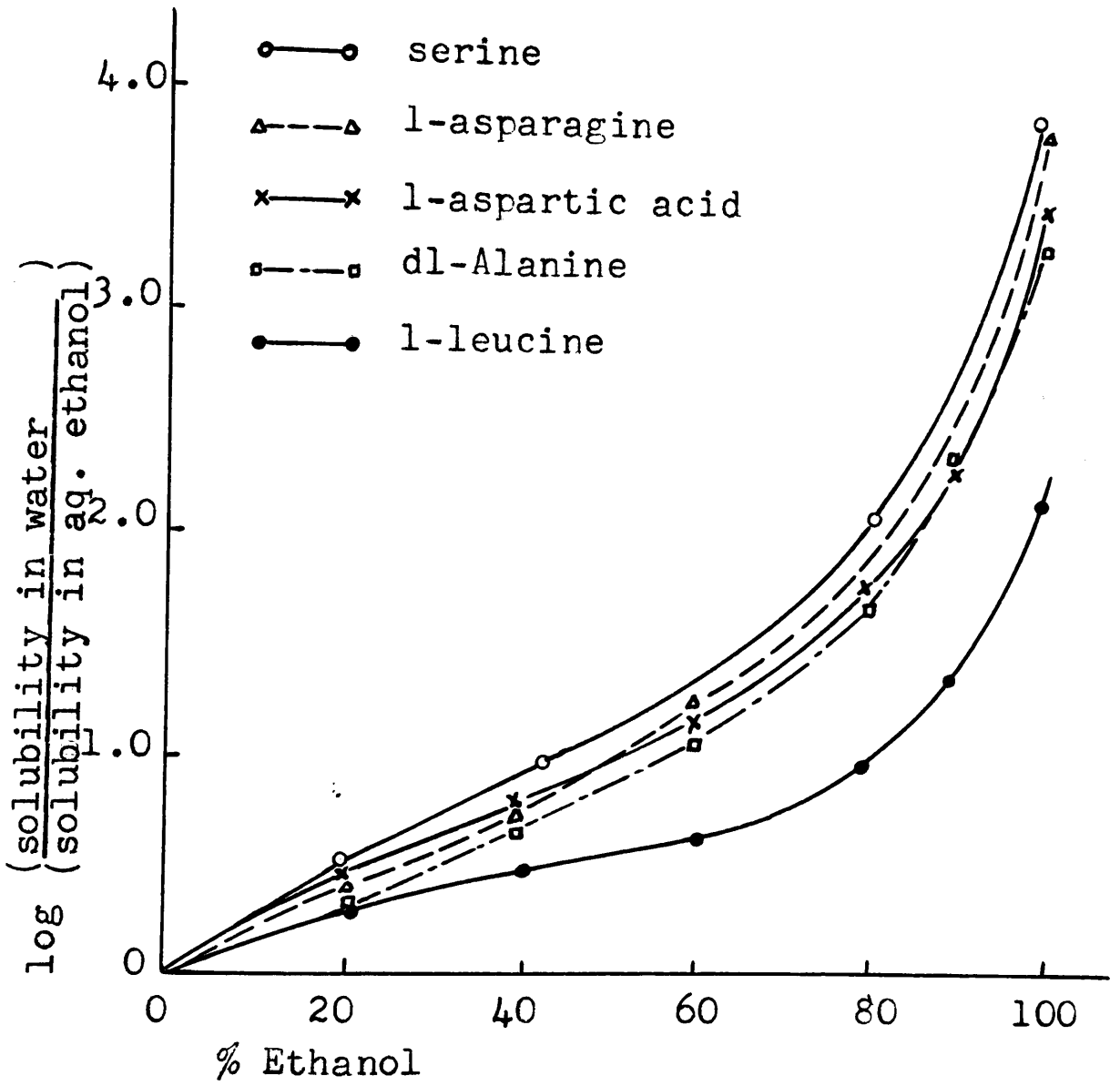
	Gliadin	Glutenin
Ammonia N	25.1 - 25.8	15.1 - 21.0
Humin N	0.8 - 1.0	1.5 - 2.1
Arginine N	5.2 - 6.2	8.5 - 12.1
Lysine N	0.6 - 1.2	4.6 - 7.0
Histidine N	3.7 - 5.8	1.5 - 3.6
Cystine N	0.5 - 1.0	0.6 - 0.9
Amino N in filtrate	51.9 - 53.6	52.3 - 54.3

for nitrogen distribution in gliadins and in glutenins prepared by acid dispersion are tabulated above. It will be seen that the gliadin is poorer in basic amino acids, and is much richer in amide groups.

An approximate idea of the effect of specific side chains on solubility of the gluten fractions may be gained from the figure below which has been calculated from data reviewed by Cohn and Edsall (211). Any deductions based on this figure must take into consideration the fact that the side chain group of the individual amino acids is attached to a relatively small, divalent, ionized dipoles. Therefore, although the relative effects of side chain groups on solubility can reasonably be deduced, the solubility of proteins in aqueous ethanol of varying concentrations, certainly is determined largely by the size of the molecule and its dipole moment.

According to the figure below, the amidation of the terminal carboxyl of a dicarboxylic amino acid does not increase the solubility in strong aqueous ethanol relative to the solubility in water. The amide group however does increase the absolute solubility in both water

Effect of Side Chain Substituents on Amino Acid Solubility in Aqueous Alcohol



and ethanol (217), but this is quite probably due to a lower crystal lattice energy for the less polar molecule. Also, a comparison of the leucine and proline contents of several proteins (211) indicates that the proline components of zein and gliadin probably contribute much less to their solubility in aqueous-alcohol than does the leucine. It is advisable, therefore, that the term "prolamine"

should not be correlated strictly with solubility in alcohol, but rather with the structure of the molecules.

The solubility relations of the prolamines in aqueous alcohols seem to be dominated by the polarity or dielectric constant of the medium. Thus the solubility curves for extraction of the zein fraction from corn proteins show a maximum at about 67 per cent (by volume) ethanol or 62 per cent (by volume) isopropanol (187). These media represent very different mole fractions, but their dielectric constants, calculated from the data of Akerlof (213) with the assumption of partial specific volumes equal to 1.0, are 50.6 and 45.6 respectively. Similarly, the maximum solubility for wheat-flour proteins occur in 55-60 per cent methanol, 55 per cent ethanol, or 40 per cent propanol.

The optimum concentration of alcohol in the dispersant is not easy to specify, because of the very broad flat maximum in the solubility curves; consequently, the differences in dielectric constants, calculated for different dispersants, cannot be considered as being significant. The consistent increase in the proportion of total protein peptized by the organic solvents with longer carbon chains shows that there is a solubility effect which is specific to molecular weight or length of carbon chain, or size of the dielectric increment of the organic molecule. Other common organic substances, which form aqueous solutions capable of peptizing zein, also have negative dielectric increments in water, e.g., aniline -7.6, dioxane -8.3, ethylene glycol -1.8, and glycerol -2.6.

Approximate data for 25°C

Soluble Fraction	Dispersant	Organic Solvent(1) Moles/litre	Dielectric Constant(2)	% Total Protein Dispersal	S(2)
Zein	67% Ethanol (3)	11.4	50.6	41 (3)	-2.6
	62% Iso-propanol (3)	8.1	45.6	52 (3)	-4.3
	65% Acetone (3)	8.85	52.2	--	-3.2
Gliadin	57% Methanol (4)	14.1	60.6	51.4 (4)	-1.4
	55% Ethanol (4)	9.4	56.0	59.3 (4)	-2.6
	40% n-propanol (4)	5.35	59.0	63.5 (4)	-4.0

(1) Calculated with assumption of specific molal volume = 1.0

(2) Calculated from data of Akerlof (213)

(3) Estimated from data by Swallen and Daneghy (187)

(4) Quoted from Bailey (51) page 38.

This simplified view of prolamine peptization is not supported by the work of Evans (148-150,153) who lists a large number of organic substances capable of dispersing zein. Apparently there is an effect specific to the molecule, since all solvents contained amino, amide, hydroxyl or carboxyl groups. Some of these primary solvents, their critical peptization temperatures and their dielectric constants are listed below. These data are not consistent with the view that, at room temperatures and below, the dielectric constant of a primary solvent has to be between about 35 and 65 in order to peptize the prolamine fraction and that media with a dielectric constant outside this range have a higher C.P.T. The real mechanism of prolamine

Solvent	C.P.T.	Dielectric Constant
Glycerol	139	56.2
"		29.5 @ 130°C
Acetamide	82	60.3 @ 83°C
Ethylene glycol	18	47.0 @ 0°C
" "		35.2 @ 20-50°C
Benzyl alcohol	-18	15.0 @ 0°C
" "		13.0 @ 20°C
Aniline	gels	7.7 @ 0°C
"		5.4 @ 100°C

peptization and the effect of such factors are little understood.

MILLING PROCESSES FOR RECOVERING STARCH FROM STRIPPER STARCH

Stripper Starch

The study to be described herein was undertaken to find out whether the gluten in stripper starch can be removed and the starch recovered without alteration. The scope of the study obviously must be limited since so many problems connected with large-scale production cannot be studied on anything less than a pilot-plant scale. Also, since the cost of commercial milling must be kept as low as possible, the methods of separation must be limited to simple mechanical processes, or to chemical steps involving dilute aqueous media.

Stripper-starch is a fraction of the steeped starch slurry which contains about 3-10 per cent gluten (dry basis). It represents the intermediate fraction which is obtained industrially during the separation of corn into the commercial products, "prime" starch (0.3 per cent gluten) and corn gluten (40-60 per cent gluten), which is sold as "gluten feed". Where tabling has been replaced by centrifuging, the primary centrifugals deliver one effluent containing 1 per cent gluten, and another containing about 40-60 per cent gluten. The former effluent yields a fraction containing 10-12 per cent gluten in a secondary centrifuging operation. This stripper-starch is recirculated through the separation procedure.

Apparently no studies have been reported which deal specifically with the structure or properties of the gluten in stripper-starch. The common assumption is that this gluten is chemically identical with the gluten of the corn-gluten fraction,

and that both these fractions simply represent an imperfect separation of the starch and gluten by the grinding technique. This hypothesis probably is true, since the corn-gluten fraction is only 40-60 per cent protein. This view implies the further assumption that the acid-steeping used is adequate for thoroughly softening the gluten. The separation is incomplete because the acid-steeping waters do not readily penetrate as far as the centre of the endosperms and because the Buhr mills used for grinding the slurry must not be set to grind too fine, to avoid damaging the moist granules. In this case, a further acid-steeping treatment should thoroughly penetrate the exposed gluten residues. Since a finer grinding operation is likely to damage the granules, a violent agitation process such as may be observed with a Waring Blendor, or a homogenizer, might break apart the hydrated gluten and starch.

It is equally possible, however, that the stronger adherence of the gluten fraction in stripper-starch is due to a greater resistance to softening and hydration during steeping. Local variations in either deposition of zein or of lipid in the endosperm would be expected to produce local variations in resistance to hydration. It is more likely, however, that the kernels on an individual cob, or on different cobs, may differ significantly in amounts of lipid and zein contained, and hence may differ considerably in the ease of dissociation of the gluten from the starch during steeping and crushing. If the remaining starch-gluten masses are made up of a more resistant type of gluten, a further acid-steeping treatment is not advisable. During the original steeping period of some 40 hours, the endosperm is protected by the

seed-coat, but after crushing there is an appreciable period during which the exposed endosperms are suspended in a medium of pH 4.0-4.2. This is followed by a thorough grinding operation in the Buhr mill. If the gluten-starch residues are too resistant to be broken apart with such treatment it is unlikely that a further acid-steeping treatment will be effective. For further separation it probably would be advantageous to use a different type of mechanical operation, steeping in a different medium, or some chemical process not based entirely on hydration, for example, enzyme digestion, fermentation, or repeated extraction.

Fermentation processes

Fermentation in various degrees is usually employed in the milling of starch. Corn ordinarily contains a wide variety of bacteria and molds, which become active if sufficient moisture is present.

The Halle process for wet milling of wheat starch has been described by Frehwald (215). Fermentation and enzymatic action were kept to a minimum during the preliminary steeping process by changing the water frequently. After crushing, water at 30°C is added to convert the wheat pulp to a very heavy slurry, and some liquid from a previous fermentation is added to accelerate fermentation. The initial fermentation apparently is glycolytic, since there is an odour of alcohol, accompanied by much frothing. A second stage occurs in which lactobacilli predominate, the froth collapses and an "odour of rancid butter" is evident. The last stage of fermentation produces the odour of putrefaction usually associated with proteolytic

breakdown. Kerr (41) states that higher steeping temperatures, about 50°C, favour the acid-producing fermentation and impair the alcoholic and putrefactive reactions. The complete fermentation required 5-6 days at 30°C. It was found that the acetic and lactic acid formed during the second stage helped to solubilize and hydrate the gluten, and that the proteolytic action solubilized gluten by breakdown of the protein mass itself. The fermentation was stopped in the initial stages of proteolysis. Longer action was found to yield mucinous gluten, so excessively solubilized that it would not readily precipitate during levigation of the starch, and that it had lost the strongly adhesive properties of the normal gluten product.

There has been a growing tendency to use a regulated fermentation by acid-forming organisms in the acid-steeping processes. Associated with this action is a liberation of a small amount of phosphate by phytase, and a slight proteolysis of the soluble proteins. Low concentrations of phosphate aid the separation of starch in tabling, and the presence of organic acids and peptones reduces coagulation and scale formation during evaporation of steepwater. During the alkaline steeping, any action by acid-producing organisms either will neutralize the alkali or will require the addition of relatively large amounts of alkali to maintain the pH at the desired level.

The use of fermentative processes as the primary means of softening the gluten was discontinued many years ago. Fermentation takes much longer than the usual steeping treatment, and is attended by an offensive putrefactive odour which is objectionable throughout the neighbourhood. Because of the bacterial putrefaction, the protein

could not be recovered for feed or for extraction of zein. Only a small portion of the starch was lost by enzyme action in the fermentation.

Enzymic Digestion of Gluten

Enzymic digestion of the gluten of stripper-starch has been recommended. In fermentative steeping of corn there was little loss of starch, due to the relative inability of the enzymes and bacteria to penetrate the intact starch granule (216). Undamaged starch granules differ greatly in their resistance to attack by amylases. Potato starch granules are quite resistant, but, as Sandstedt (217) has shown, wheat-starch granules are extensively attacked by α-amylase at pH 4.7 within 120 hours; β-amylase has relatively little effect on intact granules. During periods up to 5 hours only the damaged wheat-starch granules are attacked. Corn-starch granules are chemically more similar to wheat-starch than to potato-starch, but are more resistant than wheat starch to gelatinization and swelling. Only a very small proportion of starch granules are damaged during the grinding operation that precedes tabling. Both the α-amylase and β-amylase of malted barley have their optimum pH about 4.4, and have appreciable amylolytic action up to about pH 6.5 (218). Ungerminated maize has only slight α-amylase activity and no detectable β-amylase activity (219).

It would not be desirable to carry the proteolysis of gluten very far. As has been mentioned, only a very limited degree of proteolysis of the soluble proteins will prevent coagulation and deposition of protein in the evaporators. Extensive breakdown of the

proteins to small polypeptides, on the other hand, would so solubilize the gluten as to make its recovery for feed difficult. For this reason an enzyme preparation rich in proteinase, but without peptidase activity is to be desired. The well-knonw peptidases of plant and animal origin almost all have optimum activity about pH 7.0-8.6 (220). Since purification sufficient to eliminate the peptidases is expensive and since it is unwise to add common enzyme inhibitors to food materials, it would be convenient if digestion of the gluten could be carried out at about pH 4-5, to decrease peptidase activity.

The solubility of gluten is a minimum near pH 5 however, and increases sharply above pH 7. Unless an enzyme in solution is capable of attacking undispersed gluten, it would be expected that proteolysis of the gluten would occur more rapidly at pH levels above pH 7. With papain, which is remarkably stable to alkali, or with trypsin, the optimum pH for proteolysis may be quite high.

Apparently there is some action on an insoluble substrate by a dissolved enzyme. Laine (221) suspended solid zein in an aqueous solution of pepsin or trypsin and studied the course of proteolysis. There was an initial step during which the molecule split into two water-insoluble fractions, with the liberation of two sulphydryl groups. Further digestion by pepsin yields soluble tetrapeptides with glutamine as the terminal amino acids. Trypsin yields soluble octapeptides with alanine at one end. Studies by Smorodintsev and Zhigalov (222) suggest that papain does not split corn gluten to peptides as small as those produced with pepsin.

Steeping in Acid and Alkali

The softening of the pericarp layers and loosening of the germ are relatively easily accomplished even in water. The primary object of steeping must be regarded as the hydration and loosening of the gluten in the endosperm. Sharp and Gortner (125) concluded from viscosity measurements that hydration of wheat gluten occurs principally in the range of pH 3-4 and 10.5 -11.5. Measurements of solubility of wheat gluten at different pH values indicates a closely similar relationship (126,223). It has long been known that corn proteins are less soluble, especially in acid media, than wheat proteins (142,151). It would be expected therefore that alkaline media would solubilize and hydrate the corn proteins more thoroughly than acid media.

The penetration of the corn grain by the steeping liquid is very gradual. Kerr (41) describes a counter-current steeping system in which water enters at a pH of 3.6 and passes over 5 lots of corn before it is altered to pH 4.0. The corn entered the system at a pH of 5.8 and was treated with steep-water of pH 4.0-4.2 for about 24 hours before the pH reached 4.5. The complete steeping time for corn is usually about 40 hours. It would be expected that alkali or acid would penetrate stripper-starch very much more quickly than whole corn kernels. The usual steeping treatment for wheat grain is about 24 hours at 100°F (224). In contrast to this the alkali-steeping procedure for wheat flour requires only 30 minutes with stirring at room temperature with an additional 2 hours for settling (223).

From the experience gained in fractionation studies, wheat proteins are known to disperse more easily and completely in aqueous alkaline solutions than do corn proteins. In a study of alkali-steeping of wheat, (223), it was observed that mixing 0.01 N sodium hydroxide with wheat flour resulted in a pH of 10.4-10.8, and dispersed 95 per cent of the protein. The pH of the mixture appeared to depend on the ratio of protein to alkali, and to be relatively unaffected by dilution. The solubility of the flour proteins increased abruptly from about 30 per cent of total protein at pH 9.0 to over 95 per cent at pH 10.5. Dispersion was effected in 10 minutes. The dispersed protein could be recovered by precipitation most completely (75 per cent) at pH 6.0-6.5, but at this pH the protein frequently formed a stable suspension and failed to settle. Adjustment to pH 5.5 recovered 70 per cent of the protein with dependable precipitation. The precipitate did not pack well by gravity settling, but centrifuging at 800 times gravity gave a soft protein cake containing 70-80 per cent moisture. If the supernatant from one precipitation was used, with added alkali, to steep a fresh lot of flour, precipitation was less complete, and eventually the concentration of salt and proteins soluble at pH 5.5 became so high that no protein could be precipitated.

Hydration of the gluten, rather than dispersion, is the object of acid steeping. Only a small proportion of the gluten protein is dispersed in the steep water in corn milling. The recovery of the insoluble gluten is relatively simple; only mechanical processes such as centrifuging and tabling are necessary. Therefore, and alkaline-steeping treatment designed primarily to hydrate, rather than disperse

corn gluten may be advantageous.

Effects of Steeping on Starch

One of the main disadvantages to acid-steeping procedures is the loss of potential hot-paste viscosity of the starch. Schoch and Jensen (65) have shown that in aqueous media starch is most stable between pH 6.0 and 6.5. As the medium becomes more acid there is a rapid increase in hydrolysis as measured by the lability to alkali. The commercial practice is to evaluate potential hot-paste viscosity by means of the Scott viscosity test (41,225). Starch prepared from the first steep of an acid-steeping system usually has a Scott viscosity value of 120 or slightly higher, but after steeping at pH 4.0-4.5 the Scott viscosity value usually drops to about 85 (41). Most commercial uses of starch are adapted to pastes with this potential viscosity, and the further drop in potential viscosity that would be caused by a further acid-steeping treatment would make it useless.

The degrading effect of dilute alkali on starch is not well understood. The alkali apparently has a negligible affect on the maltose linkages (226). Hot aqueous alkali, however, has been shown to degrade starch with the production of formic, acetic and lactic acids (227). With the relatively mild conditions of alkaline steeping used in industry, little or no alteration in the starch occurs. The steeping conditions which aid hydration of gluten also tend to gelatinize the starch. The maximum alkali concentrations permissible when steeping wheat flour at various temperatures have been determined (223). Wheat starch may be steeped at 50°C in 0.01 N sodium hydroxide,

but at no more than 25°C in 0.1 N sodium hydroxide. Corn starch is slightly more resistant than wheat starch (72).

In a laboratory study of alkaline steeping, the recovered starch was found to contain 4-6 per cent impurities, as compared with about 1 per cent of impurities in commercial starches (223). This was attributed to failure of the alkali to remove cellulose in the form of cell-wall fragments and bran fibres. However, cellulose constituents are usually less easily extracted by weak acid than by dilute alkali. Unfortunately no control experiments were done by the same laboratory techniques using acid steeping. In view of these facts it seems very probable that the greater purity of commercial starches is merely a reflection of the greater efficiency common to large scale operations.

There is a widespread prejudice among commercial and technological personnel against the use of alkaline steeping because it is supposed to alter the starch. Such opinions appear to be based on the high viscosity observed in starches produced by alkaline steeping when the alkali has not been neutralized afterwards. Another reason for thinking that alkali alters the starch is the swelling and gelatinizing effect of alkali. Neutralization of the alkali, followed by washing would be expected to return ungelatinized starch to its normal volume and normal potential viscosity. While no experimental data have been reported on viscosity of neutralized alkali-steeped starch, brief statements may be found supporting the idea of reversibility (215,223).

Separation Processes

A starch slurry of about 10-12°Be at 85°F and pH 3.8-4.0 is commonly used in tabling. This slurry is discharged at as constant a rate as possible into the upper end of long flat-bottomed troughs. These troughs or "tables" are ordinarily about 120 feet long and 2 feet wide, and slope very slightly. The starch settles out on the table, very heavily at first and then less densely. The liquid running off the end of the table is called "gluten-overflow water". The overflow water is held in tanks while the solids settle, or is put through centrifuges. The recovered solids are known as "corn gluten meal". The starch on the table may be given a surface wash to remove some of the fine sediment of gluten generally found on the surface. The starch is flushed off the tables and washed by various combinations of vacuum filters and continuous centrifuges to remove the soluble protein. The capacity of these tables to handle slurry would be reduced greatly by the slow sedimentation rate of starch in alkaline medium. It seems rather doubtful, in fact, whether existing tables could be used with an alkaline medium at the low rate of flow that would be necessary to permit sedimentation of the starch on the table.

The separation of starch from gluten by continuous liquid centrifuges today is replacing the tabling method. A great saving in floor space is effected and there is a greater yield of starch. The centrifuges have a vertical rotor shaft, on which are mounted many parallel flanges, conical in vertical section. A heavy slurry

tation rate of starch relative to gluten; the changes in over-all specific gravity of the medium are secondary. The particle size therefore is very important. In centrifugal separation the separation is primarily on a basis of specific gravity, while size of the particle is relatively unimportant. The separation of alkaline slurries of starch by centrifuging should present no difficult problems. The smaller particle sizes found in alkaline slurries will not be as important as in tabling, and the rate of rotation, the initial density of slurry, and the rate of inflow afford greater flexibility of operation in the centrifuging than in the tabling method.

Kerr (41) observed that small quantities of dissolved electrolytes improved the separation of starch and gluten in the tabling process. This effect is greater in concentrated slurries. Separation was improved especially by phosphate ion, and was hindered by lactic acid. The pH of the slurry affects the completeness of separation. With ordinary electrolyte concentrations of 50-150 grains per gallon, the separation is best at pH 3.8-4.2, is less complete at pH 3.0 and improves again at pH 1.5.

The effect of electrolytes is easily understood in terms of their influence on the hydration of the gluten since the concentrations generally used are such as to cause salting-in and increased hydration. It is difficult, however, to understand the effect of the lactate ion, since it should tend to increase hydration (125). According to the data Kerr presents, it seems probable that the addition of the poorly-dissociated lactic acid decreases the effective

electrolyte concentration, whereas the phosphate ion is added to the system as the acid salt.

It is difficult to explain the effect of pH on the separation of starch and gluten. Near pH 3.0 the hydration of gluten should be at a maximum (125) and the difference in specific gravity between starch and gluten should be at its greatest. This statement assumes that since the ionizable groups in corn proteins are similar in nature to those in wheat proteins, the effect of pH on hydration will be similar.

The pH of the medium also has a marked effect on the rate of sedimentation of starch. Wiegel and Schüler (228) found that the sedimentation rate of potato starch increased steadily as the pH was lowered from 7.0 to 2.5. They attributed this effect to an increasing aggregation of starch granules in the more acid media, rather than to an increased density of the individual granules. In view of these observations, it seems likely that the inferior separation of starch and gluten at pH 3.0 is caused primarily by an effect on the forces of coacervation or aggregation between the starch granules and the gluten, rather than by an effect on the density of starch relative to the density of gluten.

Waste

A great deal of work has been done on the elimination of waste process materials in commercial processing. Several publications have appeared recently on the subject (229,230). Waste waters containing as little as 2-4 per cent of the dry matter of the corn

formerly were considered objectionable (231) since the effluent from many plants produces a stream pollution problem comparable with the sewage pollution from a city with a quarter to half a million inhabitants. To solve this problem a process has been developed by which as much as possible of the process waters is recirculated through the separation process and the rest is purified by evaporation.

A flow-sheet (229) of the water-balance for such a re-circulated system shows that the steep tanks contain about 9.5 gallons of water per bushel of dry corn. The proportion of water is increased greatly during crushing, and removal of the germ, and the mixture for tabling usually contains 40-45 gallons of water per bushel of corn. The total process-water loss from the factory can be reduced to 6-7 gallons per bushel of corn, of which 4-5 gallons come from the steepwater.

The water drawn from the "steeps" contains up to 6 per cent of non-volatile solids. If fermentation by acid-producing bacteria has been kept to a minimum the soluble proteins tend to coagulate as the SO_2 is drawn off; also, insoluble salts-- chiefly carbonates of calcium and magnesium -- precipitate out on the heating surfaces of the evaporators as a scale and may seriously restrict the milling capacity of the factory. Kerr advocates a controlled fermentation to produce non-volatile acids such as lactic acid, which will keep the minerals and proteins from forming scale during evaporation. It should be observed that alkaline steeping water would dissolve little of the calcium or magnesium out of the corn, or the stripper-starch. Further, in the evaporation of alkaline steepwater, the proteins

should tend less to coagulate than in acid medium.

EXPERIMENTAL SECTION

Preparation and Storage of Material

Samples of the various end-products from the wet-milling of corn starch were obtained from the Canada Starch Co., of Montreal, from their plant at Cardinal, Ontario. These fractions included five different lots of undried stripper starch, two samples of commercial corn starch and one sample of stripper starch which had been dried in a tunnel-kiln as is the usual commercial practice with all corn products except the two main grades of starch.

The stripper starch lot no. 6 was collected as corn gluten at the factory. The protein content is rather low for what is normally considered as corn gluten and in this study it has been designated as a stripper starch. It differs from the other stripper starches in its higher content of nitrogen and in the fact that when it was received from the factory it contained a considerable proportion of corn husks and fibres. A portion of the gluten was made up to a thick, paste-like slurry with water, and passed through a sieve into a large Buchner suction filter. The filtrate was used to slurry another portion of the gluten. In this manner, all husks and fibres were removed from the gluten without dissolving a significant amount of protein.

The major part of all samples of stripper starch was slurried in a mixture containing 10 per cent toluene and 90 per cent water, the former being added as a preservative against bacterial action. When

well mixed, the slurry was filtered on a large Buckner by suction. The treatment with toluene will be discussed in detail later (page 106). The filter-cakes of stripper starch containing about 70 per cent moisture, were crumbled and allowed to air dry for several hours, with frequent mixing. The samples, dried to about 45 per cent moisture content to prevent condensation on the walls of the container, were then packed loosely into wide-mouthed flasks, and stored at 5°C. The kiln-dried sample of stripper starch was in the form of large, hard lumps which were lightly ground in a mortar to pass a sieve of about 30 mesh. The corn starch was white and powdery, and needed no treatment.

Analyses of Stripper Starches

The various raw materials used in this study were analyzed for moisture content, ash, total nitrogen and amide nitrogen.

Moisture was determined by the loss in weight after drying in an oven at 103-105°C for 24-48 hours. Preliminary tests showed that drying under these conditions was about 98 per cent complete in 12 hours, and complete in 20 hours. Drying starch in vacuo, over phosphorous pentoxide at 25°C was slow and incomplete. When samples, which had already been dried in the oven at 103°C and atmospheric pressure, were further dried at 103°C in a high vacuum, they lost only 0.025-0.057 per cent of their weight. This extra loss of moisture was not considered important in this study.

Ash was determined by combusting samples in open crucibles in an muffle furnace at a bright-red heat. The samples were moistened

first, in order to cake the starch and thus reduce the chance of loss by air currents in the furnace. After 5 hours at the maximum temperature, the oven was allowed to cool partly and then the warm crucibles were transferred to a desiccator, and weighed when cool.

Total nitrogen was determined by a semi-micro modification of the Kjeldahl-Gunning-Arnold method (139). The samples were usually 100-200 mg. and repeated analyses indicated that no special precautions were necessary to obtain uniform sampling.

Amide nitrogen was determined by hydrolysis according to the conditions prescribed by Vickery et al (193) followed by the distillation procedure of Pucher et al (232) with slight modificatio: Because of the base-combining capacity of the hydrolyzed starch, it was necessary to add an extra 4.5 ml. of normal sodium hydroxide. The ammonia was distilled at 45-48°C and a pressure of approximately 50-60 mm. Hg. with no aeration, and was collected in ice cold 4 per cent boric acid solution. A few drops of caprylic alcohol were placed in the distillation flask to control frothing. Recoveries with standard solutions of ammonia were 96-90 per cent. The analyses, based on the moist crude material, are summarized in Table I.

The commercial corn starch samples have been dried in a continuous rotary drier. The stripper starch # 1 had been dried in trays in a tunnel-kiln at about 107°C for 24 hours. The moisture content of the corn starch samples, the stripper starch # 1 and the wheat flour all represent an equilibrium determined by the hygroscopic nature of the material and the temperature and humidity

TABLE I: Partial Analyses of the Types of Starch Used in This Study

Types of Starch	% Moisture	% Ash	Total Nitrogen	Amide Nitrogen as % of Total Nitrogen
corn starch (Canada Starch Co.) # 1	9.92	.0724	.0727	---
corn starch (Argo.) # 2	10.3	.055	.0755	---
stripper starch # 1	8.79	.307	1.008	12.5
stripper starch # 2	41.7	---	0.350	---
stripper starch # 3	28.1	.117	0.656	12.1
stripper starch # 4	23.9	.196	0.622	12.2
stripper starch # 5	46.1	.280	1.124	12.6
stripper starch # 6	45.0	.591	3.20	12.0
wheat flour (Ogilvie's)	8.36	.53	2.45	---

of the laboratory air. The moisture content of stripper-starch lots 2 to 6 indicates the different degrees to which the materials have been dried before they were stored in the cold. The amount of protein in the commercial corn starch samples is about 0.4 per cent (N x 5.7). The amount appearing in the stripper-starch fraction varies greatly as the operating conditions in the plant are varied. Thus stripper starch # 1 came largely from a tabling overflow, whereas stripper starches # 5 and # 6 came from two different stages in the centrifugal process for removing gluten.

The amide nitrogen content of the stripper starches shows little variation. The variation does not seem to be correlated with total nitrogen content or ash, and is probably mainly due to varietal or seasonal differences in the corn being milled.

Histochemical Study of Stripper Starch

To get a rough idea of the nature of the association between the gluten and the starch granules in the stripper starch, a visual examination was made with the microscope after applying various staining and colour reactions for proteins. The usual histochemical methods for colouring protein matter involve the use of reagents which gelatinize the starch granules, or else are suitable only for proteins which are less inert than gluten. Various modifications of some of the common staining techniques were investigated. Stripper starch # 1 was used in the initial experiments. The gluten of this sample seemed to be the most unlikely to dissociate from the starch during the treatment with staining reagents.

Staining procedures which require repeated treatments are very difficult to perform if the granules have not been mounted or fixed to the glass slide in some way. Gentle heating, in the manner used to fix moist bacteria will not fix the granules of moderately dry stripper starch. To get around this difficulty, a film of collodion in ether was spread on the surface of a glass slide and allowed to solidify until it was "tacky". The dry stripper-starch granules were then dusted onto the surface. Only a certain proportion of the granules adhered to the collodion, the loose granules being

readily washed away. The collodion base, however, was found to be unsatisfactory, because, when soaked for several hours in alkaline or acid reagents, it became detached from the slide.

A thin film of paraffin on the surface of the slide provided a better mount. The sieved starch was dusted onto a flat surface, and the paraffin-coated surface of another slide pressed onto the granules. A certain proportion of the granules became embedded sufficiently to withstand gentle staining operations.

When viewed with the microscope, the granules in a suspension of unstained starch appear to have a thin outer layer of colour. This colour varies somewhat with the colour of the illumination, and with the colour of the liquid medium when staining reagents were used. The appearance was considered to be an optical effect at the interface between starch and water. With most of the staining methods which were investigated this optical rim had a colour that would be expected from a weak staining reaction with protein. However, the changes observed in this rim as the focal plane is altered slightly, lead to the conclusion that it is an optical effect.

Haematoxylin, either alone or preceded by a mordant, did not selectively stain any part of stripper starch. Various modifications of the Biuret test gave a definite Biuret colour when viewed without the microscope, but when viewed with the microscope all the colour appeared to be in the liquid phase. Stripper starch # 2 could be soaked in 1.0 N sodium hydroxide for up to 20 minutes, or 0.2 N sodium hydroxide for several hours without gelatinizing most of the granules. The nitroprusside stain described by Lison (253) did

not indicate any protein even when the starch was left to soak for long periods in the reagents.

Millon's reagent gave a definite selective staining. The nitric acid, mercuric nitrate and sodium nitrate were used in the proportions described by Lison (233) but the long standing-periods which he recommends, did not appear to improve the staining reaction, and hence were eliminated. When dry stripper starch was treated with Millon's reagent for 1 to 2 hours, little or no staining was visible; after 4 to 6 hours the pink colour reached a maximum and faded with longer treatment. With moist stripper starches the colour developed more quickly, reaching a maximum in about one hour.

When viewed at high magnification, with oil immersion, most of the single granules were free of any protein. A few granules had small irregularly-shaped pink patches on their surfaces. In a few cases a small fibre or lump of protein could be seen with granules imbedded in the surface. The greatest proportion of the pink material appeared to be in the numerous large clumps of granules. The larger clumps appeared to be made up of 30-50 granules, cemented together by the protein granules. Typical formations are represented in figure 1 below. In no case did the protein appear to penetrate into the starch granule, or to form an envelope around it.

Moist stripper starches were stained by placing a few small lumps of the stripper starch in a pool of the Millon's reagent on a slide, and distributing the starch by gentle pressure with a spatula. The microscopic appearance of the moist stripper starches was identical with the dried stripper starch, except that slightly higher proportions

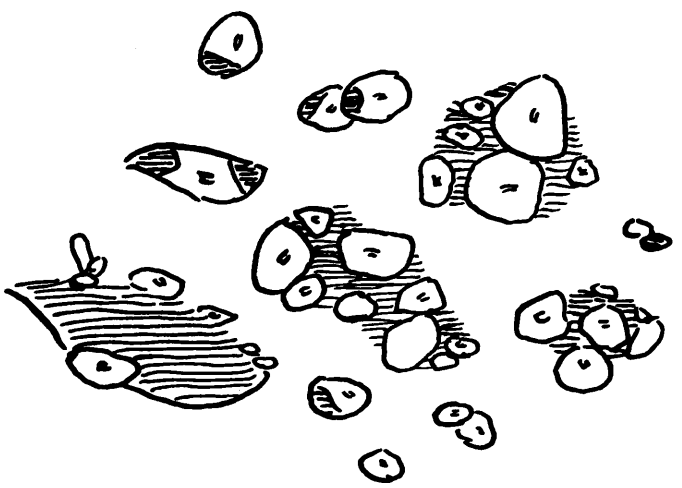


Figure 1. Microscopic Appearance of Stripper-Starch Under Oil Immersion After Staining with Millon's Reagent

of free granules and small clumps were observed in the former. These proportions were affected greatly by the thoroughness with which the original lumps were broken apart with the spatula.

The colour of the stained gluten varied from yellowish-brown to a dull pink. The contrast between the starch and the stained gluten may be improved by viewing in slightly bluish light. Occasionally, small fibres of bran may be seen, which stain a brilliant pink, i.e. they are quite different from the usual colour obtained with gluten.

The Effect of Drying on Stripper-Starch Properties

The first sample of stripper-starch (# 1) had been dried in a tunnel-kiln at 107°C at the factory at Cardinal, Ontario. The material is dried promptly in order to prevent putrefaction and fermentation. Preliminary experiments on a batch of this material showed that the gluten was very resistant to separation from the starch. Suspecting that denaturation had occurred as a result of the drying, some stripper-starch (# 2) which had not been dried was obtained and stored in the moist condition with toluene as a preservative. The following experiment was carried out to compare the two types of stripper-starch with respect to the separability of the gluten and the starch during sedimentation in aqueous media at various pH levels.

Four suspensions of 200 gm. of stripper-starch # 1 in 50 ml. of M/20 buffer were prepared. The suspensions were buffered respectively at pH 3.0 and pH 4.0 with sodium acetate, at pH 7.0 with potassium phosphate and at 9.0 with sodium borate. The mixtures were stirred continuously for 3 hours in a water bath at 40°C. They were then diluted to 180 ml. with buffer and sedimented in a vertical glass tube about 50" long x 1/2" diameter. A fine stream of air bubbles entered the tube about 4" from the bottom, and agitated the suspension just enough so that only the heavier particles settled. Over a period of two hours the rate of bubbling was gradually decreased and finally stopped, so that all the particles sedimented. The bottom section of the tube, containing the sediment was removed and

centrifuged. The supernatant was decanted, and the sedimented column of starch was extruded and divided into layers and dried. In figure 2 the nitrogen content of the layers is plotted against "mid-point" of the layer. The latter is on the abscissa which has a scale in terms of the dry weight of the material between any given point and the top of the column expressed as a percentage of the total dry weight of the sedimented column.

There was very little separation of gluten and starch at any pH used. At pH 4.0 the content of protein increases almost linearly from top to bottom of the column. This was interpreted to be evidence that gluten was associated more with the larger aggregates of granules. The shape of the curves for sedimentation at pH 3.0, pH 7.0 and pH 9.0 was interpreted as evidence that some dissociation had occurred. The high content of nitrogen at the bottom of the column probably indicates that at pH 7.0 and pH 9.0 more starch than gluten is removed from the larger aggregates.

Moist stripper-starch from lot # 2 was sedimented in a similar manner. Two suspensions, buffered at pH 4.0 with M/20 sodium acetate and at pH 8.6 with M/20 equimolar phosphate-borate mixture, were fractionated. The results are shown in figure 3.

It is evident that the gluten of the undried stripper-starch is separated much more easily than after it has been dried. The separation of gluten from starch is slightly greater at the more alkaline pH. In suspensions of moist stripper-starch, the gluten is relatively high in the largest aggregates. At pH 4.0 the smaller aggregates of granules, found in the upper half of the

Figure 2

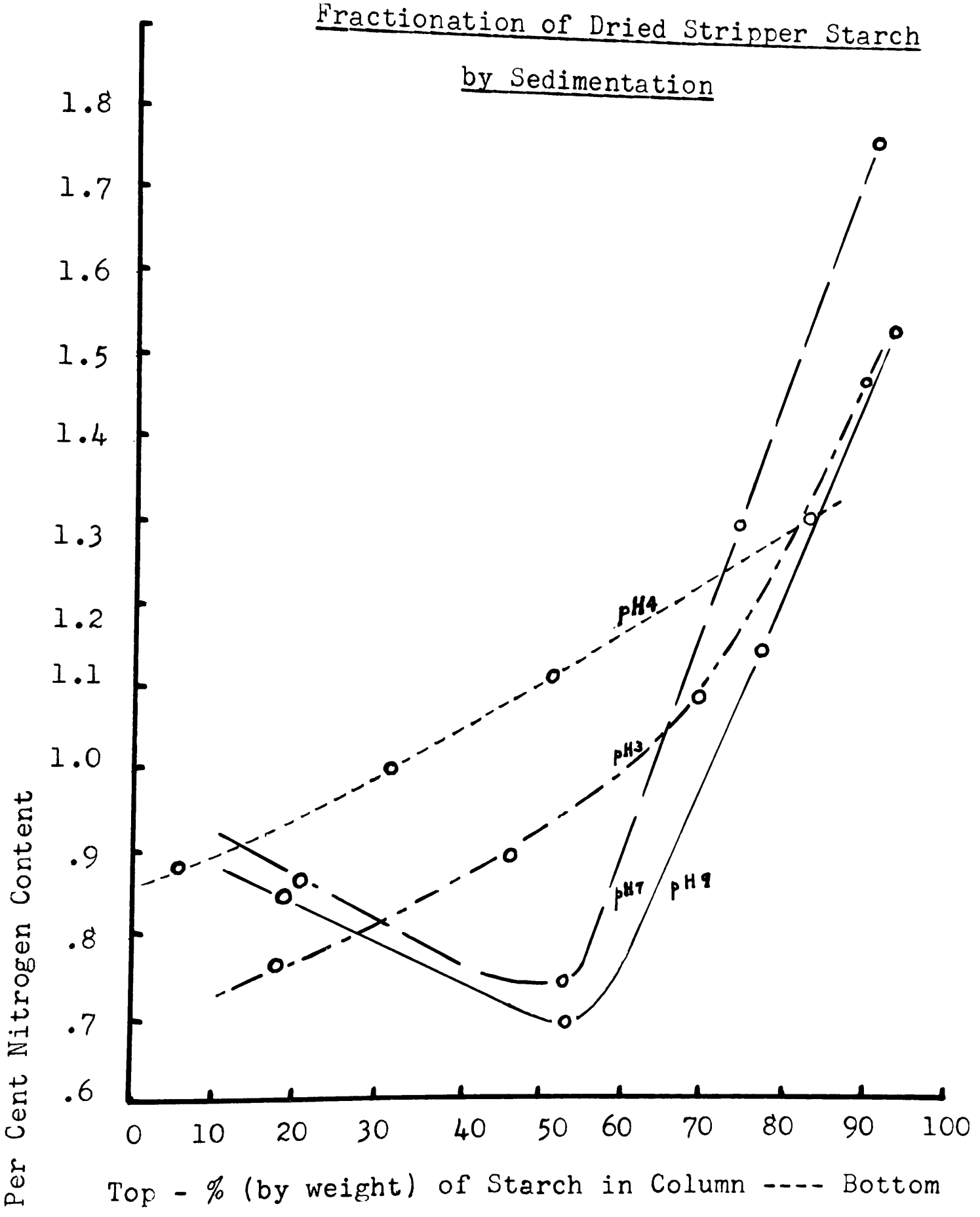
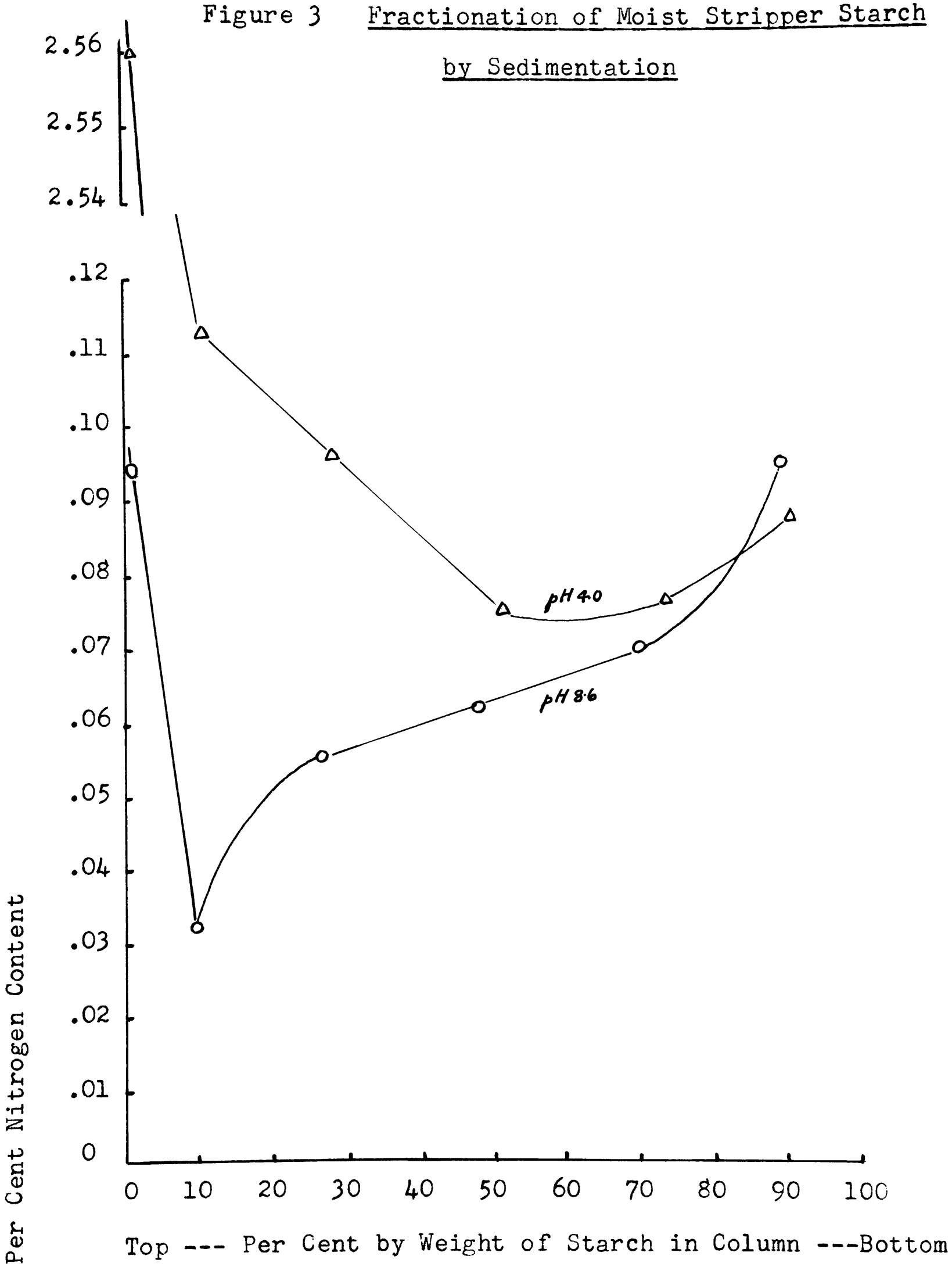

Fractionation of Dried Stripper Starch
by Sedimentation
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
.9
.8
.7
.6
pH4
pH3
pH7
pH 9
0
10
20
30
40
50
60
70
80
90
100
Per Cent Nitrogen Content
Top - % (by weight) of Starch in Column ---- Bottom

Figure 3 Fractionation of Moist Stripper Starch by Sedimentation



column contain considerable amounts of gluten. At pH 8.6 this gluten largely has dissociated from the smaller aggregates; the loss of gluten from the larger aggregates in the lower half of the column is not so marked.

The rate at which gluten dispersed in the liquid phase of a stripper-starch slurry also was greatly altered by drying. Four suspensions were prepared as shown in Table 2. The starch was calculated on the basis of 8.8 per cent moisture content. The buffers used were an equimolar solution of acetate and phosphate for pH 5.5

TABLE 2: The Effect of Drying on Dispersion of Protein in a Suspension of Stripper-Starch

	Dried Stripper-Starch # 1		Moist Stripper-Starch # 2	
	pH 5.5	pH 7.6	pH 5.5	pH 7.6
Starch per 100 ml.	20.0	20.0	20.0	20.0
Buffer conc.	.025 M	.025 M	.025 M	.025 M
Nitrogen conc. mg/100 ml.	201.5	201.5	109.3	109.3
% Total nitrogen initially soluble	7.65	9.38	8.76	15.6
% Total nitrogen soluble after steeping	8.90	11.7	9.78	20.5
Time to disperse (hrs.)	5.2	6.7	1.2	1.5

and an equimolar solution of phosphate and borate for pH 7.6. The suspensions were steeped at 40°C with constant stirring. Toluene was added to prevent bacterial growth. Aliquots were withdrawn at inter-

vals, centrifuged, and the supernatant analyzed for nitrogen. The graphs obtained from plotting these readings indicated that, in the case of starch # 1 the dispersed nitrogen increased at a constant rate, and abruptly reached a maximum and constant value. Analyses on sample # 2 were not done frequently enough to be sure of this constant rate of dispersion, but the readings fit well on such a plot. The time taken for dispersion is listed in Table 5.

It is obvious that drying greatly decreases the rate of dispersion in aqueous medium. At the time that this experiment was carried out, the necessity of keeping the concentration of undispersed nitrogen constant when comparing the solubilities of complex protein systems was not appreciated. Therefore, by this experiment it is impossible to estimate the effect of drying on the absolute solubility of the gluten. By comparing the ratios of nitrogen dispersed at pH 7.6: nitrogen dispersed at pH 5.5 it is obvious that the relation of solubility to pH has been altered by drying.

The Use of Air-Dried Stripper-Starch

In view of the observation that drying had drastically denatured the gluten of stripper-starch # 1, it became of interest to determine whether stripper-starch could be dried in the laboratory air without denaturation. Wheat gluten is apparently not denatured by a similar drying process. If stripper-starch could be air-dried in this manner, storage and handling procedures would be much simplified.

Three portions of moist stripper starch # 2 were weighed accurately onto watch glasses and left to dry in the laboratory air.

After 48 hours, they had lost 31.7 -32.5 per cent moisture (wet basis). The air-dried stripper-starch therefore ranged in moisture content from 13.6 to 14.6 per cent. Three samples of stripper-starch # 2 on watch-glasses were slurried with varying amounts of water to give paste-like mixtures. These were left to dry in the laboratory air for 50 hours, and had a final content of 14.0-14.2 per cent moisture. These samples were weighed at intervals during the next month. During this period there was a slow decrease in weight, and later an increase. The maximum changes observed were 0.34-0.44 per cent of the weight, or 2.4-3.1 per cent of the moisture.

From these observations it appears that stripper-starch could be used in an air-dried form as long as accuracy greater than 0.5 per cent was not required. One disadvantage is that drying periods of 48 hours would be required whenever the starch was recovered from suspensions, but the final moisture content seems to be reproducible to $\pm$ 0.5 per cent.

The effect of air-drying on the solubility of gluten was investigated. Samples of stripper-starch were thoroughly dried in the laboratory air, or in an oven at 100°C. Aliquots containing 50.0 mg. of nitrogen were selected from the moist stripper-starch and from both of the above-mentioned dried forms. These were suspended in 100 ml. of 5 per cent potassium sulphate solution. The suspensions were agitated gently for 4-6 hours at room temperature, then centrifuged for 30 minutes at 1500 r.p.m., and the supernatant removed with a pipette. Salts were removed from the solid phase by shaking the latter for

a few minutes with 100 ml. of water followed by centrifuging at 1500 r.p.m. for 30 minutes. The washed residue was then suspended in 50 per cent aqueous ethanol, and the suspensions were shaken for 1 hour, left to stand overnight and centrifuged as above.

TABLE 3: The Effect of Drying on the Solubility of Gluten

Lot No. of Stripper-Starch	Drying Procedure		% Total Nitrogen	
	Temp. °C	Time Days	Soluble in Saline	Soluble in Alcohol
# 4	not dried		17.5 17.5	15.9 12.5
"	25	7	17.8 18.3	12.7 13.8
"	100	2	16.5 17.5	10.6 8.9
# 6	not dried		12.1 11.7	18.1 16.9
"	25	11	11.9 11.7	13.4 14.3
"	100	4	9.60 10.7	11.0 12.0
# 6	not dried		9.82	24.3
"	25	25	9.63	22.4
"	100	5	8.05	18.0

The results from these experiments are collected in Table 3. The figures show that the protein which is soluble in 5 per cent potassium sulphate is not affected by the drying in the air, but is

slightly affected by drying at 100°C for the longer periods. The prolamine fraction is slightly decreased by drying in air, and greatly decreased by heating at 100°C.

Drying also affects the coloured components of stripper-starch. The intense yellow colour of fresh, moist stripper-starch is mostly lost when the material is dried in the air or in a furnace, and is not regained upon subsequent moistening. With the fresh stripper-starch, the colour is extractable to a considerable degree with water and especially with aqueous alcohol, but not with ether. After drying, very little of the colour is extractable by water or alcohol. It is probable, therefore, that the various components of the colour are part of a protein complex, and both solubility and colour are destroyed partially by drying.

Storage of Stripper-Starch

Several methods of storing stripper-starch were investigated. It seems desirable to store gluten and stripper-starch without drying them by any of the usual procedures, since these procedures are all known to cause some degree of denaturation of the gluten. It is necessary however, to prevent the action of the bacteria, moulds and enzymes which are certain to be present in the crude material.

Method 1. Moist crude stripper-starch (86 gms.) was thoroughly mixed with anhydrous sodium sulphate (80 gms.) and stored in a tightly capped bottle at room temperature for several months. At the end of this period, there was no odour and no growth of mould was detectable.

To remove the salt, the mixture was slurried in 200 ml. of distilled water, allowed to stand for 15 minutes and filtered. After several extractions in this manner, the filtrate gave only a faint test for sulphate ion. Kjeldahl analysis of the collected filtrates showed that 1.5-2.0 per cent of the initial nitrogen had also been extracted. Hence, this method of storage permits almost complete recovery of the corn-protein system. However, washing out the salt is tedious, and the dry protein may be partially denatured.

Method 2. Crude, moist stripper-starch (40 gm.) was covered with 15 ml. of 40 per cent sucrose solution. After two weeks, strong alcoholic fermentation was evident. Since the starch was found to contain 41.7 per cent moisture (wet basis), the sucrose was undoubtedly diluted below an effective bacteriostatic level. Any suspension or solution of gluten prepared from corn endosperm will contain appreciable amounts of carbohydrate, which must be removed or taken into consideration. The use of carbohydrate as a bacteriostatic agent which need not be removed is therefore attractive. This method was not investigated further, however, because the syrupy suspension makes quantitative procedures extremely difficult.

Method 3. Moist crude stripper-starch was stored at 5°C in a tightly-capped bottle. Filter-cake usually contains enough moisture that condensate forms on the walls of the bottle. This may be avoided by crumbling the filter-cake, and leaving it to dry in the air to a moisture content of about 45 per cent (wet basis) before storing. After 2-3 weeks at 5°C, the odour indicated that the stripper-starch had become definitely more mouldy, and many of the

lumps in the semi-powdered stripper-starch did not disintegrate easily when pressed lightly with a spatula. After about 6 weeks a green, brown, or red growth of mould became noticeable both in the centre of lumps and on their surfaces.

Method 4. Toluene is a common bacteriostatic agent, and because of its inert and volatile nature it is unnecessary, ordinarily, to remove it from the system. Furthermore, no denaturation of gluten should occur from its use.

Successive thin layers of moist stripper-starch were loosely packed in a bottle. Each layer was sprayed with a little toluene, so that the mixture had a strong odour of toluene. When stoppered and stored at 5°C, the stripper-starch seemed to keep slightly better than when no toluene vapour was present.

A slurry containing approximately 40 per cent stripper-starch (dry basis) was stirred vigorously and toluene added to the surface. Enough toluene was added to give a final concentration of 5 per cent, and was rapidly incorporated into the slurry. When the slurry was filtered by suction, a greenish, clear filtrate was obtained. The yellow filter-cake had a strong, yeasty odour quite different from that of the original stripper-starch or of the stripper-starch which was sprayed with toluene. During the drying period, much of the yeasty odour disappeared and the odour of toluene became more evident. When stored in a bottle at 5°C, this material kept for 2-3 months without any sign of mould. The yeasty odour and the toluene odour both gradually decreased, however, and after about 4 months a growth of mould was visible.

The loss of protein during the treatment with toluene is small. Three lots of stripper-starch were made into slurries containing 2-3 gm. nitrogen per litre, and the loss of protein in the filtrates represented only 2.85-3.35 per cent of the original protein. The batch of stripper-starch may be divided into several portions, and the filtrate from one portion used to slurry the next. The loss of protein is then less than 2 per cent.

A sample of stripper-starch was treated with toluene and then allowed to stand for several periods at 5°C or at 25°C. Formol titrations were carried out on aliquots at the end of each period. The use of the formol titration with material such as stripper-starch is discussed in Appendix I. The results are summarized in Table 4.

TABLE 4: Changes in Stripper-Starch During Storage

Length of Storage Period	Temp. during Storage Period	Formol Titration at End of Storage Period
Days	°C	m. eq. NaOH/gm. starch
0		.0713
8	5	.0703
3	25	.0832
27	5	.0805
14	5	.0845
14	25	.2605

It is evident that there is a definite increase in titrable groups when stored at 25°C for even a short time. The final 14 day storage period at 25°C resulted in a heavy moist growth of mould. In this

case the sample was dried in the laboratory air, ground, and an aliquot taken. No definite changes in titration value at the end of the cold-storage periods can be deduced; the variations shown in the table are of the same order of magnitude as differences between checks, and probably represent the difficulty of obtaining representative samples from the lumpy starch.

Two portions of a stripper-starch sample were stored at 5^{o}C; one lot had been treated with toluene. At the end of 10 weeks there was still a strong yeasty smell to the sample treated with toluene; there was no lumpiness, odour or appearance of mould in the starch. The other sample had a strong smell of mould, was quite lumpy, and careful inspection showed some small greenish spots on the surfaces of the lumps.

It was concluded that stripper-starch may be stored at 5^{o}C for periods of several weeks, after treatment with toluene, without significant attack by moulds or proteolytic bacteria.

Nitrogen Distribution in Stripper-Starch

The histochemical study indicated that the proportion of gluten to starch in the various clumps varied greatly. The following fractionation according to specific gravity was performed on stripper-starch # 2 in order to grade both the free granules and the clumps according to their nitrogen content.

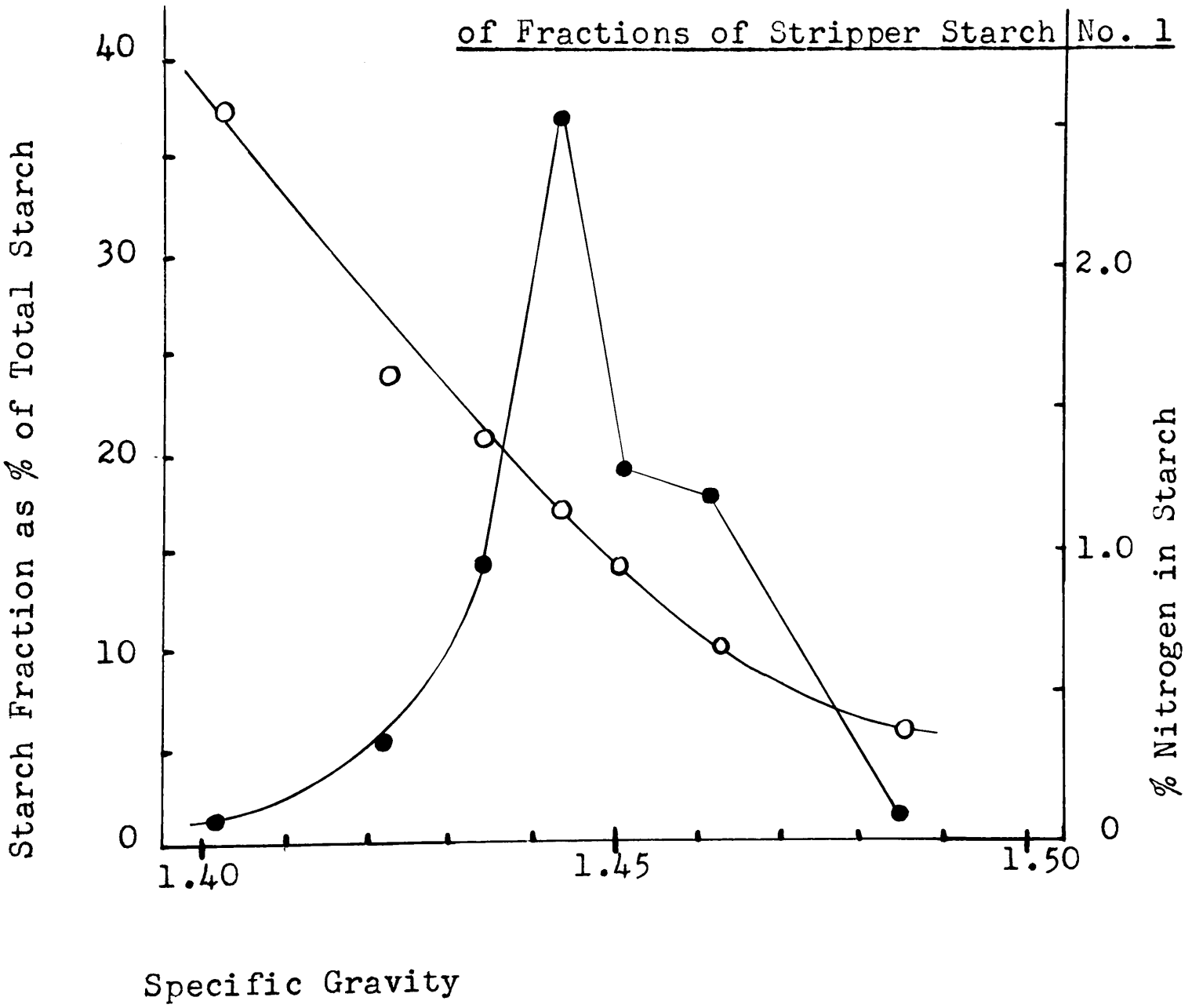
Samples of sieved stripper-starch # 1 were suspended in carbon tetrachloride, in tetrachloroethane and in chloroform respectively, for several hours. In the first two liquids all of

the starch in the sample floated. In chloroform, a small amount of the sample precipitated, and the rest floated.

About 9.0 gm. of sieved stripper-starch # 1 were suspended in 50 ml. of chloroform in a stoppered separatory funnel and shaken vigorously for 5 minutes. After standing for 30 minutes, most of the sample had floated to the top of the liquid. The cloudy liquid was withdrawn and clarified by centrifuging in a corked tube. The clear liquid was then returned to the separatory funnel, and the precipitated solids were dried in the oven. A second fraction was precipitated by carefully adding ethyl ether from a burette until part of the starch was observed to sink. The mixture was then shaken vigorously and the fractionation repeated as before. In this manner the original sample was divided into seven fractions. The specific gravity of the solvents was checked by pyknometer, and the specific gravity, at which each fraction precipitated, was calculated. The results are shown in figure 4. In a control experiment with commercial corn starch, fractionated in carbon tetrachloride-ethyl ether mixtures, all the starch precipitated between specific gravity 1.51 and 1.53. Of this sample 80 per cent precipitated sharply at specific gravity 1.52.

From the data of figure 4, it is apparent that over 75 per cent of the starch in the stripper-starch # 1 contains 3.4-6.8 per cent gluten (N x5.7). This observed distribution of nitrogen in the starch refers only to the aggregates which have been produced by kiln-drying, followed by gentle grinding and sieving. It is difficult to explain how these mechanical operations could produce such a distribution,

Figure 4 Specific Gravity and Nitrogen Content of Fractions of Stripper Starch No. 1



however, and it seems reasonable to conclude that they represent a somewhat similar distribution of nitrogen in the clumps found in moist stripper-starch.

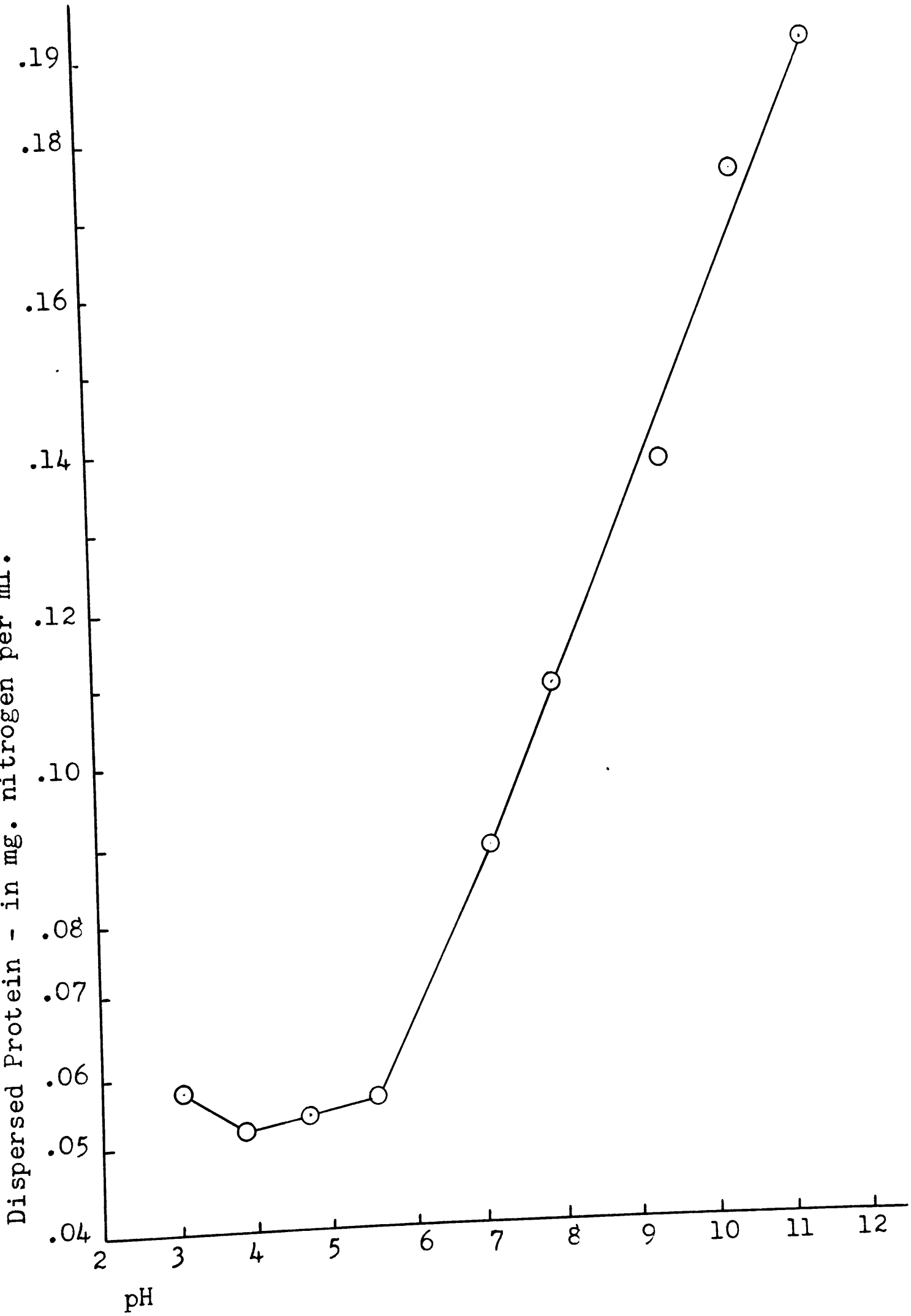
The Effect of pH on the Solubility of Corn Gluten

In an aqueous suspension of stripper-starch, such as an enzyme digest, or a mixture for steeping, a small proportion of the gluten will dissolve. The following experiment was performed to determine the effect of pH on the solubility of the most soluble fraction of corn proteins.

Into each of nine 50 ml. flasks was placed 5.00 gm. of moist stripper-starch of lot # 2 and 35 ml. of distilled water. The flasks were shaken thoroughly, and adjusted to various pH's between 3 and 11 with 0.5 N hydrochloric acid or 0.5 N sodium hydroxide. The pH levels were measured with a glass electrode (Beckman). The flasks were shaken continuously for 10-12 hours at room temperature, and sodium hydroxide added at intervals to adjust for the slow uptake of alkali. The slurry from each flask was centrifuged for 35 minutes; the supernatant was decanted, and a 5 ml. aliquot analyzed for nitrogen.

The results are plotted in figure 5 and show a low and nearly constant solubility in the range from pH 3.5 to pH 5.5. In more alkaline solutions the solubility increases sharply. The increase is approximately linear with respect to pH, up to pH 11. It should be emphasized that a more-or-less different solubility curve would be expected when the concentration of total nitrogen is

Figure 2 Solubility of Corn Proteins at Different pH Levels



lower than that used in this experiment.

Comparison of Solubility of the Proteins of Different Lots of Stripper-Starch

The technique for the division of corn gluten into a fraction soluble in 5 per cent potassium sulphate, and another fraction soluble in 50 per cent aqueous ethanol has already been described (page 104). Following the same procedure, the different types of stripper-starch used in this study have been fractionated, and the collected results are presented in the table below.

TABLE 5: The Fractionation of Different Types of Stripper-Starch According to Solubility

Lot No. of Stripper-Starch	Total Nitrogen Content	Nitrogen Distribution in Fractions as % of total Nitrogen		Insoluble Residue as % of Nitrogen Insoluble in Salt Solution
		Soluble in Salt Solution	Soluble in Aq. Ethanol	
2	.350	22.7	21.7	71.9
4	.622	17.5	(15.8)	(81)
5	1.124	13.8	26.9	68.8
6	3.20	10.5	25.3	71.5

The only systematic difference observed between the different lots of stripper-starch is in the proportion of nitrogen that is soluble in 5 per cent potassium sulphate. This fraction is a lower proportion of the total proteins in the stripper-starch lots with higher total content of nitrogen. A considerable variability was observed in the

nitrogen distribution even in the case of duplicate fractionations, performed simultaneously, and the differences observed in proportion of alcohol-soluble nitrogen do not warrant any conclusion. The figure for the alcohol-soluble fraction of the protein of stripper-starch of lot # 4 is probably erroneous - the result of faulty technique.

It is noteworthy that, in the protein residue left after extraction with salt solution, the alcohol and alcohol-insoluble portions are relatively constant. This conclusion has been established for wheat protein also.

The Exhaustive Extraction of Stripper-Starch

A process suggested for the wet-milling of wheat (223) advocates the removal of gluten by dispersion in alkali. The following experiment was performed to gain some idea of the ease of removal of proteins from stripper-starch by alkali, with and without added electrolyte.

Two mixtures containing 6.00 gm. of moist stripper-starch of lot # 2 and 100 ml. of distilled water were prepared in each of two flasks; one mixture was left at pH 4.1 and the other was adjusted to pH 10.0 with 0.5 N sodium hydroxide. Two similar mixtures were prepared using 5 per cent potassium sulphate solution instead of distilled water. The four flasks were shaken by machine for 8 hours at room temperature. They were then centrifuged, the supernatants were decanted and aliquots were analyzed for nitrogen. The aqueous extraction was repeated five times. The residues from the mixtures with potassium sulphate were then shaken continuously in neutral 60 per cent aqueous ethanol for 10-12 hours. The mixtures were

centrifuged and aliquots of the supernatants were analyzed for nitrogen. The alcoholic extraction was repeated three times. The results are summarized in table 6 below.

TABLE 6: The Dispersion of Nitrogen of Stripper-Starch by Repeated Extractions

% of Total Nitrogen Dispersed	Extraction in water		Extraction with 5% Potassium Sulphate	
	pH 4	pH 10	pH 4	pH 10
By One Extraction	5.05	29.6	17.8	23.0
By Three Extractions	7.60	45.7	31.2	32.5
By Five Extractions	--	56.1	40.5	39.1
Residues Extracted with 60% Ethanol				
By One Extraction			34.1	34.9
By Three Extractions			41.5	42.0

It is interesting to observe that, compared to wheat gluten, the corn proteins are resistant to extraction with alkali at pH 10, even when they have not been dried. While it is well known that salts decrease the solubility of proteins in alkali, it is interesting to observe that in 5 per cent potassium sulphate, the pH of the medium has very little effect on solubility. The division of gluten into salt-soluble, alcohol-soluble and insoluble fractions is accomplished usually by a single extraction with each solvent. It is obvious from the results above, that a single extraction gives very incomplete extraction.

The removal of protein from stripper-starch extraction with alkali, in concentrations safely below the level which produces gelatinization, does not seem promising as a basis for a commercial process.

Enzymatic Digestion of the Protein of Stripper-Starch

The purpose of these experiments was to explore the possibility of removing the gluten of stripper-starch by enzyme hydrolysis without affecting the starch. Moderate amounts of powdered trypsin ("Trypsin AG" of Takamine Laboratory, Clifton, New Jersey, U.S.A.) and powdered papain (Merck & Co., Montreal) and a very small amount of powdered ficin (Merck & Co., Montreal) were obtained.

Each enzyme was suspended by adding approximately 100 mg. to 90 ml. of buffer solution, and dispering the mixture for 1 minute in a Waring Blendor. The mixing bowl of the Waring Blendor was washed out with 10 ml. of buffer, and the wash solution added to the buffer. Papain and ficin were buffered with M/20 sodium acetate at pH 5.0, trypsin was buffered at pH 8.0 with M/20 dibasic potassium phosphate. To the papain suspension was added 2 ml. of 0.5 M potassium cyanide, and the suspension was warmed for 1.5 hours at 40°C.

Digests with a total volume of 250 ml. were made up containing 80 gm. moist stripper-starch (3, and 25 ml. of enzyme suspension, and buffer to give a final concentration of twentieth molar. Toluene was added as a bacteriostatic agent. These suspensions were

stirred continuously in closed digest bottles at 40°C in a water bath. Aliquots were taken when first mixed, after 5 hours, and after 8 1/2 hours. Free-amino nitrogen in the supernatants was measured by formol titration (trypsin digest) or Van Slyke deamination technique (papain and ficin digests).

TABLE 7: Hydrolysis or the Protein of Stripper-Starch by Enzymes

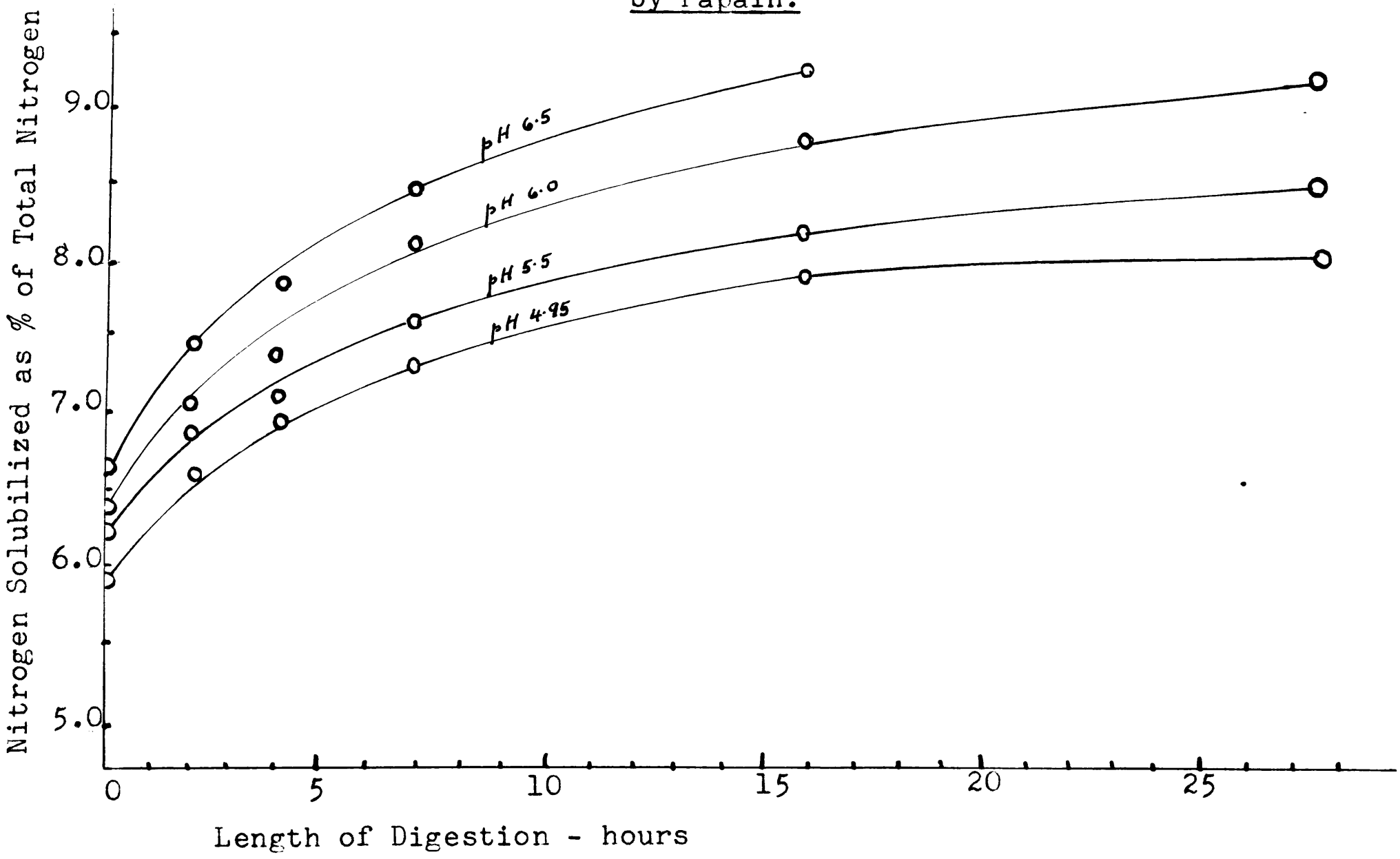
	Free Amino Nitrogen m.eq. per 100 ml.		
Enzyme	0 hrs.	5 hrs.	8.5 hrs.
Trypsin	.262	.560	.688
Papain	.264	.364	.422
Ficin	.264	.497	.618

The three suspensions of crude enzyme all hydrolyze gluten at approximately the same rate and to the same extent.

Papain was selected for a study of the amount of gluten that could be solubilized by a prolonged treatment with enzyme. Preliminary experiments showed that the presence of cyanide did not inhibit acid-producing organisms which, at 40°C for 25-30 hours, produced large amounts of acid. Sodium fluoride was used to inhibit the activity of the acid-forming bacteria.

A suspension containing 1 per cent powdered papain was prepared as described in the preceding experiment. Four 240 ml. suspensions, each containing 78.7 gm. of stripper-starch N 3, and approximately M/5 buffer, were adjusted to pH 4.95, 5.5, 6.0 and 6.5 respectively. The buffer was an equimolar mixture of sodium acetate

Figure 6 The Solubilization of the Nitrogen of Stripper-starch by Papain.



and monobasic potassium phosphate. These suspensions were stirred continuously in a water-bath at 40°C for 3 hours to saturate the liquid phase with protein (see page 102). Then 10 ml. of freshly prepared papain suspension and 0.25 gm. of sodium fluoride was added to each digest. At intervals 30.0 ml. aliquots were withdrawn and centrifuged for 15 minutes. The volume of the supernatant was 20.0 ml., and 5 ml. aliquots were analyzed for total nitrogen. The per cent of total nitrogen solubilized was calculated, and the results are plotted in figure 6.

The optimum pH of papain for proteolysis is usually about pH 4.2-5.5. It is possible that the increased degree of proteolysis observed in this experiment at pH levels up to 6.5 indicates that the optimum pH for this system is outside the usual range. It is more probable, however, that the increased degree of proteolysis is due to the greater solubility of gluten or its products at higher pH levels. The reaction in each digest was close to completion at the end of 28 hours; the proportion of nitrogen solubilized was only 8-9 per cent of the total nitrogen. No further experiments with enzymatic digestion were performed, since complete removal of protein by this method did not appear to be commercially practicable or economical.

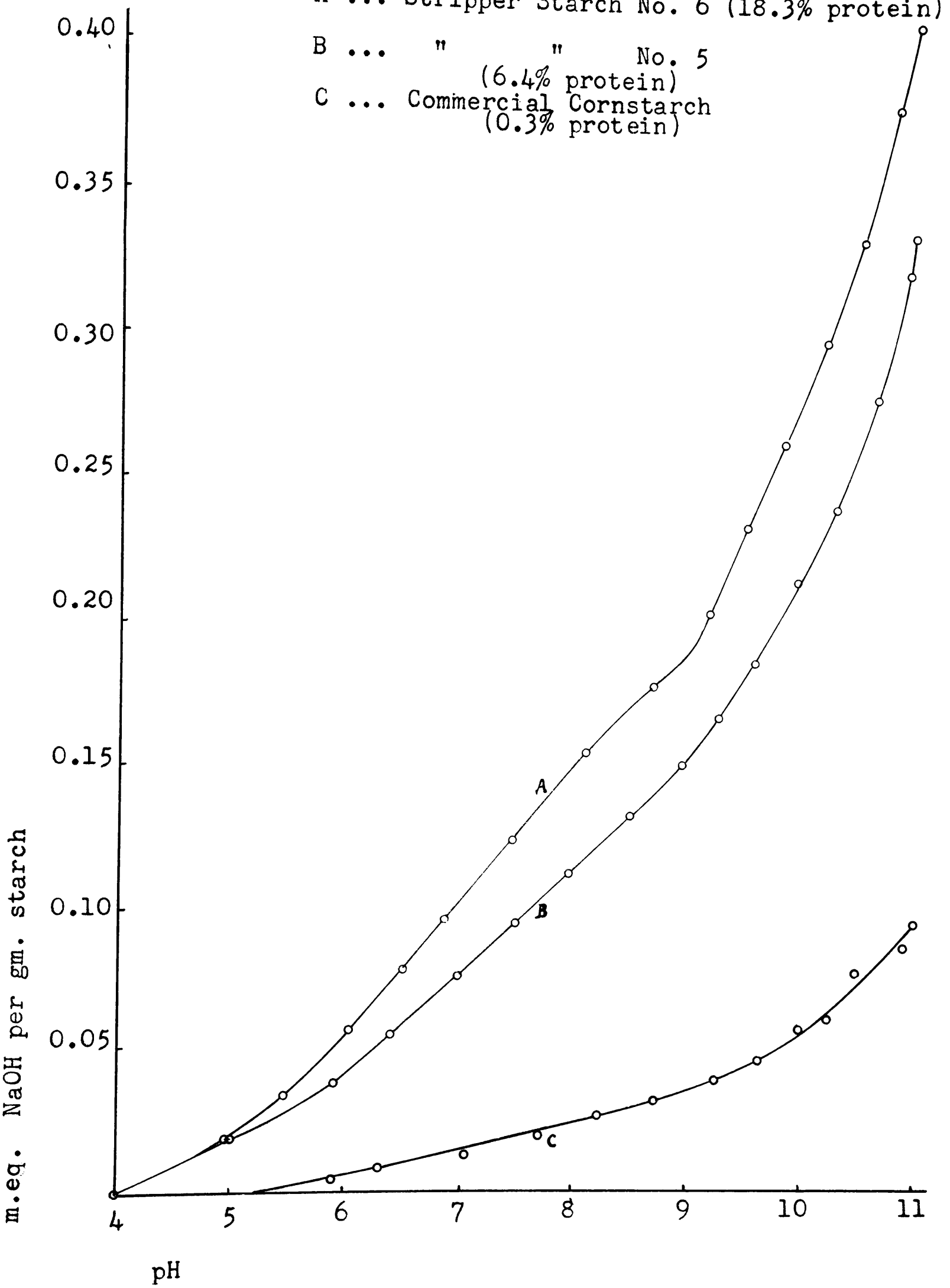
The Base-Combining Power of Stripper-Starch

When alkali is added to an aqueous suspension of stripper-starch, a large proportion of the alkali is quickly "bound" by the protein. This rapid uptake of alkali occurs presumably by interaction with free, dissociable groups in the protein molecule. The pH, which results from the initial addition of base, undergoes a gradual decrease, indicating a relatively slow hydrolysis, with the liberation of acid groups. The following experiments were designed to differentiate as much as possible these two effects, and determine their importance in the removal of the corn gluten from stripper-starch, and in the peptization of the free gluten.

Three suspensions containing 5.00 gms. starch (dry basis) and 20.0 ml. total volume of water were titrated with approximately N/10 alkali. The three suspensions contained respectively, commercial corn starch, stripper starch # 5 and stripper starch # 6 and therefore contained different amounts of protein. The suspensions were all adjusted to pH 4.40. Each was then titrated by adding from a burette, at two-minute intervals, enough alkali to give an increase of about 0.3-0.4 of a pH unit. After each addition the pH of the solution was measured. The results are plotted in figure 7.

Corn starch binds relatively little base. The stripper-starch of lots # 5 and 6 bind a considerably greater amount of alkali at any given pH, Approximately 4.5 times as much protein is present in the suspension of stripper-starch # 6 as in the suspension of stripper-starch # 5, so that the increased alkali uptake is not at all proportional to the nitrogen content. The increased uptake

Figure 7 Base Combining Capacity of Stripper Starch



probably is nearly proportional to the exposed dissociable groups in the protein, and must be influenced to a considerable degree by the surface area of the gluten particles.

It is interesting to observe that, except with the stripper-starch of lot # 6, there is no irregularity of the titration curve in the region pH 6 to pH 8.5. This indicates that only a small part of the alkali is bound by free phosphate groups.

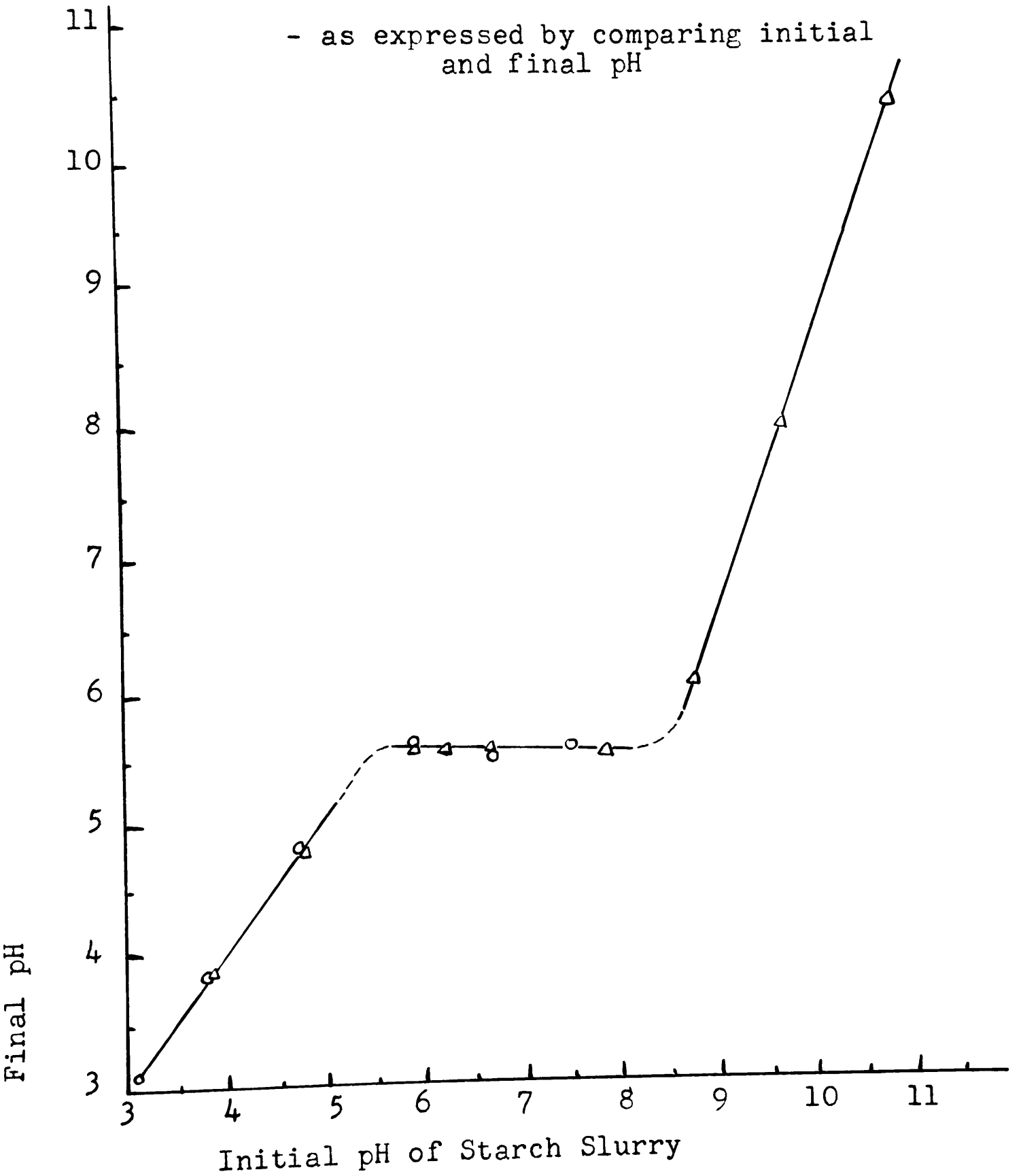
When a stripper-starch suspension was brought to an alkaline pH and allowed to stand, the pH decreased slowly. This decrease did not appear to be proportional to the initial pH. The following experiment was performed to investigate the relation between initial and final pH.

Into each of several 50 ml. flasks was placed 5.00 gm. of moist stripper-starch of lot # 2 and 35 ml. of distilled water. The suspensions were adjusted to various pH levels between 3.5 and 11 with 0.5 N hydrochloric acid or 0.5 N sodium hydroxide. The pH levels were measured with a glass electrode (Beckman). The pH was determined again after shaking continuously for 10-12 hours. The results from two such experiments, plotted in figure 8, show good agreement.

There is no gradual uptake of alkali below pH 5.5. At initial levels between 5.5 and 8.5 a gradual uptake of alkali occurs and stops sharply when the pH has been reduced to pH 5.5. The relatively sharp break in the curve at pH 8.5 indicates that there is a maximum amount of alkali which can be taken up completely, and when more than this amount is present, the excess alkali raises

Figure 8 Absorption of Alkali by Stripper Starch During Steeping

- as expressed by comparing initial and final pH



the pH above 5.5. The sharpness of the break at pH 8.5 also indicates that 10-12 hours is more than sufficient to complete the reaction.

The interpretation of this curve is made difficult by the fact that no record was kept of the amount of alkali initially added to the system. However, from the data in figure 7, it is justifiable to assume that in the region of pH 5.5 to pH 9.0, the increase of alkali in the system is directly proportional to the increase of initial pH. The lack of data just at the points of inflection at pH 5.5 and pH 8.5 is regrettable also. The theoretical significance of this curve will be discussed and correlated with the other experiments in a later section.

In the above section, no control experiment was performed with an equivalent amount of protein-free starch. To differentiate between amount of alkali taken up by the starch and amount taken up by the protein, the following simple experiment was performed. Three suspensions, each containing 5.00 gm. of commercial corn-starch (dry basis) and a total of 20.0 ml. of water, were adjusted quickly to pH 7.07, pH 8.60 and pH 10.65 respectively, with 1 N sodium hydroxide. Also, three suspensions were made up from stripper-starch lot # 5, to contain 500 gm. of starch (dry basis), 20.0 ml. of water, and 0.669 gm. of corn protein. These suspensions were also adjusted quickly to pH 7.07, pH 8.60, and pH 10.65 respectively. At intervals during the next 30 hours, the suspensions were shaken well and N/10 sodium hydroxide added from a burette to maintain a constant pH. As shown in table 8, the gradual uptake of alkali

TABLE 8: The Slow Uptake of Alkali by Stripper-Starch

pH	Uptake of Alkali by Stripper-Starch	Uptake of Alkali by Commercial Corn-Starch	Uptake of Alkali by Corn Protein
	M.eq./gm. starch	M.eq./gm. starch	M.eq./gm. protein
7.07	.0124	.00515	.0109
8.60	.0325	.00615	.0394
10.65	.0690	.0295	.0987

appears to be a function mainly of some component of the stripper-starch which has been removed from the commercial corn starch. It seems probable that this component is the protein, and the slow alkali uptake per gram of corn protein has been calculated for the three levels of pH.

Alkali-Steeping of Stripper-Starch

In an early section of this study, data were presented (figures 2 and 3) showing a better separation of gluten and starch in alkaline than in acid medium. With fresh moist stripper-starch of lot # 2, steeping in buffer at pH 8.6 (figure 3) resulted in a column with the lower 90 per cent of the starch averaging less than 0.4 per cent protein. This part of the starch would be acceptable commercially as "prime starch".

Preliminary experiments showed that steeping at an alkaline pH with no buffer or added electrolyte gave a good recovery of starch low in nitrogen. Suspensions containing moist stripper-starch from lot # 2 were steeped at pH 10-10.5 for 4 hours, at 22°C and at 40°C respectively. After steeping, the solids were

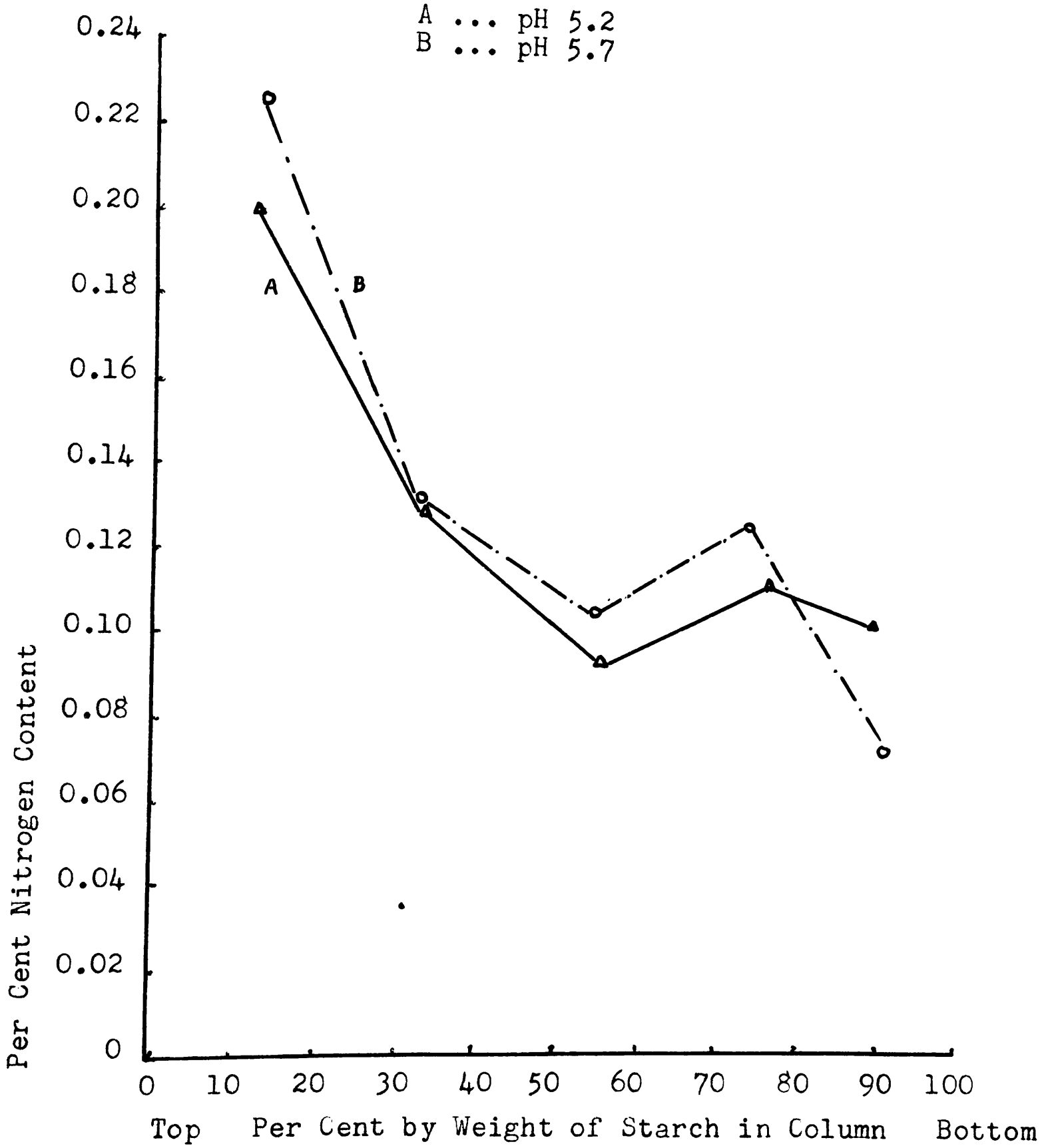
washed with three 100 ml. portions of water at pH 10. From the suspensions steeped at 22°C, 55 per cent of the original starch was recovered with a nitrogen content of only .076 per cent; from the suspensions steeped at 40°C, 72 per cent of the starch was recovered with a nitrogen content of only .065 per cent.

The Sedimentation of Alkali-Steeped Starch

In view of the large uptake of alkali observed at pH 5.5 it appeared possible that the binding of alkali, by stripper-starch, may considerably improve the separation of gluten even when the suspension is not maintained at a pH more alkaline than pH 5.5. It is obvious that in a commercial process, a very important saving of alkali would be effected. Each of two large centrifuge tubes was filled with a suspension of 5.00 gm. of moist stripper starch from lot # 4, and 20 ml. of water. The suspensions were adjusted to approximately pH 9.0 and steeped at 50°C for 4 hours. The final pH's were 5.2 and 5.7 respectively. The solids were then washed by centrifuging in 3 portions of distilled water. The sedimented columns of starch from the last centrifuging were removed carefully in layers which were dried separately. In figure 9, the nitrogen distribution in the different layers has been plotted against the mid-point of the layer, in the manner explained on page 101.

The slight difference in nitrogen distribution between the two columns is probably due to the slight difference in the final pH levels after steeping. This conclusion was supported by

Figure 9 Nitrogen Distribution in Columns of Sedimented Starch with Added Alkali



the observation that the material which caused turbidity in both supernatants settled out in a few hours at pH 5.2 but remained in suspension for several days at pH 5.7.

From figure 9, it is evident that only a small fraction of the starch contains less than 0.10-0.12 per cent nitrogen. This is almost twice the nitrogen content that is acceptable in commercial corn starch. It appears, therefore, that simple uptake of alkali by the stripper-starch with separation and washing at pH levels near neutrality will not adequately remove the gluten. In later work a constant pH was always maintained during steeping.

The Effect of Steeping Conditions on Separation of Starch and Gluten

The effect of pH, time, and type of alkali on the separation of starch from gluten in stripper-starch was studied. In a commercial process, the steeped stripper-starch would be centrifuged or tabled as a heavy slurry, and the fraction low in nitrogen would be washed thoroughly later to remove soluble protein. Therefore, in the comparative studies which follow, the steeping mixture is sedimented and analyzed for nitrogen distribution without washing out the soluble protein.

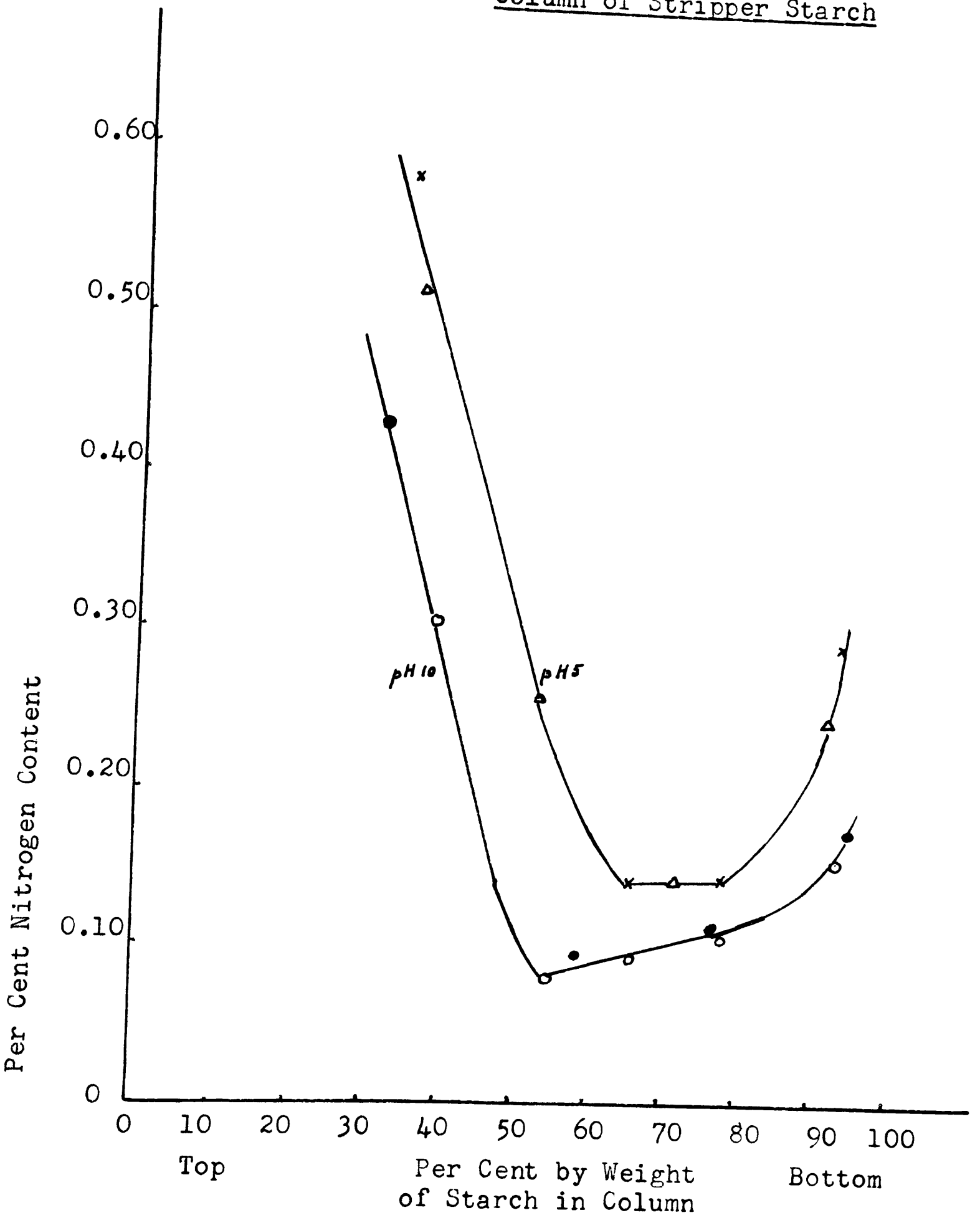
For the subsequent experiments involving sedimentation, special centrifuge tubes were used. These consisted of a transparent plastic cylinder 15 cm. long by 1.5 cm. diameter, fitted with a rubber stopper at each end. When a suspension was centrifuged in one of these tubes the solids were packed tightly against one stopper and the supernatant could be decanted from the other end.

If desired, the stopper in contact with the starch could be replaced by a small disc of filter paper and a stopper with a hole through the center, so that by further centrifuging most of the remaining moisture could be removed from the starch column. Whether the starch was moist or semi-dry, the stopper adjoining it could be removed, and the column extruded onto a glass plate, and cut into layers.

When a stripper-starch suspension was sedimented in one of these centrifuge cylinders the horizontal bands of colour which were observed through the transparent wall of the cylinder were usually quite irregular in shape. This irregularity appeared to be formed mainly in centrifuging. If the centrifuge reached maximum speed quickly, or if the suspension was allowed to settle part way in the vertical tubes by gravity alone, before packing the sediment by centrifuging, the bands were much more regular. During sedimentation it is desirable to insulate the tubes from temperature gradients which set up convection currents in the suspension. The irregularities exist mainly on the surface; when the extruded column is cut into layers the interior is quite uniform.

Four suspensions, each containing 5.00 gm. of moist stripper-starch from lot # 4 and 20 ml. of distilled water, were mixed in these tubes, and two were adjusted to pH 4.0, the other pair to pH 10.0. The suspensions were steeped for 5 1/2 hours at 40°C, and changes in pH were corrected at intervals. The suspensions were then sedimented carefully, cut into layers, dried and analyzed. In figure 10 the nitrogen contents of the different layers are plotted as described on page 101. The points for each pair of suspensions show excellent

Figure 10 Nitrogen Distribution in Sedimented Column of Stripper Starch



reproduceability. The greater separation of gluten and starch in the alkaline suspension is clearly evident at all points in the column.

The effectiveness of different alkalies was compared. Suspensions prepared as above were adjusted to pH 10.0 with 1.0 N sodium hydroxide, 1.0 N sodium carbonate, and saturated calcium hydroxide solutions. These suspensions were steeped for 3 hours at 50°C, then sedimented and divided into layers for analysis. The layers did not all contain the same amount of starch, but have been recalculated to correct for this. The figures presented in the table below indicate that sodium hydroxide is superior to sodium carbonate and calcium hydroxide as a steeping agent. In addition, relatively less of the sodium hydroxide is required. The lower effectiveness of relatively large amounts of sodium carbonate and calcium hydroxide may be related to the observation that less gluten was dissolved at pH 10 in a 5 per cent potassium-sulphate solution than in water (see page 114).

TABLE 9: A Comparison of Different Alkalis for Steeping

Alkali	Amt. of Alkali Soln. Required ml/gm starch	% Nitrogen Content of Layers: Top 1	2	3	4	Bottom 5
1.0N sodium hydroxide	.07	1.81	0.161	--	.049	0.188
1.0N sodium carbonate	.27	1.53	0.286	0.236	0.259	0.244
Satd. calcium hydroxide	.80	1.61	0.292	0.242	--	0.209

The Effect of Time and pH of Steeping

For most of the studies on steeping, the suspensions have been adjusted to pH 10. The advantages of using this pH level are that it exerts a strongly alkaline action, yet it is far enough below the gelatinization point at the higher steeping temperatures to give a good margin of safety, and it inhibits the action of bacteria and moulds. However, it is quite possible that an effective dissociation and hydration of gluten may occur in the region pH 7-9. Less alkali would be required at the lower levels of pH, provided the acid producing bacteria could be inhibited. Kerr (41) mentions some organic halogen compounds which may be effective bacteriostatic agents in alkaline medium. In the experiment reported in this study, three to five drops of toluene per 10 ml. of suspension have been added usually. Three lots of duplicate suspensions, each containing 5.00 gm. of stripper starch of lot # 4 and 20 ml. of water were steeped at 50°C and pH 7.0, pH 8.0 and pH 10.0 respectively. At intervals they were shaken thoroughly and any changes in pH corrected. After steeping for 15 hours, the suspensions were sedimented, and the columns divided into six nearly equal layers of which the bottom five were dried and analyzed for nitrogen. The average result for each pair of duplicates is presented in the table below; any differences in the weights of the corresponding layers has been adjusted approximately by calculation. The analyses indicate a steady improvement in removal of nitrogen from the lower part of the column as the pH is raised from pH 7 to pH 10.

TABLE 10: The Effectiveness of Steeping at Different Alkaline pH Levels

pH	% Nitrogen Content of Layer				
	Top 1	2	3	4	Bottom 5
7.0	1.40	.410	.107	.114	.212
8.0	1.05	.376	.096	.106	.187
10.0	1.00	.310	.079	.090	.125

In a suspension of moist stripper-starch, the supernatant reaches a constant concentration of protein in 1-5 hours; steeping for as little as 3 hours has been found to give considerable separation of the starch and gluten. On the other hand, the slow-uptake of alkali by stripper-starch continues well over 10 hours. Several suspensions were maintained at each of pH 3.7 and at pH 9.0, with vigorous shaking intermittently. At intervals during the first 10 hours one of the suspensions at each pH level was sedimented and the column analyzed for distribution of nitrogen. In both cases there was steady improvement of separation throughout the first 10 hours. The separation was better in alkaline medium at all times up to 10 hours.

Steeping in alkali must be assumed, therefore, to be a slow process, and the improvement in the separation of gluten and starch probably parallels the alkali uptake. The saturation of the liquid phase with dispersed protein apparently bears no relation to the dissociation and hydration of undispersed protein.

The Effect of Alkali on The Starch Granule

When two suspensions containing the same amount of commercial corn starch, but with one adjusted to pH 10.0 and the other left at pH 4.2, are allowed to sediment by gravity, it is evident that the rate of settling is many times more rapid at pH 4.2. However, the final volume occupied by the starch in each suspension was found to be the same. When the suspension at pH 10 was readjusted to pH 4.0, the rate of settling was approximately the same as a sample to which an equivalent amount ofdilute sodium chloride solution had been added.

Suspensions of commercial corn starch were made up in sodium hydroxide solution of increasing concentration. Gelatinization began in .05 N sodium hydroxide (pH 11.2) at 22°C, and in .03 N sodium hydroxide (pH 10.5) at 40°C. When the various suspensions were centrifuged, no change in volume of the sedimented granules was observed in any alkali concentration below that necessary to cause gelatinization.

The effect of alkali-steeping on hot-paste viscosity was determined using a modification of the viscosimeter suggested by Hoeppler (234-236). A vertical glass tube, approximately 35 cm. long x 1.9 cm. diameter was supported in a water bath at 90°C. The starch suspension to be tested was gelatinized by stirring at about 200 r.p.m. with a constant-speed stirrer in a bath of boiling water. Evaporation was carefully controlled. The hot paste was then poured into the vertical glass tube, and stirred gently for 3

minutes. At the end of this period a very small metal sphere was dropped into the paste from a height of 1-2 cm. A strong, diffuse, white light behind the glass tube silhouetted the sphere as it fell through the paste. The rate of fall was kept close to 1 cm. per second. No spiral movement was observed (236), and the same time of fall was obtained when the sphere was dropped from a height of 1 cm. above the surface of the paste as when it was released below the surface. During the first 5 minutes, the falling-times for 10 spheres were determined and agreed well, but after this time a progressively shorter falling-time was observed. The average radius of several spheres was 0.0786 cm., the average density of a sphere was 7.96 and the density of the starch suspensions was calculated as approximately 1.02. From these figures the absolute viscosity of the starch paste could be calculated by applying Stokes' formula for the velocity of falling spheres.

Three suspensions containing 5.0 gm. of corn starch and 90 ml. of distilled water were adjusted to pH 3.8, pH 6.75 and pH 10.0 respectively, with a few drops of 0.1 N hydrochloric acid or 0.1 N sodium hydroxide. Triplicate samples of such suspensions were steeped for 24 hours in a water bath at 50°C. Sufficient 0.1 N sodium chloride was then added, drop by drop, to each suspension to give a total amount of 45 drops. Each mixture was then carefully neutralized with 0.1 N hydrochloric acid. Hot-paste viscosities were determined on the suspensions as described above. They are calculated in table 10 for the three levels of pH.

TABLE 10: Effect of Steeping on Hot-Paste Viscosity of Corn Starch

Steeping pH	Average Time of Fall	Hot-Paste Viscosity of Starch
	Secs.	Centipoises
3.8	8.0	5.18
6.75	13.7	8.76
10.0	12.1	7.85

The corn granules have been shown to be most resistant to change at pH levels near pH 6.75. The steeping treatment at pH 3.8 is approximately the same as the corn receives in normal milling and is considered to be mild. Alkali steeping, therefore, causes comparatively little decrease in the potential hot-paste viscosity of the granule.

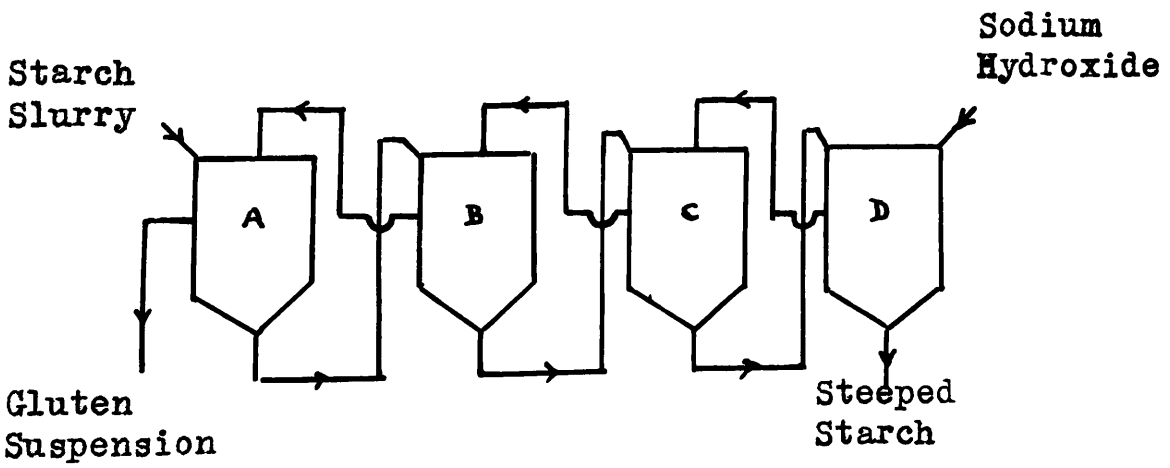
Laboratory Model for a Counter-Current Steep.

Laboratory models of certain commercial processes that give continuous separation were investigated. Usually the physical and mechanical differences between the process as conducted in the model, and what it would be like in large-scale equipment made the value of such experiments problematical.

A model was constructed to study a process for continuous separation of the hydrated gluten. The starch, after thorough steeping in alkali, was passed slowly into a vertical column at a point well below the upper surface of the suspension. A slow stream of water or dilute sodium hydroxide at pH 10 was forced in at the

bottom of the column. With the proper rates of flow the hydrated gluten was carried upwards, and drawn off in an effluent from the top of the column. A heavy slurry of starch, containing little protein, could be collected from the bottom of the column. Recovery of up to 78.5 per cent of the protein in the effluent at the top was obtained, but the rates of flow had to be so low that the capacity of starch production was very small. Convection currents usually occurred in the column and interfered with the separation.

A laboratory model of a counter-current steeping system was more successful. The laboratory model simulated the four-phase steeping system shown in the flow sheet below. Each steep



contains two volumes of thick stripper-starch slurry and one volume of gluten-NaOH suspension from a previous steep. The mixture was steeped two hours and left to settle for one hour. One volume of the supernatant was transferred then from each tank to the tank shown on the left (see diagram), and the two volumes of slurry remaining are transferred to the tank to the right.

The supernatant fraction which entered the system as dilute sodium hydroxide solution at D, became more saturated with protein in C and B, and left the system as a thick gluten suspension at A. Freshly mixed starch slurry, adjusted to pH 10 entered at A and was withdrawn at D, after four steepings.

In a laboratory model, using a slurry of approximately 50 gm. stripper-starch (dry basis) per 100 ml. of water, the results shown in the table below were obtained. In setting up the model system four suspensions of stripper-starch were prepared. After steeping and settling, the supernatant from the suspension in the A position, and the "bottom slurry" from the D position were discarded. The appropriate transfer of each supernatant and each bottom slurry was then carried out; a fresh lot of moist stripper-starch was added to position A and fresh sodium hydroxide to position D. After five such cycles, the system was considered to be in equilibrium, and the contents of each tube were centrifuged. The solids of each tube had a surface layer of gluten which was very sharply defined in C and D. In commercial practice, the suspension could be centrifuged so as to separate such a sharply defined gluten layer; in the laboratory model the layer was carefully removed with a spatula. Both starch and gluten were dried and analyzed for content of nitrogen. The data indicate that recoveries of starch equalling 85 to 90 per cent of the initial charge, with a nitrogen content as low as 0.03 per cent may be expected. In case the stripper-starch contained more protein initially, good recovery of starch with 0.08 per cent nitrogen should still be possible.

TABLE 11: Recovery of Starch and Gluten from Model of Counter-Current Steeping System

Steep No.	Supernatant	Gluten Layer		Starch Layer	
	mg. Nitrogen / ml.	% of Initial charge	% Nitrogen	% of Initial charge	% Nitrogen
A	1.46	9.8	3.88	87.5	.111
B	1.50	9.6	4.06	90.8	.100
C	1.14	8.0	3.03	88.0	.059
D	0.76	8.4	2.28	- -	.038

The Solubility of Corn Protein in Neutral Dispersants

The lack of a single neutral medium which will disperse the complete corn protein system restricts greatly the study of corn gluten. The choice of a dispersant is restricted greatly also by the fact that the corn gluten cannot be mechanically separated from the corn starch, and that many dispersing media gelatinize starch.

The dispersing action of sodium salicylate on corn protein of stripper-starch was investigated. Aqueous solutions containing 4, 8 and 12 per cent sodium salicylate at pH 6.2 were prepared. Into 50 ml. aliquots of these solutions were weighed 3.00 gm. of stripper-starch „ 4 (= 18.7 mg. nitrogen). Similar mixtures containing 18.7 mg. of nitrogen per 50 ml. were made up from stripper-starch „ 6. The tubes containing these mixtures were all shaken continuously for 8-10 hours. The tubes were then centrifuged for 30 minutes at 1500 r.p.m., and the supernatants decanted. Aliquots were analyzed for dispersed nitrogen, and the results are collected in the table below. In order to have a basis for comparison

TABLE 13: Dispersal of Corn Gluten by Aqueous Salicylate

Concn. of Sodium Salicylate	Dispersed Nitrogen as % of Total Nitrogen		
	Stripper-Starch # 4	Stripper-Starch # 6	Wheat Flour
0	- -	6.7	- -
2.0	- -	5.1	- -
4.0	10.6	9.0	52.0
6.0	- -	11.1	66.0
8.0	11.4	13.0	72.5
10.0	- -	17.0	79.0

of this technique with similar observations which have been reported in the literature, the experiment was repeated with aliquots of wheat flour containing 18.7 mg. of nitrogen.

The corn proteins obviously are much less soluble in sodium salicylate solutions than are wheat proteins. In both cases the starch was gelatinized in solutions more concentrated than 10 per cent sodium salicylate. By comparing the observed solubility of the wheat proteins with the studies reported by McCalla and Rose (112), it appears that the technique used here for dispersing the proteins results in a lower proportion of dispersed nitrogen. This is probably due to the centrifuging, since the studies reported in the literature allowed the starch to settle out overnight. In support of this idea is the observation that, when stripper-starch was shaken with 8 per cent sodium salicylate in 40 per cent aqueous ethanol, and left to settle by gravity overnight, approximately 85 per cent of the protein remained in dispersion. But when the mixture was separated by centrifuging for 30 minutes at 1500 r.p.m.,

only 55-60 per cent of the protein remained in dispersion.

Solubility of Corn and Wheat Proteins in Salicylate-Ethanol Solutions

A series of aqueous solutions adjusted to a final concentration of 10 per cent sodium salicylate and containing 0-70 per cent ethanol was prepared. Similar series adjusted to final sodium salicylate concentrations of 12 per cent, 15 per cent and 20 per cent were also prepared. To 50 ml. aliquots of each were added moist stripper-starch # 4 or # 6 containing 18.7 mg. of nitrogen. These mixtures were agitated gently at room temperature for 10-12 hours and then centrifuged at 1500 r.p.m. for 30 minutes. The supernatants were then removed by pipette and aliquots analyzed for nitrogen. The results are shown in figures 11 and 12.

From the data in figure 11 it is apparent that the salicylate greatly increases the solvent action of the lower concentrations of alcohol. This increase in solubility is greater the higher the sodium salicylate concentration. The data suggest that in the higher concentrations of alcohol, the effect is reversed, and salting-out occurs. The net effect of adding sodium salicylate is to shift the point of maximum solubility to lower alcohol concentrations without changing the general proportions of the curve.

Figure 11 does not indicate whether the dispersed nitrogen can be increased significantly above 70 per cent by using 15 per cent or 20 per cent sodium salicylate in a suitable alcohol concentration. The data in figure 12 show that not much more than 70 per cent of

Figure 11 Solubility of Corn Protein from Stripper Starch No. 4 in Sodium Salicylate-Aqueous Ethanol Solutions

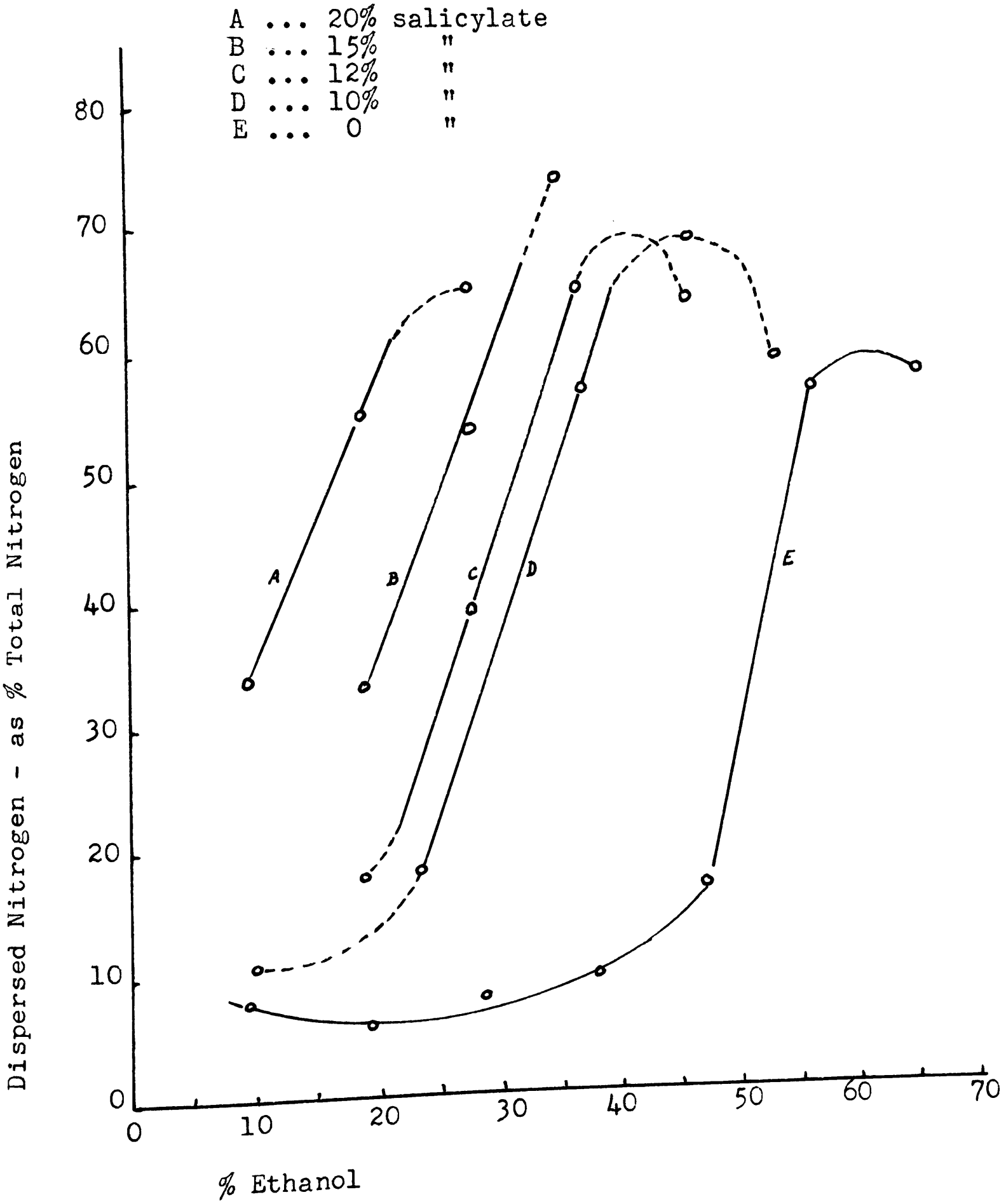
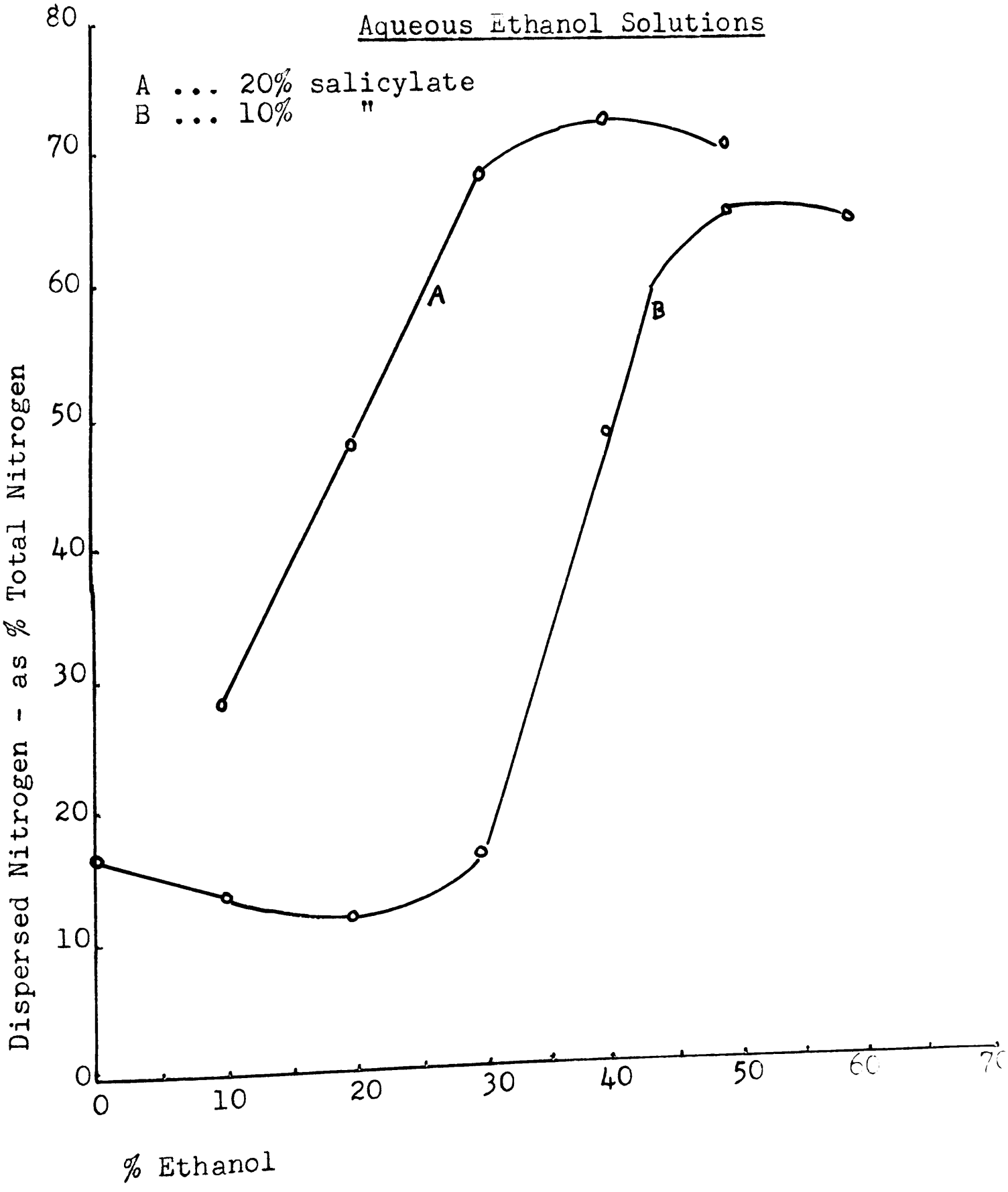


Figure 12 Solubility of Corn Protein of Stripper Starch No. 6 in Sodium Salicylate - Aqueous Ethanol Solutions



the protein can be dispersed by 20 per cent sodium salicylate solution. In 25 per cent sodium salicylate containing 15 per cent ethanol, the starch was gelatinized. The curves of figure 12 are similar to those of figure 11, but shifted to slightly higher alcohol concentrations. The specific gravity of the ethanol used for the determinations reported in figure 11 was checked; the ethanol used in the work for figure 12 was not checked.

The solubility of aliquots of wheat flour containing 18.7 mg. of nitrogen in 50 ml. volumes of 10 per cent and 20 per cent sodium salicylate in 10-40 per cent aqueous ethanol solutions was measured using the technique described above. The results are tabulated below.

TABLE 14: Dispersal of Wheat Proteins in Alcoholic Salicylate

Conc. of Ethanol	Per Cent of Total Nitrogen Dispersed	
	10% Sodium Salicylate	20% Sodium Salicylate
%		
0	79.0	- -
10	69.0	57.3
20	66.0	70.7
30	66.0	69.0
40	68.0	61.5

The solubility of the wheat proteins in salicylate-ethanol mixtures differs considerably from the solubility of corn proteins. It seems safe to assume that aqueous 20 per cent sodium salicylate disperses 100 per cent of wheat proteins (109,172). Hence, ethanol in low concentrations decreases the solubility of wheat proteins in

both 10 per cent and 20 per cent sodium salicylate. In the latter solution, however, there was a slight maximum in the solubility when the solution contained 20-30 per cent ethanol. There was no evidence for such a maximum with 10 per cent sodium salicylate in ethanol concentrations up to 40 per cent. These observations may be summarized as follows. Wheat proteins are dispersed much more completely by sodium salicylate solutions than are corn proteins. When so dispersed the solubility of wheat proteins is decreased more by low concentrations of ethanol, and increased less by medium concentrations of ethanol, than is the solubility of corn proteins.

The Use of Alcoholic Salicylate to Extract Corn Proteins from Starch

Preliminary experiments have been conducted to study the use of a solvent containing 20 per cent sodium salicylate and 30 per cent ethanol for extracting the total proteins from stripper-starch or corn meal. This solvent was chosen because it contains the lowest concentration of alcohol with which maximum extraction was shown to be possible.

In the previous work, a gradual denaturation and precipitation was observed when the dispersed protein was allowed to stand at room temperature. At the same time, the liquid phase, which was still cloudy after centrifuging at 1500 r.p.m. for 30 minutes, became clear. Whether the precipitation is denaturation, or just a normal settling of fine colloidal particles has not been determined. A slightly slower precipitation was observed when the extraction and

DISCUSSION and CONCLUSIONS

The Role of Corn Gluten in Stripper-Starch

In the introductory review, corn gluten was defined as the protein in the main waste-fraction obtained from the overflow waters in tabling or from centrifuging. The division between protein which becomes corn gluten and protein which becomes part of stripper-starch appears to be quite arbitrarily determined by the conditions of milling. There is no known reason for assuming that the proteins in corn gluten and stripper-starch differ in any way except the amount of starch granules with which they are associated. Thus a sample, collected at the factory as 'corn gluten', has been listed in this study as stripper-starch lot # 6 because it contained a proportion of protein lower than is considered normal for corn gluten. The study of the protein in stripper-starch therefore is essentially a study of corn gluten.

At the beginning of this study, it seemed quite possible that the gluten of stripper-starch was distributed more-or-less evenly with each granule. Such a distribution would be reasonable, since the particles which make up stripper-starch have already been selected on a basis of sedimentation rate, and the density of the particle is an important factor in this method of fractionation.

The histo-chemical study of stripper-starch showed that there was actually a wide variation in the ratio of gluten to starch in each particle. The particles appeared to vary in composition continuously from single starch granules with no gluten attached,

to relatively large masses of gluten with only a few starch granules attached. The major part of the gluten appeared to be in moderate-sized clumps of granules where it probably acts as a cementing substance between granules.

These observations were confirmed by the results of a procedure which fractionated stripper-starch according to specific gravity. A considerable range in specific gravities was observed, the heavier particles containing less than 1 per cent gluten and the lighter particles over 15 per cent gluten. About 60 per cent of the particles in the stripper-starch sample which was observed contained a relatively constant proportion of gluten, between 5 and 7 per cent. The absolute values and, to a lesser extent, the relative proportions of the fractionation curve would be expected to vary in the different stripper-starches produced by changes in milling conditions.

The samples of undried stripper-starch in suspension showed a higher proportion of single granules, free of gluten, and of gluten masses free of granules. Presumably these become attached at random to one another and to the larger gluten-starch aggregates on the filter presses, and are very difficult to dissociate after drying the moist filter cake at a high temperature. In accordance with this is the observation that when a suspension of dried, ground filter-cake was allowed to sediment, little separation of gluten and starch was observed, and the gluten was found mainly at the bottom of the sediment (figure 2). The latter fact was considered to indicate that the larger-aggregates contained more gluten. A moist stripper-starch, when sedimented, showed a much larger degree of separation of the components (figure 3). The top layers were found to contain

15-25 per gluten, while the particles in the middle of the sediment contained usually 0.4-0.8 per cent gluten. The bottom layers of the sediment had an increased content of nitrogen, indicating that the larger aggregates contained the most gluten.

These conclusions all agree with the following summary of the relation of stripper-starch to "prime" starch and corn gluten. The starch granules to be found in the "prime" starch fraction are determined not merely by their high specific gravity which results from being nearly free of nitrogen, but also by their inclusion in the relatively large aggregates of granules which are formed in slightly acid media (228). Granules which do not become aggregated, although they may be free of nitrogen, sediment so slowly that they are included in the stripper-starch fraction. Also included in the stripper-starch fraction are all aggregates with a rate of sedimentation intermediate to that of the prime starch and the gluten fractions. This rate of sedimentation results from the smaller aggregates containing a small percentage of gluten, or the larger aggregates containing a large percentage of gluten. The smaller aggregates of granules, containing a high percentage of gluten, sediment so slowly that they become part of the corn-gluten fraction.

The study of denaturation by drying is very important. The ordinary drying procedures used in industry employ temperatures near 100°C either in continuous rotary driers or in tunnel-kilns. It was demonstrated in this study that the gluten becomes quite

resistant even to alkali after such treatment. Prolonged drying at 100°C or even at room temperature was shown to alter the solubility of gluten in aqueous solutions of salt or in aqueous alcohol.

The observation that drying wet stripper-starch in the laboratory air can cause some denaturation of the gluten necessitates keeping the material moist. Since most experimental work requires that the nitrogen content of the material be already known, some method of storing the moist material becomes essential. The method which has been adopted in this study is a preliminary attempt to solve this problem, but much more work is desirable to determine how adequately it does so, and whether some other method is better.

In the introductory review the possibility was noted that the starch granules of stripper-starch were associated with portions of gluten which were more resistant chemically than the rest of the gluten. In support of such an idea, it was observed that stripper-starch which was richer in gluten contained less protein that was soluble in 5 per cent potassium sulphate. There did not seem to be any difference in the zein and glutenin fractions. However, since the experimental procedure was such that total mineral associated with the starch differed, it cannot be concluded that there is any chemical difference among the glutens of the various lots of stripper-starch. The work of Rich (126,137) on the pentization of wheat protein illustrates this point. More work should be done on this question.

The Action of Alkali on Gluten

The different experiments concerning the effect of alkali on gluten may be correlated as follows. When either commercial corn starch or fresh, moist stripper-starch is suspended in water, the mixture usually has a pH between 4 and 5. If alkali is added to this suspension to give pH 5.5, considerable buffering power is evident. The buffer capacity of the gluten is very much greater than the capacity of the starch per unit weight (figure 7). In the region pH 3.5-5.5 the solubility of gluten is essentially constant (figure 5), and when the pH of the suspension has been adjusted it is stable (figure 8).

These properties are altered sharply above pH 5.5. As the pH becomes more alkaline, the solubility of gluten increases greatly. When the pH of a suspension is maintained at a level above 5.5 there is a gradual uptake of alkali over a period of several hours. The uptake of alkali however, appears to be limited, and if enough alkali is added initially, the absorption of alkali will become almost negligible after a period of about 1 day, while the pH is still well above pH 5.5 If less than this amount of alkali is added, the uptake of alkali will cease when the pH has decreased to pH 5.5.

The titration curves showing rapid binding of alkali do not show any irregularity at pH 5.5. Moreover, the buffering power is not proportional to the amount of protein present. It is suggested therefore, that these curves represent a titration of dissociable groups present on, or near, the surface of the gluten masses.

Phosphate does not appear to be a major buffering group in this case. If this interpretation of the titration curves is correct, then the relatively rapid saturation of the liquid phase with respect to dissolved protein is primarily dependent on the surface of the protein mass.

The slow uptake of alkali does not appear to be related to the dissolving of the gluten. The liquid phase of an undried stripper-starch suspension becomes saturated with nitrogenous compounds in an hour or two. The slow uptake of alkali, which goes on for periods over 10 hours, appears to be correlated with a slow separation of gluten from starch which process also continues for many hours. Both the slow absorption of alkali and the separation of gluten from starch appeared to be greater at pH 10 than at pH 8.0. Whatever may be the exact mechanism involved, it seems safe to assume that the slowness of the process results from the slowness with which alkali penetrates the gluten masses.

The effects associated with the slow uptake of alkali suggest that dissociation of the gluten-starch aggregates occurs when some force of attraction becomes unstable at pH 5.5. The solubility curve suggests that the gluten complex is effectively iso-electric over the whole range pH 3.5 to 5.5, and that sharply at pH 5.5 there is a slow dissociation into charged molecules or units. These charged units then pair off with alkali, and become more soluble as the pH is raised. As a result of the dissociation, a considerable portion of the starch is released.

Although the properties of the gluten itself suggest coacervation, there are several observations which indicate that the linkage between starch and gluten is not coacervation. The visual inspection of the stripper-starch showed discrete patches of protein attached to the sides of single granules. The ability of the force of coacervation between two surfaces to hold together two relatively large masses is problematical. In most of the aggregates, the starch granules appeared to be embedded partially in the gluten, so that a dissociation or softening of the gluten would be expected to free the starch withou any electrical forces being involved. On the other hand, all artificial starch-protein coacervates have been shown to dissociate completely above pH 7. The separation of gluten from starch during steeping was found to be progressively better as the pH was raised from 7 to 10.

When stripper-starch is suspended in a cold solution of salicylate in aqueous ethanol at pH 6.9, much of the gluten in the starch sediment was observed to be at the bottom of the sediment indicating that the gluten was part of fairly large aggregates. Violent stirring with a Waring Blendor separated much of the starch from the gluten, and allowed the latter to sediment as a sharply defined layer on top of the starch.

The Milling of Stripper-Starch

In the modern milling procedures, stripper-starch largely is recirculated through the centrifuges and eventually passes into either the prime-starch fraction or the corn-gluten fraction.

There is probably considerably breakdown of the stripper-starch during the recirculation.

It is probable that a considerable amount of the corn gluten could be separated as a fraction containing less than 8-10 per cent gluten. At certain times the recovery of the starch from this fraction and the stripper-starch fraction might be economically justifiable. The use of a counter-current steeping system such as is described should give good recovery of starch containing less than 0.08 per cent nitrogen even when the raw material contains much higher proportions of protein than the sample used for the experiment. The alkaline steeping suspension, furthermore, should be easily separable by centrifuging. The amount of acid needed to neutralize the starch is small. The loss of potential hot-paste viscosity is also small and could be compensated for by leaving the starch slightly alkaline to pH 4.5.

If desired the gluten may be recovered. The recovery of zein from the proteins extracted from corn-gluten has already been patented (237). The neutralization of the material, by mixing it with a large amount of the acidic corn-gluten (pH 4.0) would make it acceptable for use in cattle feed.

The preliminary experimental work on enzymatic digestion of gluten and exhaustive extraction of the stripper-starch did not appear likely to result in a sufficient removal of gluten to yield 'prime' starch. In addition, both processes seemed relatively costly, and therefore they were not studied extensively.

Salicylate in Aqueous Ethanol as a Dispersant for Gluten

In the introductory review, dispersing media for gluten were considered in several groups. One of these groups was organic solvents which alone or in aqueous solutions selectively dispersed the prolamine fraction. Some degree of correlation between the low dielectric constant of these media and their dispersing action could be shown, although dispersing forces peculiar to the molecular structure were also noted. Another group was called neutral dispersants. This group includes a great variety of molecular structures, which have a very non-specific dispersing action, and peptize proteins, carbohydrates and fats. Substances such as glycine, urea, urethane, ammonium thiocyanate and salts of salicylic acid have been classed together as non-specific dispersing agents for many years. Their non-specific dispersing action is probably related to their strongly dipolar nature, and positive dielectric increment in aqueous solution. This view is supported by the similarity between their dispersing action and that of lyotropic salts such as potassium iodide.

It seemed possible that the solubility of wheat and corn-gluten might be greater in an aqueous solution containing both organic solvent and dipolar molecules, than in an aqueous solution containing only one of these. The dispersing action of molecules such as the salts of naphthalenebutyric acid and phenylacetic acid (238) and certain alkyl sufonates, which would not be expected to be strongly dipolar ions, may depend on just such a balance of non-

polar and polar structures in the molecule.

Lejeune (72) has shown that starch granules may be exposed to higher concentrations of aqueous sodium salicylate without being gelatinized, if small percentages of ethanol or acetone are added to the solution. The organic molecules apparently antagonize the dispersing action of the salicylate. The theory that this antagonism would be less, or even reversed, in the dispersal of gluten seemed reasonable, since alcohol tends to precipitate dispersed starch, but tends to peptize certain fractions of gluten.

The above considerations are based almost entirely on studies involving wheat gluten. The experimental observations, discussed at some length on page 136, are interesting because the theory appears to apply well to corn gluten, but applies very poorly to wheat gluten.

SUMMARY

1. A method of mounting and staining stripper-starch for microscopic examination of the gluten was described.

2. A denaturation of the corn-gluten of stripper-starch resulting in decreased solubility and greater resistance to separation from the starch was shown to result from drying procedures.

3. A method was described for preserving undried stripper-starch by storage in the cold after treating it with a volatile bacteriostatic agent such as toluene which is easily removable and does not affect the gluten.

4. The distribution of the ratio of protein to starch in the particles of a dried sample of stripper-starch was studied.

5. Corn gluten was shown to have a relatively constant-solubility between pH 3.5 and pH 5.5, with a sharply increasing solubility above pH 5.5.

6. The proportion of gluten soluble in dilute salt solution appeared to vary inversely with the total proportion of gluten in the stripper-starch. This may have been caused by a variable concentration of salt under the experimental conditions.

7. The complete removal of gluten from stripper-starch by repeated extraction with water and dilute salt solutions at different pH levels, or by enzymatic digestion, was attempted. The results indicated that these procedures would not be practical for industrial purposes.

8. Two separate processes were distinguished in the absorption

of alkali by gluten. One process appeared to be a rapid titration of dissociable groups near the surfaces of the gluten masses. The other process was a slow absorption of alkali at pH levels above ph 5.5, which suggested dissociation of a coacervate.

9. A good recovery of starch, nearly protein-free, was obtained by steeping stripper-starch in alkali. The degree of separation of gluten from starch after steeping was determined by analyzing the distribution of nitrogen in sedimented columns of the steeped starch. Of the alkalis tested, sodium hydroxide was found to be most effective; the separation was greater at higher pH levels, and increased up to at least 10 hours.

10. The decrease in hot-paste viscosity of starch was found to be less after steeping at pH 10 than after steeping at pH 3.8-4.0.

11. The solubility of corn gluten in aqueous solutions containing sodium salicylate and ethanol was determined. A high solubility of gluten in solutions of the proper concentration, without gelatinization of the starch, was demonstrated.

12. A method for isolating the complete corn gluten, substantially free of starch, by extraction with an aqueous solution containing approximately 20% salicylate and 30% ethanol, and recovery by salting-out, is proposed.

Claims To Original Research

The following are considered to be the original contributions to knowledge in this study.

1. The application of a histochemical method for investigating the form of the protein component in fractions obtained in the milling of corn.

2. The demonstration that gluten exists as discrete particles. An unexpectedly great heterogeneity in composition, specific gravity, and size of the individual particles in stripper-starch (has been observed).

3. A demonstration that denaturation of gluten to a very resistant form is caused by drying at the elevated temperatures used industrially and slight denaturation is caused by drying at room temperature.

4. The description of a method for preserving moist stripper-starch for moderate periods by storing it at 5°C after treating it with a neutral water-insoluble substance such as toluene which ordinarily does not need to be removed.

5. The differentiation of alkali absorption into two separate processes, the one a slow absorption of alkali which occurs above pH 5.5 only, the other a rapid binding of alkali which shows no significant change at pH 5.5.

6. The conclusion that the rapid binding of alkali by gluten is a titration of dissociable groups near the surface of the gluten particles.

7. Evidence on which is based the view that associative forces similar to coacervation exist in the native corn gluten complex between pH 3.5 and 5.5.

8. Observations which lead to the conclusion that in stripper-starch or other corn fractions which are made up of intact starch granules and relatively large masses of undispersed gluten, the forces of coacervation are not important in holding together the starch and gluten.

9. The demonstration that the optimum pH of papain in a digest containing undispersed substrate such as corn gluten greatly altered according to the effect of pH on the solubility of the substrate.

10. A study of the effect of pH, time, and choice of alkali on effectiveness of alkali-steeping and a study of a model of a counter-current steeping system is described.

11. The demonstration that steeping starch at pH 10 causes a smaller decrease in potential hot-paste viscosity than does steeping under the common industrial conditions at pH 3.8-4.0.

12. The study of the solubility of corn gluten in aqueous solutions containing various amounts of sodium salicylate and ethanol.

13. The extraction of the major portion of the corn gluten from stripper starch by a chilled aqueous solvent containing 20 per cent sodium salicylate and 30 per cent ethanol. A method of preparing undenatured starch-free corn gluten by salting out the dispersed protein is possible.

BIBLIOGRAPHY

1. Mangelsdorf, P.C. and Reeves, R.G. Texas Agr. Expt. Sta. Bull. No. 574, 1939.

2. Hector, J.M. Introduction to the Botany of Field Crops; Vol. 1, Cereals. Central News Agency Ltd., Johannesburg, South Africa.

3. Sandstedt, R.M. Cereal Chem. 23, 337, 1946.

4. Weatherwax, P. Bull. Torrey Bot. Club 53, 381, 1926.

5. Gordon, M. Proc. Roy. Soc. (Victoria) 34, 105, 1922.

6. Osborne, T.B. The Vegetable Proteins. Longmans Green & Co. 1909.

7. Wassilieff, N. Ber deut. botan. Ges. 26, 454, 1908.

8. Schulze, E. and Winterstein, E. Z. physiol. Chem. 66, 431, 1910.

9. Schulze, E. Z. physiol. Chem. 71, 31, 1911.

10. Sharp, P.F. and Elmer, R. Cereal Chem. 1, 83, 1924.

11. Sharp, P.F. Cereal Chem. 2, 12, 1925.

12. Zaleski, W. Botan. Centr. 117, 57, 1911.

13. Teller, G.L. Plant Physiol. 10, 499, 1935.

14. Zeleny, L. Cereal Chem. 12, 536, 1935.

15. Suzuki, W. Botan. Centr. 75, 289, 1898.

16. Meunscher, W.C. Bot. Gaz. 75, 249, 1923.

17. Baldwin, E. Dynamic Aspects of Biochemistry; Cambridge Univ. Press, Eng. 1947.

18. Eckerson, S. Wash. Agr. Exp. Sta. Bull. 133, 1917.

19. Thatcher, R.W. Wash. Agr. Exp. Sta. Bull. 111, 1913.

20. Thatcher, R.W. J. Am. Soc. Agron. 7, 273, 1915.

21. Harlan, H.V. J. Agr. Research 19, 393, 1920.

22. Brenchley, W.E. Ann. Bot. 26, 903, 1912.

23. Blish, M.J. J. Agr. Research 19, 181, 1920.

24. Loew, O. Angew. Botan. 15, 518, 1933.

25. Jodidi, S.L. and Markely, K.S. J. Am. Chem. Soc. 45, 2137, 1923.

26. Jodidi, S.L. J. Agr. Research 30, 587, 1925.

27. Jodidi, S.L. and Wangler, J.G. J. Agr. Research 30, 989, 1925.

28. Landis, Q. and Frey, C.N. Cereal Chem. 15, 91, 1938.

29. Jorgensen, H. Biochem. Zeit. 280, 1, 1935.

30. Jorgensen, H. Biochem. Zeit. 283, 134, 1935.

31. Balls, A.K. and Hale, W.S. Cereal Chem. 13, 656, 1936.

32. Balls, A.K. and Hale, W.S. Cereal Chem. 15, 622, 1938.

33. Elion, E. Cereal Chem. 20, 234, 1943.

34. Read, J.W. and Haas, L.W. Cereal Chem. 16, 60, 1939.

35. Sandstedt, R.M. and Fortmann, K. Cereal Chem. 20, 517, 1943.

36. Ford, W.P. and Maiden, A.M. J. Soc. Chem. Ind. 57, 278, 1938.

37. Olcott, H.S., Sabirstein, L.A. and Blish, M.J. Cereal Chem. 20, 87, 1943.

38. Sullivan, B., Howe, M., Schmalz, F.D. and Astleford, G. Cereal Chem. 17, 507, 1940.

39. Miller, E.C. Plant Physiology (p. 536) McGraw Hill, 1938.

40. Zirkle, C. Am. J. Botany 13, 301, 1926.

41. Kerr, R.W. Chemistry and Industry of Starch; Academic Press, New York, N.Y.

42. Lindet, L. and Nottin, P. Compt. rend. 176, 149, 1923.

43. Sjostrum, O. Ind. Eng. Chem. 28, 63, 1936.

44. Alsberg, C.L. Plant Physiol. 13, 295, 1938.

45. Badenhuizen, N.P. Protoplasma. 33, 440, 1940.

46. Ostwald, W.O. and Hertel, R.H. Kolloid.Z. 47, 258, 1929.

47. Ostwald, W.O. and Hertel, R.H. Kolloid. Z. 47, 357, 1929.

48. Sande-Bakhuyzen, H.L. van de. Proc. Soc. Exp. Biol. Med. 23, 302, 1926.

49. Sjostrum, O. and Katz, J.R. Z. physik. Chem. 182, 73, 1938.

50. Hanson, E.A. and Katz, J.R. Z. physik. Chem. A 168, 339, 1934.

51. Bailey, C.H. Constituents of Wheat and Wheat Products; Reinhold Publishing Corp., New York, N.Y.

52. Sair, L. and Fetzer, W.R. Ind. Eng. Chem. 35, 205, 1944.

53. Sair, L. and Fetzer, W.R. Ind. Eng. Chem. 36, 316, 1944.

54. Rakowski, A. Kolloid. Z. 9, 225, 1911.

55. Katz, J.R. Kolloidchem. Beihefte 9, 65, 1917.

56. Katz, J.R. and Hanson, E.A. Z. physik. Chem. A 168, 321, 1934.

57. Hanson, E.A. and Katz, J.R. Z. physik. Chem. A 168, 339, 1934.

58. Rask, O.S. and Alsberg, C.L. Cereal Chem. 1, 7, 1924.

59. Haller, R. Helv. Chim. Acta. 28, 450, 1945.

60. Samec, M. Kolloid. Z. 195, 72, 1928.

61. Samec, M. and Mayer, A. Kolloid. Chem. Beiheft 13, 272, 1921.

62. Koets, P. J. Phys. Chem. 40, 1191, 1936.

63. Alsberg, C.L. Proc. Soc. Exp. Biol. Med. 36, 127, 1937.

64. Schoch, T.J. J. Am. Chem. Soc. 60, 2824, 1938.

65. Schoch, T.J. and Jensen, C.C. Ind. Eng. Chem., Anal. Ed., 12, 531, 1940.

66. Schoch, T.J. Cereal Chem. 18, 121, 1941.

67. Schoch, T.J. Advances in Carbohydrate Chem. 1, 247, 1945.

68. Samec, M. Colloid Chem. 4, 167, 1932.

69. Schoch, T.J. J. Am. Chem. Soc. 64, 2954, 1942.

70. Samec, M. Kolloidchem. Beihefte 3, 126, 1912.

71. Ostwald, W.O. and Frenkel, G. Kolloid. Z. 43, 296, 1927.

72. Lejeune, G. Compt. rend. 216, 217, 1943; C.A. 39, 829, 1945; original not available.

73. Bear, R.S. and Samsa, E.G. Ind. Eng. Chem. 35, 721, 1943.

74. Buswell, A.M., Gore, R.C. and Rodebush, W.H. J. Phys. Chem. 45, 543, 1941.

75. Ceasar, G.V. Chap. 9 in Kerr: c.f. ref. (41).

76. Haworth, W.N. J. Soc. Chem. Ind. (London) 58, 917, 1939.

77. Samec, M., Minaeff, M. and Ronzin, N. Kolloid. Beihefte 19, 203, 1924.

78. Samec, M. Biochem. Z. 186, 337, 1927.

79. Samec, M. Biochem. Z. 218, 249, 1930.

80. Clayson, D.H.F. and Schryver, S.B. Biochem. J. 17, 493, 1923.

81. Schryver, S.B. and Thomas, E.M. Biochem. J. 17, 497, 1923.

82. Ling, A.R. and Nanji, D.R. J. Chem. Soc. 127, 652, 1925.

83. Kerr, R.W. and Trubell, O.R. Cereal Chem. 18, 530, 1941.

84. Taylor, T.C. and Werntz, J.H. J. Am. Chem. Soc. 49, 1584, 1927.

85. Taylor, T.C. and Lehrman, L. J. Am. Chem. Soc. 48, 1739, 1926.

86. Evans, J.W. and Briggs, D.R. Cereal Chem. 18, 443, 1941.

87. Taylor, T.C. and Lehrman, L. J. Am. Chem. Soc. 52, 808, 1930.

88. Schoch, T.J. J. Am. Chem. Soc. 64, 2954, 1942.

89. Taylor, T.C. and Walton, R.P. J. Am. Chem. Soc. 51, 3431, 1929.

90. Lehrman, L. J. Am. Chem. Soc. 64, 2144, 1942.

91. Sherman, H.C. and Baker, J.C. J. Am. Chem. Soc. 38, 1885, 1916.

92. Tanret, C. Bull. soc. chim. France 17, 83, 1915.

93. Ling, A.R. and Nanji, D.R. J. Chem. Soc. 123, 2666, 1923.

94. Kerr, R.W. and Severson, G.M. J. Am. Chem. Soc. 65, 193, 1943.

95. Samec, M. and Hairdtl, H. Kolloidchem. Beiheft 12, 281, 1920.

96. Samec, M. Trans. Faraday Soc. 31, 395, 1935.

97. Stamberg, O.E. and Bailey, C.H. Cereal Chem. 16, 309, 1939.

98. Posternak, T. Helv. Chim. Acta 18, 1351, 1935.

99. Samec, M. Kolloid. Beihefte 40, 449, 1934.

100. Von Przylecki, St. J. and Grynnberg, M.Z. Biochem. Z. 248, 16, 1932.

101. Von Przylecki, St. J. and Dobrowolska, S. Biochem. Z. 245, 388, 1932.

102. Samec, M. and Blinc, M. Kolloidchem. Beihefte 30, 163, 1930.

103. Samec, M. and Antonovic, Z. Kolloidchem. Beihefte 23, 377, 1926.

104. Samec, M. Kolloidchem. Beihefte 33, 95, 1931.

105. Kepner, B.H. Ind. Eng. Chem. 6, 481, 1914.

106. LeClerc, J.A. J. Assoc. Offic. Agr. Chemists 4, 180, 1920.

107. Dill, D.B. and Alsberg, C.L. Cereal Chem. 1, 222, 1924.

108. Spencer, E.Y. and McCalla, A.G. Can. J. Research C, 16, 483, 1938.

109. McCalla, A.G. and Gralen, N. Can. J. Research C, 20, 130, 1942.

110. Curtis, J.J. and Earle, F.R. Cereal Chem. 23, 88, 1946.

111. Hansen, D.W., Brimhall, B. and Sprague, G.F. Cereal Chem. 23, 329, 1946.

112. Osborne, T.B. and Mendel, L.B. J. Biol. Chem. 37, 557, 1919.

113. Jones, D.B. Cereal Chem. 3, 194, 1926.

114. Osborne, T.B. J. Am. Chem. Soc. 19, 525, 1897.

115. Osborne, T.B. and Clapp, S.H. Am. J. Physiol. 20, 477, 1907.

116. Hoffman, W.F. and Gortner, R.A. Colloid Symposium Monographs II, 209, 1925.

117. Osborne, T.B. and Liddle, L.M. Am. J. Physiol. 26, 295, 1910.

118. Dakin, H.D. J. Biol. Chem. 44, 499, 1920.

119. Dakin, H.D. Z. Physiol. Chem. 130, 159, 1923.

120. Osborne, T.B. Proteins of the Wheat Kernel. Carnegie Institution of Washington (No. 84, 1907).

121. Herzner, R. Biochem. Z. 202, 320, 1928.

122. Staker, E.V. and Gortner, R.A. J. Phys. Chem. 35, 1565, 1931.

123. Blagoveschenski, A.V. and Yurgenson, M.P. Biochem. J. 29, 805, 1935.

124. Blagoveschenski, A.V. and Sossiedov, N.I. Biochem. J. 28, 1575, 1933.

125. Sharp. P.F. and Gortner, R.A. Minn. Agr. Exp. Sta. Tech. Bull. 19, 1923.

126. Rich, C.E. Cereal Chem. 13, 522, 1936.

127. Blish, M.J. Ind. Eng. Chem. 8, 138, 1916.

128. Olson, G.A. Ind. Eng. Chem. 6, 211, 1914.

129. Jones, D.B. and Gersdorf, C.E.F. J. Biol. Chem. 58, 117, 1923.

130. Hoffman, W.F. and Gortner, R.A. Cereal Chem. 4, 221, 1927.

131. Gortner, R.A., Hoffman, W.F. and Sinclair, W.B. Colloid Symposium Monograph V, 179, 1928.

132. Gortner, R.A. Hoffman, W.F. and Sinclair, W.B. Cereal Chem. 6, 1, 1929.

133. Herd, C.W. Cereal Chem. 8, 1, 1931.

134. Harris, R.H. Cereal Chem. 8, 47, 1931.

135. Pascoe, T.A., Gortner, R.A. and Sherwood, R.C. Cereal Chem. 7, 195, 1930.

136. Geddes, W.F. and Goulden, C.H. Cereal Chem. 7, 527, 1930.

137. Rich, C.E. Cereal Chem. 10, 222, 1933.

138. Csonka, F.A. J. Biol. Chem. 118, 147, 1937.

139. Official and Tentative Methods of Analysis of the Association of

Official Agricultural Chemists; George Banta Publ. Co., Menasha, Wisconsin.

140. Osborne, T.B. Z. Anal. Chem. 41, 25, 1902.

141. Harris, L.J. Proc. Roy. Soc. Lond. B, 94, 441, 1922.

142. Cohn, E.J., Berggren, R.E.L. and Hendry, J.L. J. Gen. Physiol. 7, 81, 1924.

143. Hoagland, R. Ind. Eng. Chem. 3, 838, 1911.

144. Sharp. P.F. and Herrington, B.L. Cereal Chem. 4, 249, 1927.

145. Schleimer, V.A. Z. ges. Getreidew. 3, 138, 1911; reviewed by Bailey (51); original paper not sighted.

146. Dill, D.B. and Alsberg, C.L. J. Biol. Chem. 65, 279, 1925.

147. Dill, D.B. J. Biol. Chem. 72, 239, 1927.

148. Evans, C.D. and Manley, R.H. Ind. Eng. Chem. 33, 1416, 1941.

149. Manley, R.H. and Evans, C.D. Ind. Eng. Chem. 35, 661, 1943.

150. Evans, C.D. and Manley, R.H. Ind. Eng. Chem. 36, 408, 1944.

151. Swallen, L.C. Ind. Eng. Chem. 33, 394, 1941.

152. Marvel, C.S., Copley, M.J. and Ginsberg, E. J. Am. Chem. Soc. 62, 3109, 1940.

153. Manley, R.H. and Evans, C.D. J. Biol. Chem. 143, 101, 1941.

154. Bailey, C.H. and Blish, M.J. J. Biol. Chem. 23, 345, 1915.

155. Blish, M.J. and Sandstedt, R.M. Cereal Chem. 6, 494, 1929.

156. Donard, E. and Labbe, H. Compt. rend. 137, 264, 1903.

157. Lindet, M. and Ammann, L. Bull. Soc. Chim. France (4) 1, 968, 1907.

158. Hangaard, G. and Johnston, A.H. Compt. rend trav. Lab. Carlsberg 18, 1, 1930.

159. Watson, C.C. , Arrkenius, S. and Williams, J.W. Nature 137, 322, 1936.

160. Elliot, M.A. and Williams, J.W. J. Am. Chem. Soc. 61, 718, 1939.

161. Gortner, R.A. and Macdonald, R.T. Cereal Chem. 21, 324, 1944.

162. Doty, D.M., Bergdoll, M.S., Nash, H.A. and Brunson, A.M. Cereal Chem. 23, 115, 1946.

163. Jones, D.B. and Csonka, F.A. J. Biol. Chem. 78, 289, 1928.

164. Csonka, F.A. J. Biol. Chem. 97, 281, 1932.

165. Blish, M.J. and Sandstedt, R.M. Cereal Chem. 2, 57, 1925.

166. Blish, M.J., Abbott, R.C. and Platenius, H. Cereal Chem. 4, 129, 1927.

167. Olcott, H.S. and Mecham, D.K. Cereal Chem. 24, 407, 1947.

168. Tague, E.L. Cereal Chem. 2, 117, 1925.

169. Blish, M.J. and Sandstedt, R.M. J. Biol. Chem. 85, 195, 1929.

170. Larmour, R.K. and Sallans, H.R. Can. J. Research 6, 38, 1932.

171. Mangels, C.E. and Bailey, C.H. Ind. Eng. Chem. 25, 456, 1933.

172. McCalla, A.G. and Rose, R.C. Can. J. Research 12, 346, 1935.

173. Harris, R.H., Olson, R.V. and Johnson, J. Cereal Chem. 19, 748, 1942.

174. Rose, R.C. and Cook, W.H. Can. J. Research 12, 63, 1935.

175. Harris, R.H. and Johnson, M. Arch. Biochem. 8, 135, 1945.

176. Cook, W.H. and Alsberg, C.L. Can. J. Research 5, 355, 1931.

177. Cook, W.H. Can. J. Research 5, 389, 1931.

178. Anson, M.L. Advances in Protein Chem. 2, 361, 1945.

179. Neurath, H., Greenstein, J.P., Putnam, F.W. and Erickson, J.A. Chem. Revs. 34, 157, 1944.

180. Sharp, P.F. and Gortner, R.A. J. Phys. Chem. 26, 101, 1922.

181. Blish, M.J. and Sandstedt, R.M. Cereal Chem. 3, 144, 1926.

182. Geddes, W.F. Can. J. Research 2, 65, 1930.

183. Cook, W.H. and Rose, R.C. Can. J. Research 12, 238, 1935.

184. Cook, W.H. and Rose, R.C. Can. J. Research 12, 248, 1935.

185. Dill, D.B. and Alsberg, C.L. J. Biol. Chem. 63, Proc. 68, 1925.

186. Gottenberg, M. J. and Alsberg, C.L. J. Biol. Chem. 73, 581, 1927.

187. Swallen, L.C. and Daneghy, J.P. Colloid Chem. 6, 1140, 1946.

188. Blish, M.J. Cereal Chem. 2, 127, 1926.

189. Pearsall, W.H. and Ewing, J. Biochem. J. 18, 329, 1924.

190. Halton, P. J. Agr. Sci. 14, 587, 1924.

191. Csonka, F.A. and Jones, D.B. J. Biol. Chem. 73, 321, 1927.

192. Tague, E.L. J. Am. Chem. Soc. 47, 418, 1925.

193. Vickery, H.B., Pucher, G.W., Clark, H.E., Chibnall, A.C. and Westall, R.G. Biochem. J. 29, 2713, 1935.

194. Woodman, H.E. J. Agr. Sci. 12, 231, 1922.

195. Neglia, F.J., Hess, W.C. and Sullivan, M. J. Biol. Chem. 125, 183, 1938.

196. Baker, J.C., Parker, H.K. and Mize, M.D. Cereal Chem. 21, 97, 1944.

197. Balls, A.K. and Hale, W.S. Cereal Chem. 17, 243, 1940.

198. Balls, A.K., Hale, W.S. and Harris, T.S. Cereal Chem. 19, 840, 1942.

199. Sinclair, W.B. and Gortner, R.A. Cereal Chem. 10, 171, 1933.

199a. Csonka, F.A. and Jones, D.B. J. Biol. Chem. 73, 321, 1927.

199b. Sørensen, S.P.L. Compt. rend trav. Lab. Carlsberg 18, no. 5, 1930.

200. Sørensen, S.P.L. Kolloid. Z. 53, 306, 1930.

201. Kunitz, M. J. Gen. Physiol. 22, 207, 1938.

202. Butler, J.A.V. J. Gen. Physiol. 24, 189, 1940.

203. Northrop, J.H. and Kunitz, M. J. Gen. Physiol. 13, 781, 1930.

204. Bungenberg de Jong H.L. and Klaar, W.J. Trans. Faraday Soc. 28, 27, 1932

205. Bungenberg de Jong, H.L. Trans. Faraday Soc. 28, 798, 1932.

206. Krejci, L. and Svedberg, T. J. Am. Chem. Soc. 57, 946, 1935.

207. Elliot, M.A. and Williams, J.W. J. Am. Chem. Soc. 61, 718, 1939.

208. Fisher, E.A. and Halton, P. J. Chem. Soc. Ind. Trans. 52, 195, 1933.

209. Sullivan, B. Cereal Chem. 17, 661, 1940.

210. McCaig, J.D. and McCalla, A.G. Can. J. Research C, 19, 163, 1941.

211. Cohn, E.J. and Edsall, J.T. Proteins, Amino Acids and Peptides. Reinhold Pub. Corp. N.Y.

212. McMeekin, T.L., Cohn, E.J. and Weare, J.H. J. Am. Chem. Soc. 57, 626, 1935.

213. Akerlof, G. J. Am. Chem. Soc. 54, 4125, 1932.

214. Small, J.C. J. Am. Chem. Soc. 41, 113, 1919.

215. Frehwald, F. Starch-Making; Scott Greenwood & Son. London.

216. Allen, P.W. Industrial Fermentation; New York Chemical Catalogue Co., N.Y., U.S.A., 1926.

217. Sandstedt, R.M. Cereal Chem. 18, 237, 1941.

218. Caldwell, M.L. and Doebbling, S.E. J. Biol. Chem. 110, 739, 1935.

219. Kneen, E., Sandstedt, R.M. and Hollenbeck, C.M. Cereal Chem. 20, 399, 1943.

220. Anderson, J.A. Enzymes and Their Role in Wheat Technology: Interscience Publishers, Inc. N.Y. 1946.

221. Laine, T.A. Ann. Acad. Sci. Fennicae. Ser. A II. Chemica, no. 11, 7, 1944; C.A. 40, 3781, 1946; original not available.

222. Smorodintsev, I.A. and Zhigalov, V.P. J. Gen. Chem. USSR 16, 125, 1946; C.A. 40, 6521, 1946; original not available.

245. Richardson, E.M. Proc. Roy. Soc. (London) B 115, 121,

246. Van Slyke, D.D. J. Biol. Chem. 12, 275, 1912.

247. Wilson, D.W. J. Biol. Chem. 56, 183, 1923.

APPENDIX I: Formol Titration

The most fundamental work on the formol titration was done by Levy. The apparently solid mathematical and physico-chemical basis of his work is likely to lead uncritical workers into applying the conditions and technique which Levy advocates to systems where they are not strictly applicable, or to overestimate the absolute accuracy of results gained while using his conditions. Hence the following discussion may be useful. In developing his theory, Levy (239) assumes that the total concentration of amino groups will be small compared with the concentration of formaldehyde, so that the free formaldehyde present at equilibrium equals approximately the formaldehyde added to the system. He further assumes that the change of dielectric constant with added formaldehyde is of no importance, contrary to the earlier opinion of Harris (240) and contrary to the opinion of Dunn and Lushakoff (241).

Activities are assumed to be expressible accurately enough by the concentrations. The calculation of values for the hydrogen ion dissociation constant of the amino group in formaldehyde (K_1) and for the dissociation constant of amino-acid anion with one and two moles of formaldehyde (K_2 and K_3 respectively) involves the assumption that at high concentrations of formaldehyde (F), the terms $K_1 K_2 F$) and (K_1) are negligible compared with ($K_1 K_3 F^2$). From the graphs presented, this assumption seems unwise for tyrosine and phenylalanine (and possibly some of the other amino-acids) at formol concentrations up to 2 or 3 M, although it appears to hold for leucine, glycine and glutamic acid.

The validity of the above assumptions and of Levy's basic theoretical treatment depends upon the linear relation of F to the function $\left[\frac{Gf}{K_1} - 1\right]\frac{1}{F}$ where pGf represents the pH at the mid-point of titration of the amino acid in the aqueous formaldehyde. The data plotted in his early paper show an excellent linear relation. However, in his selection of the titration end-point, Levy (242) arbitrarily selects a pGf of 7.9 as a fair approximation of the average pGf for an amino acid mixture. The range of measured pGf values is from about 6.65 for glycine to 8.45 for tyrosine, except arginine which has a pGf of 3.35 (243). Furthermore, Levy emphasizes throughout his work that these pGf values cannot be yet considered as describing a system where formaldehyde is reacting with the terminal amino or carboxyl groups only, but rather must be considered as applying to a system where the whole ampholyte is reacting with formaldehyde.

Furthermore, the assumption of a pGf average of 7.9 leads to the expression for the titration end-point, $pHs = 9.6 + 1/2 \log C_a$ where C_a is the total molar concentration of the amino acids being titrated. By assuming that C_a will be kept close to 0.1 M, Levy derives pH 9.1 as the proper end-point of the titration.

It is of the utmost importance to realize that this end-point is of little real significance in many practical application. The hydrolyzates of such proteins as gluten, gelatin, histones, etc., cannot be considered as representative amino acid mixtures, and the assumption of an average pGf of 7.9 is open to question in such cases. When working with intact proteins, where the α-carboxyl and α-amino groups are not being titrated, but only the side-chain groups, the

pGf values of terminal groups may be radically different, and the average pGf for these terminal groups only will probably be quite significantly different. Furthermore, the end-point is quite sensitive to concentration of amino-groups. Thus, if the concentration of free amino-groups is of the order of .01 M, as it often is with proteins, the end-point becomes pH 8.6. For such work the comparative studies of assay methods by Van Slyke and Kirk (244) and Richardson (245) are still of fundamental importance.

Whenever the formol titration was used in this study, 5.0 ml. of the solution which was being assayed was adjusted to pH 7.0 with a few drops of 1.0 N and 0.1 N sodium hydroxide or hydrochloric acid. Three ml. of 37 per cent formaldehyde, freshly filtered through basic magnesium carbonate, was added, and the solution or mixture titrated carefully to pH 9.1 with 0.1 N sodium hydroxide. This end-point has the advantage of being just past the buffering region for phosphate; if borate buffer is being used an end-point of pH 9.1 offers no advantage. With the low concentrations of amino groups and high concentrations of buffer that are common in gluten solutions, blanks to correct for the effect of buffer and starch are always advisable.

APPENDIX II: Van Slyke Deamination Technique

The estimation of free amino-groups by deamination with nitrous acid, according to the general procedure of Van Slyke, is well known (246). Certain interesting points were observed in the attempt to use it for extremely dilute solutions. In this study, the method had to be adapted to follow changes in solutions containing only 0.005 micromoles of free amino-group per ml. of solution.

The apparatus used was the Eimer and Amend Scientific Co. modification of the Van Slyke apparatus. The "macro"-model accomodating 5.0 ml. of solution for analysis, was used, except that the usual 30 ml. burette was replaced with a "micro"-burrette of 3.0 ml. capacity.

When the 35 ml. of 30 per cent sodium nitrite was allowed to run by gravity from the nitrous acid reservoir into the glacial acetic in the deamination chamber, a slight variability in degree of mixing occurred. If the reagents were mixed in a flask or cylinder by one or two quick shakes, and then quickly transferred to the deamination chamber, a more constant blank could be obtained.

The age of the nitrous acid mixture also affected the blank. A few trials indicated that both the amount of nitrogen liberated and the ratio of nitrous oxide to nitrogen in the total gas which is given off, depends on the age of the nitrous acid solution. The blank decreased with increased age of the nitrous acid mixture, as is indicated by calculating from the data by Wilson (247). According to this data the blank becomes fairly constant after the nitrous acid has aged for about 10 minutes. To shorten the method, therefore, an ageing period of exactly 3 minutes was used. The blank is only slightly higher than

with longer ageing periods, and with exact timing the blank is quite reproducible..

The addition of caprylic alcohol to prevent foaming caused considerable variability in the blanks. Also, when deamination is complete it is advisable to displace the gas from the deamination chamber by adding water. According to the usual procedure, partly spent nitrous acid solution is used for the displacement, but this causes a noticeable variability in the blanks. For the final measurement of volume, it is customary to bring the absorbing solution of permanganate over to the stop-cock of the gas burette. More accuracy is obtained by adjusting the meniscus of the permanganate to a mark on the capillary tubing near the stop-cock of the gas burette. This may be done conveniently by means of a screw pinch-cock on the rubber tubing attached to the levelling bulb.

By means of such modifications it is possible to make a complete measurement in 10 to 12 minutes, with a reproducibility between $\pm$ .01 on gas volumes of 0.50 ml (blank) to $\pm$ 0.02 for gas volumes of 3.00 ml (full micro-burette).