tion of the sterols in soybeans; in West Germany, investigations of the chemical changes occurring at the surface of the fat globules in stored foam-dried whole milk; in Poland, study of the antioxidant components of wood smoke used in meat curing, and the effects of methods of generation on the quantity and activity of the antioxidant components of the smoke; in Colombia, investigation of the stabilization of flavor concentrates of tropical fruits; and in the United Kingdom, investigations of the carbohydrase enzyme systems of alfalfa and their use in structural analysis of alfalfa polysaccharides.

Marketing Research Grants

In Finland, studies are under way on residues of insecticides and fungicides applied to different plant products after harvest, including stability of the residues during marketing, storage, food preservation, and the effects of pesticides on food quality; in Israel, development of a rapid, simple test for protein nutritive value of cereal grains and feeds; in Poland, flaxseed storage and chemical changes during storage; in Spain, basic studies on the constituents of rice influencing quality, and development of objective methods for measuring market quality of raw and precooked rice; and in Italy, determination of the persistence and fate of various insecticides in or on wheat during storage, milling, and baking or cooking of the products made from treated wheat.

Since the beginning of this program in 1958, the equivalent of somewhat more than $50,000,000 in foreign currency has been made available to finance agricultural research abroad. About 500 research grants have been executed. Publication of research results is encouraged. Up to the present time more than 400 research articles have been published on grant results, most of them in English. Reprints are available in the research divisions of the department sponsoring the research.

Results of Program

Many useful results have stemmed from this program. In the area of biological control of destructive insects, a great deal of progress has been made in finding predators for the balsam woolly aphid through grants in Pakistan and India. Several thousand of a dozen different parasites which are effective in parasitizing the balsam woolly aphid have been shipped to the United States and liberated in infested areas in the Pacific Northwest and in Southern Appalachia. The sugarcane borer is one of the worst insect enemies of sugarcane, corn, and sorghum along our Gulf Coast. Studies of several species of sugarcane borers in widely scattered areas of India resulted in the discovery of a number of parasites. Five species have been shipped to Florida for evaluation under our environmental conditions. Under a grant in Spain, two parasites for gypsy moths have been found and last year 30,000 were shipped to this country. This year we are obtaining 19,000 more for liberation in New Jersey.

African swine fever, a highly contagious disease of swine, has spread to Spain and Portugal in recent years and is a potential threat to our own hog industry. It is of special concern because of its resemblance to hog cholera, with which it may be confused. Under a Spanish grant, a rapid diagnostic test has been found for African swine fever, differentiating it readily from hog cholera. Under a grant in England on scrapie (a disease of sheep), the English investigators have been able for the first time to transmit the disease from sheep to goats and to confer it on mice. Since mice are more convenient test animals than sheep, this should facilitate progress in studies on scrapie.

Through this research program we have been able to strengthen and broaden enormously our search for new supplemental crops through evaluation of wild plants. Grants in Spain, Yugoslavia, Turkey, Israel, Pakistan, India, Korea, and Uruguay have given us world-wide coverage in this search. About 3000 specimens of wild plants have been shipped to our plant exploration group in Beltsville for evaluation of agronomic characteristics and are being screened by the Regional Research Laboratories for chemical utility. A number of promising wild plants with unusual components have been found.

Under a grant in England, the antioxidants in oats have been isolated and characterized. In Finland we have a large grant under the guidance of Virtanen on the components of plants which confer undesirable flavor to milk. Members of the brassica family—such as turnips and cabbage, and seedlings of wheat, corn, and rye—have been studied. Several novel and unusual components and their enzymic conversion products have been identified, including organic sulfoxides, thiocyanates, isothiocyanates, and hydroxylamines.

In a world-wide research program such as this, there is a serious problem of communication in bringing the results directly to the attention of American scientists. This symposium provides an opportunity for American scientists to obtain detailed information on a few of these PL 480 grants. Following papers illustrate some of the progress under these grants.

Received for review October 20, 1964. Accepted February 9, 1965. Division of Agricultural and Food Chemistry, Symposium on World-Wide Research Programs in Agricultural Chemistry, 149th Meeting, ACS, Chicago, Ill., September 1964.

WORLD-WIDE RESEARCH

Immunochemical Study of Wheat, Barley, and Malt Proteins

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It is often difficult to define or isolate a protein from a mixture and to prove its purity or homogeneity. In most cases, physical-chemical methods are used, such as solubility, electrophoretic migration, sedimentation, and chromatography, but several proteins in a mixture may have identical or similar physical properties and therefore be difficult to distinguish.

To define proteins simultaneously by two completely distinct criteria, we established a method, which we call "immunoelectrophoretic analysis" (IEA) (*15*). This allows one to define every protein in a mixture by its electrophoretic mobility, which depends on the number and charge of ionizable groups on the molecule, and by its immunochemical specificity, which is based on the steric configuration of certain groups on the molecule called "antigenic determinants or sites." In some cases, when the protein has certain prosthetic groups or possesses an enzymatic activity, a third definition of a constituent in a mixture can be introduced by convenient color reactions performed in the same experiment (*23*).

The principle of this method is simple

Salt-soluble proteins of wheat, barley, and malt extracts were analyzed by electrophoresis and immunoelectrophoresis. Ten, 20, and 12 independent constituents were enumerated and their relative mobilities were calculated. Some of the proteins were characterized by their enzymatic activities and two proteins of wheat were purified by chromatography. Changes in barley proteins during malting were observed. Immunochemical investigations indicate that β-amylase undergoes physicochemical changes during malting but retains its original antigenicity. No protein possessing even partial immunochemical identity with the α-amylase of malt could be detected in barley. Evidently, it is synthesized during germination. Gliadin and glutenin fractions obtained by chromatography were analyzed by immunoelectrophoresis; the several gliadin fractions were immunochemically identical. Glutenin migrates in agar gel containing 3M urea and gives an immunochemical identity reaction with gliadin.

and it needs no complicated apparatus. The sample to be analyzed is placed in a gel (generally 1% agar) and submitted to electrophoretic migration. Then a trough, parallel to the axis of this migration, is made in the gel and an immune serum is poured into it. The antibodies diffuse into the gel; when they meet the corresponding antigens, they give a specific precipitate which deposits in the form of arcs. The reaction being specific, every protein gives an independent arc.

Thus, IEA allows the enumeration and definition of every component of a mixture, the detection of even small amounts of impurities, or the comparison of components in different mixtures. We could, for example, follow the fate of some of the barley proteins during brewing and identify them in beer, or even in the "cold haze," because their immunological specificities were still present although their mobilities were modified (*10, 11, 14*).

The main disadvantages of IEA are: Immune sera are used to detect the various constituents; as an animal's responses to immunization are not constant, several immune sera have to be tried and the number of constituents detected is to be considered as a minimum.

Only soluble components can be determined. This is an important handicap in the case of some seed proteins which are normally not water-soluble, but by using $3M$ urea solution (*17*) we overcame this difficulty.

Materials and Methods

Materials. The wheat used is a genetically pure Magdalena variety; the barley is the Aurore variety, and the malt was always prepared in the same manner from this variety of barley. In our studies on the insoluble proteins we used commercial preparations of gliadin (Nutritional Biochemicals Corp.), a sample of poly-*dl*-alanine–gliadin, which is a soluble derivative of gliadin prepared by Sela (*22*), a sample of an alcohol-soluble fraction of wheat given to us by Mossé (*18*), and our own preparations of insoluble proteins.

Extraction of Soluble Proteins. The flour obtained from the seeds is suspended in a phosphate buffer containing $0.5M$ NaCl (pH 7 for wheat and pH 6.6 for barley and malt), the proportions being 1 gram of flour for 2 or 3 ml. of buffer. The suspension is stirred for 2 hours at 4° C., then centrifuged, filtered, and dialyzed against the same buffer in order to eliminate small molecules (*3*).

Extraction of Insoluble Proteins from Wheat. After extraction of soluble proteins from the flour by $0.5M$ NaCl, the insoluble residue was extracted either by acetic acid ($0.05M$, pH 3.5) as recommended by Coates and Simmonds (*7*) or by a phosphate buffer (pH 4.7, $\mu = 0.05$) containing $3M$ urea.

After extraction of soluble proteins, gliadin was obtained by extraction with 60% alcohol and glutenin was extracted from the residue of the gliadin preparation by a mixture of 20% isopropyl alcohol and 17% lactic acid as used by Pinckney, Greenaway, and Zeleny (*21*) and modified by the addition of 0.2% sodium bisulfite (*5*). After dialysis against water, the preparations were lyophilized and stored.

Preparation of Immune Sera. The immune sera were obtained by immunizing rabbits with increasing doses of solutions of the soluble proteins or of gliadin and glutenin in $3M$ urea.

Simple Electrophoresis (EA) or Immunoelectrophoretic Analysis (IEA) was performed in agar gel. In the case of insoluble proteins, solutions in $3M$ urea were submitted to electrophoresis in agar gel containing $3M$ urea. At the end of the electrophoretic migration, the gel is washed in order to eliminate the excess of urea, which would inhibit the formation of the specific precipitate.

Fractionation of Extracts. Several techniques have been used: ammonium sulfate precipitations, preparative electrophoresis in agar gels, Sephadex, and diethylaminoethylcellulose, and carboxymethylcellulose chromatography. As eluents in this last case and in our studies on insoluble proteins of wheat, the three following have been utilized: acetate buffer (pH 3.8) in $0.2M$ NaCl, phosphate buffer (pH 11.5) in $0.2M$ NaCl, and $0.1M$ NaOH (*8*).

Results

Total Extracts of Soluble Proteins. Simple electrophoresis in agar of barley and malt extracts shows modifications due to the malting. Figure 1 represents the results of simple electrophoresis (at the top) and a schematic representation of zones of migration in which we found proteins and also some enzymatic activities. Several differences are easily seen, such as the concentration of protein in different zones and particularly in the amylase activities. The β-amylase activity has a much larger zone with malt than with barley extracts and α-amylase activity has been observed only in malt. This activity corresponds to a mobility of around minus 3×10^{-5} sq. cm. volt^{-1} sec.$^{-1}$; in this zone we also observe a protein stain in the malt but nothing in the barley extracts. These results suggest either that the barley contains an inactive precursor of α-amylase possessing a different mobility, or that this enzyme is synthesized *de novo* during malting. This question could be answered by the use of immunochemical techniques.

IEA has allowed us to distinguish at least 20 different proteins in barley, about 12 in malt, and about 10 in wheat extracts (*12*).

Figure 2 shows the quantitative distribution of barley proteins and the position of the precipitin arcs in different zones of electrophoretic mobility. Figure 3 shows a comparative IEA of barley and malt developed with the same antibarley immune serum. The diminished number of arcs and the alterations in their positions prove that the malting process has modified several of the proteins (degradation, changes in solubility, in mobility, etc.). Thus, for example, the appearance of a new arc in zone 1 seen with the malt and not with the barley shows that it must correspond to a protein which exists in the barley, because it reacts with an antibarley immune serum, but possesses a faster mobility in malt.

Figure 4 shows that extracts of wheat grains and of flour possess the same components and that their mobilities are practically the same. The only apparent difference was that the extract of grains contained a lipoprotein in zone 1, which was stained by Sudan black. As

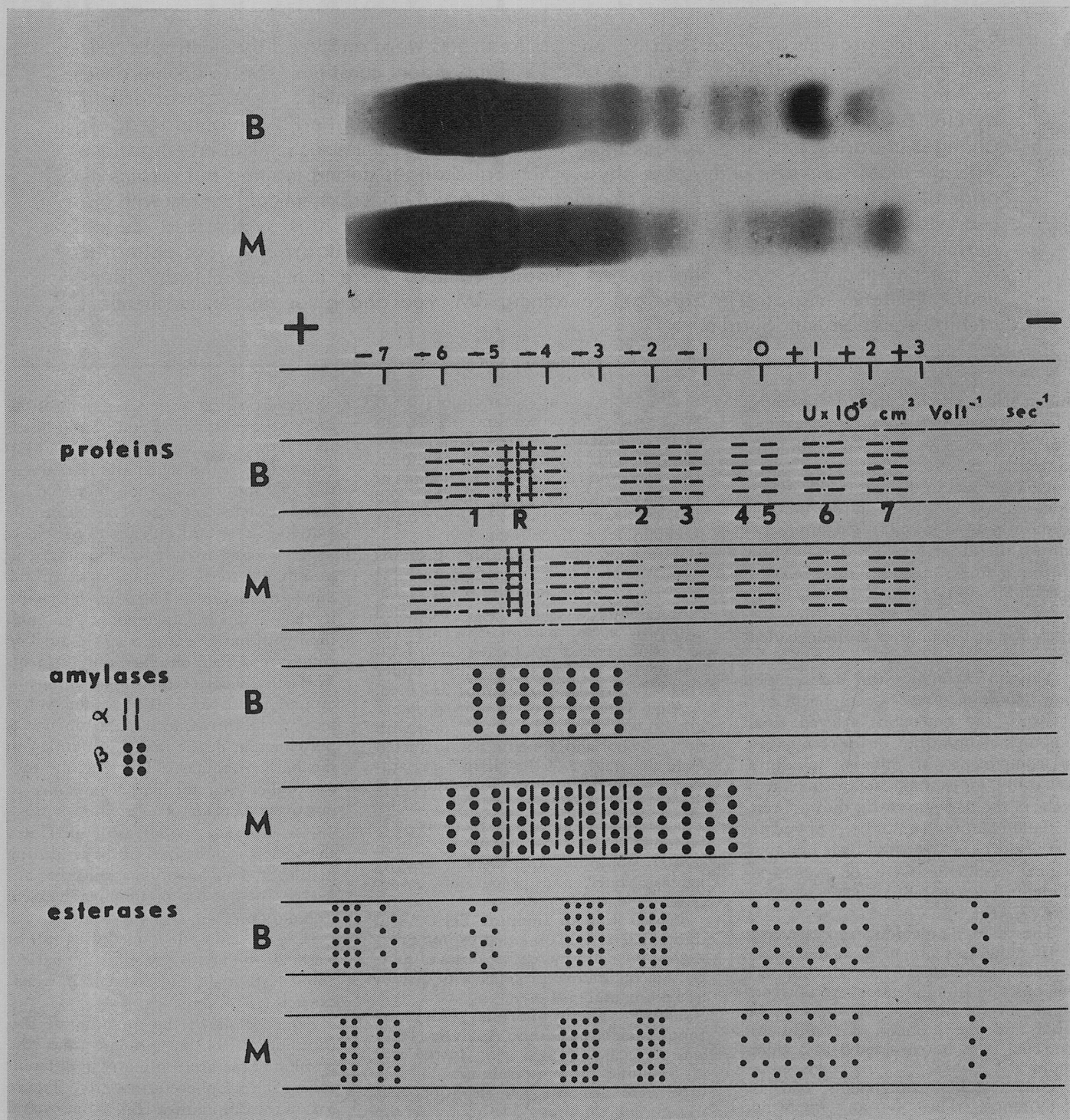

Figure 1. Total extracts of soluble proteins

Top. Photograph of electrophoresis in agar gel, veronal buffer, 0.05*M*, pH 8.2, of extracts after staining with amido black
B. Barley
M. Malt
Partition of proteins, amylase, and esterase activities
1, 2, 3, etc. Electrophoretic migration zones
R. Starting reservoir

it was not observed in the flour, it probably originates from the peripheral part of the grain which is absent from the flour.

When extracts of barley and wheat are compared, it is particularly striking that the number of different proteins is much larger in barley extracts than in wheat.

Some years ago, Hall (*16*) observed in this laboratory by IEA that hybrids of wheat and rye contain proteins of both parents. Some comparative examinations of our extracts from barley and wheat have been performed using immune sera against barley and against wheat. Each extract was analyzed with the homologous immune serum and also immune sera specific for the other species. Figure 5 shows that, in each case, several of the proteins react with the antibodies specific for the other extract. Thus we may conclude that these proteins are similar and probably even identical in these two seeds.

Studies on Fractions. Various techniques of fractionation of barley and wheat proteins have been published

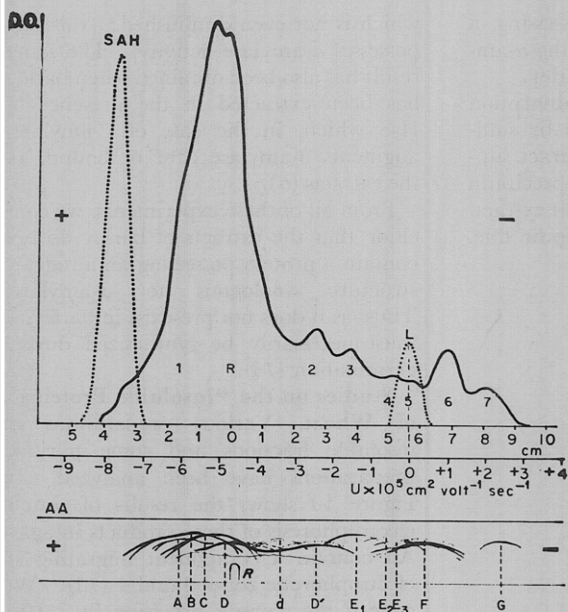

Figure 2. Distribution of barley proteins and position of precipitin arcs

pH 8.2
Upper. Densitometric curve of electrophoretic diagrams after staining. ——— Barley extract. – – – Levan (L) and human serum albumin (SAH). *R*, starting reservoir. 1, 2, migration zones.
Lower. IEA, letters designate groups of precipitin arcs with same mobility

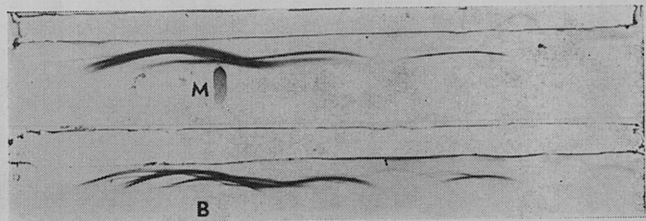

Figure 3. Immunoelectrophoretic analysis of barley and malt extracts performed under same conditions with anti-barley serum

pH 8.2 *B.* Barley *M.* Malt

(*12, 14*). Using our method of analysis, we have established that fractions obtained by ammonium sulfate precipitation of barley extracts, as described by Quensel, are all heterogeneous. The fraction which precipitates at 15% saturation could be subfractionated. Two proteins from wheat have been isolated in an immunologically pure state, as shown in Figure 6. Their diffusion coefficient has been determined by the immunochemical method of Allison and Humphrey (*1*).

Moreover, we have proved that, for barley as well as for wheat, the characterization of the proteins as albumins and globulins by their solubility in water is not a sufficient criterion (*12*).

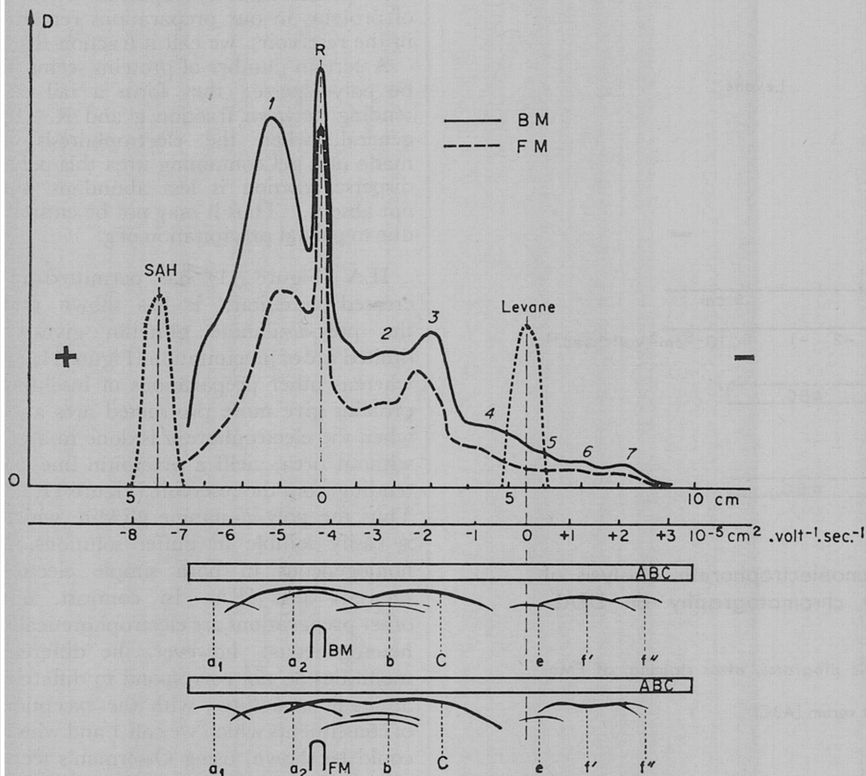

Figure 4. Electrophoretic and immunoelectrophoretic comparison of extracts

pH 8.2
BM. Wheat
FM. Wheat flour
Upper. Densitometric curve of electrophoretic diagram after staining of BM, FM, human serum albumin (SAH), and levan (L)
1, 2, etc. Migration zones
Lower. IEA of BM and FM with antiwheat serum (ABC)

Studies on Amylases in Barley and Malt. The following properties of α- and β-amylases have been used for their identification: α- and β-amylases hydrolyze amylose, which is colored blue by iodine, giving, respectively, dextrin and maltose which do not stain (*4*). The activity of β-amylase is more easily inhibited by small amounts of heavy metal salts than is the activity of α-amylase (*9*).

Using these properties and an antimalt immune serum, we could identify those arcs of specific precipitation in the IEA which correspond to the α- and β-amylases in malt extracts and a β-amylase in barley extracts (Figure 7).

The mobilities of these amylases have been calculated, as has the diffusion coefficient of the α-amylase from malt, and it has been confirmed that the β-amylase from malt is soluble in distilled water whereas the α-amylase is not. On the other hand, the β-amylase from barley is less soluble in water and migrates faster than that from malt (Figure 7). But these β-amylases possess the same antigenic properties, which proves that they have similar stereochemical configurations. Thus, it seems that it is the same molecule which undergoes during malting, and probably during the first days of germination, transformations which modify some of its physical-chemical properties.

We could not detect an α-amylase activity in barley extracts. If an inactive precursor of this amylase were present in barley, it should have an antigenic structure analogous to the active α-amylase of malt. This possibility has been tested in several experiments. Figure 8 shows that a specific precipitin line possessing α-amylase activity is formed between a malt extract and an antimalt immune serum. This line is not deflected by an extract of barley, as would happen if this extract contained

even very small amounts of an antigenic substance of analogous configuration. Moreover, this experiment shows that the antibarley immune serum does not contain antibodies which can react with the α-amylase. Even small amounts of an antigen may provoke formation of antibodies, and as that did not occur here, it may be used as an additional argument that a protein possessing a structure analogous to that of the α-amylase of malt is absent from barley.

As shown in Figure 9, the absorption of an antimalt immune serum by sufficient quantities of a barley extract suppresses the formation of all the precipitin bands which it forms with a malt extract, with the exception of one precipitin line, which is not even diminished; this line possesses α-amylase activity. The same result has also been obtained when barley has been extracted in the presence of H_2S which, in the case of β-amylase, augments β-amylase (free or bound) in the extracts (*6*).

From all of these experiments, we conclude that the extracts of barley do not contain a protein possessing an antigenic structure analogous to α-amylase. Thus, as it does not pre-exist in barley, it must necessarily be synthesized during germination (*13*).

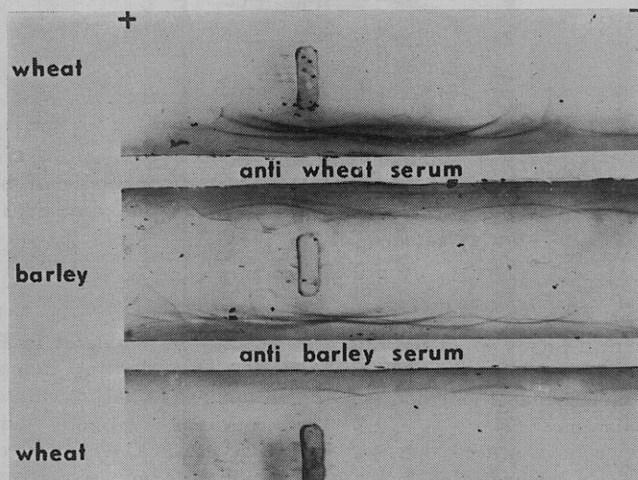

Figure 5. Immunoelectrophoresis of barley and wheat extracts performed with antibarley and antiwheat sera at pH 8.2

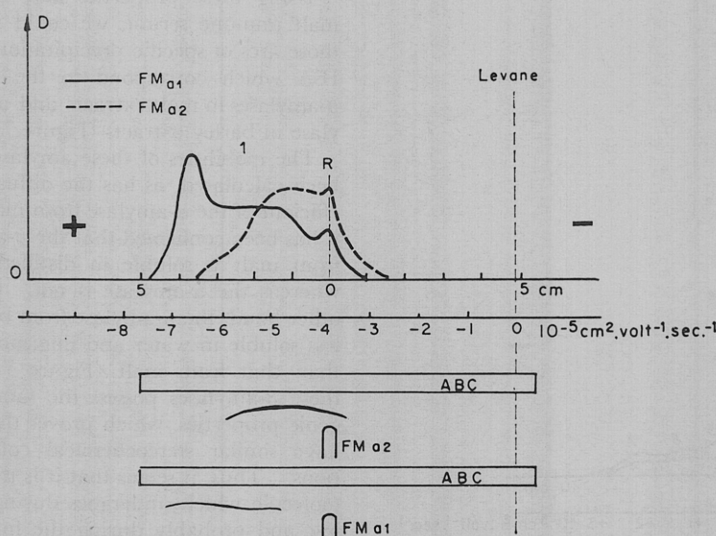

Figure 6. Electrophoretic and immunoelectrophoretic analysis of wheat flour protein separated by chromatography on DEAE-cellulose

Upper. Densitometric curve of electrophoretic diagrams, after staining, of FMa_2 (second peak) and FMa_1 (third peak)
Lower. IEA of FMa_2 and FMa_1 with antiwheat serum (ABC)

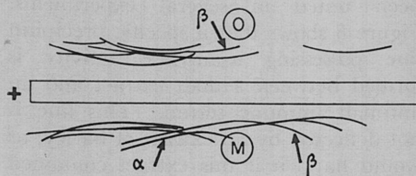

Figure 7. Immunoelectrophoretic analysis of saline barley (O) and malt (M) extracts developed with antimalt serum

Arrows indicate precipitin lines corresponding to α- and β-amylases

Studies on the "Insoluble Proteins" of Wheat. Various preparations of insoluble fractions and some purified preparations have been analyzed (*2*). Figure 10 shows the results of simple electrophoresis of these products in agar. All contain a component migrating in electrophoretic zones 3 and 4 (*12*). We gave it the name g provisionally. It is the unique component of the poly-*dl*-alanine gliadin and the main constituent of all the other preparations. But they contain also in varying proportions:

A component, which we designate provisionally as 1 and which migrates in zone 1 (*12*), was shown to be at least partially composed of proteins present in the "soluble protein" fraction. Its presence is due to incomplete extraction of soluble proteins during preparation of the insoluble proteins.

A second relatively important fraction of proteins in our preparations remains in the reservoir; we call it fraction R.

A certain number of proteins seems to be polydisperse; they form a tail extending between fraction g and R. In general, when the electrophoresis is made in a gel containing urea this polydisperse fraction is less abundant but not absent. Thus it may not be entirely due to partial precipitation of g.

IEA (Figure 11) has permitted increased precision; it has shown that the poly-*dl*-alanine glutenin gives a limited arc of precipitation (Figure 11, 3), whereas other preparations of insoluble proteins give more protracted arcs and, when the electrophoresis is done in a gel without urea, yield a precipitin line extending from the reservoir (Figure 11, 2). Thus the poly-*dl*-alanine gliadin, which is easily soluble in buffer solutions, is homogeneous in both simple electrophoresis and IEA. In contrast, our other preparations are electrophoretically heterogeneous; however, the different mobilities do not correspond to different antigenic structures, with the exception of constituents which we call 1 and which could be shown, using Osserman's technique (*19*), to correspond to "soluble proteins."

The antigenic identity of the components of our various preparations of insoluble proteins could be confirmed by the double diffusion method (*20*), as shown in Figure 12; the commercial gliadin (1), our preparation of insoluble

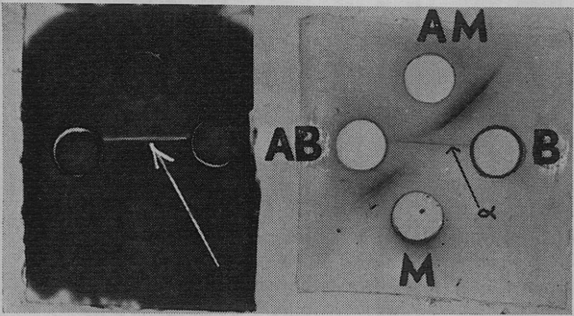

Figure 8. Double diffusion of barley (B) and malt (M) extracts with antibarley (AB) and antimalt (AM) sera

Left. Characterization of α-amylase
Right. Protein staining of precipitin lines
Same configuration used for both parts of figure

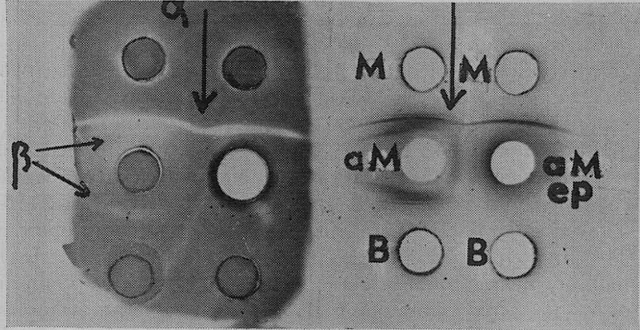

Figure 9. Double diffusion of barley (B) and malt (M) extracts with antimalt serum (AM) and same antimalt serum absorbed with barley flour (AMep)

Left. Characterization of α-amylase
Right. Protein staining of precipitin lines
Arrows indicate precipitin lines corresponding to α- and β-amylases

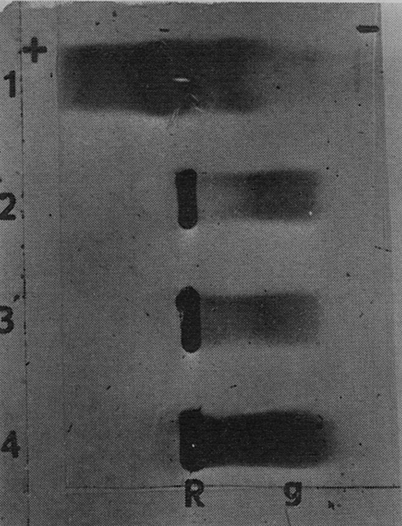

Figure 10. Electrophoresis in agar gel with veronal buffer and 3M urea of proteins of wheat

pH 8.2
1. Salt solution extract
2. Alcohol extract
3. Gliadin NBC
4. Extract in phosphate buffer at pH 7 with 3M urea

g. Major constituent of "insoluble proteins"
R. Starting reservoir

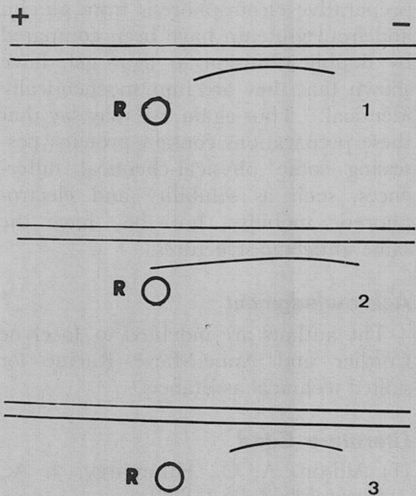

Figure 11. Immunoelectrophoretic analysis in veronal buffer developed with anti-"insoluble protein" serum

pH 8.2
1. Poly-*dl*-alanine gliadin
2. Extract of insoluble proteins
3. Same as 2 but electrophoresis performed in veronal buffer with 3M urea

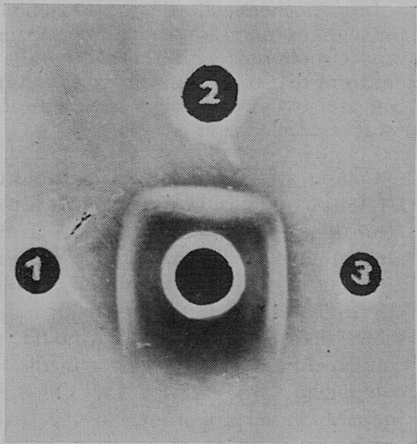

Figure 12. Double diffusion according to Ouchterlony, developed with gliadin serum

1. Commercial gliadin NBC
2. Extract of insoluble proteins
3. Alcohol extract according to Mossé

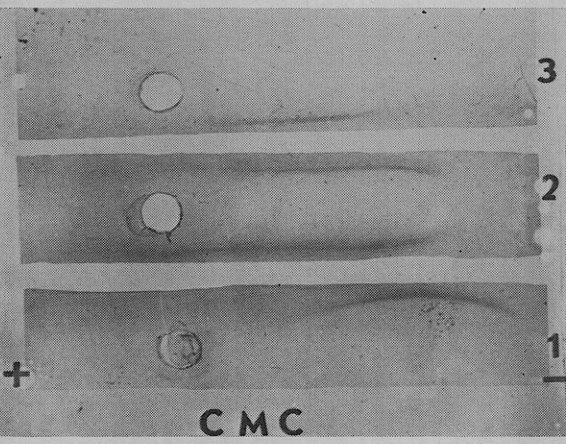

Figure 13. Immunoelectrophoretic analysis developed with antigliadin serum with 3M urea of three fractions of gliadin obtained by chromatography on carboxymethylcellulose

1, 2, 3. Fractions eluted with buffers of corresponding numbers

Figure 14. Determination of gliadin (GLI) and glutenin (GLU) in veronal buffer

Left. EA
Right. IEA
pH 8.2, 3M urea

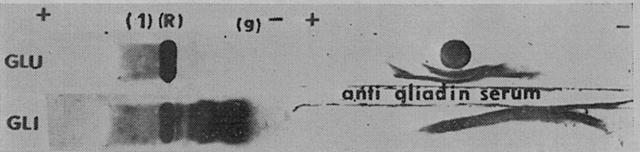

Figure 15. Determination of gliadin (GLI) and glutenin (GLU) in phosphate buffer

Left. EA
Right. IEA
pH 8.2, 3M urea

proteins (2), and a preparation of alcohol-soluble proteins of Mossé (3) give confluent precipitin arcs. The same result is obtained with other preparations of insoluble proteins, gliadin, and poly-*dl*-alanine gliadin.

Chromatography on carboxymethylcellulose of a commercial gliadin has allowed us to obtain three subfractions. The first eluate (Figure 13, 3) has a mobility nearer to the reservoir; however, all three subfractions are antigenically identical.

From these experiments, we conclude that:

All preparations of insoluble proteins and of gliadin contain a common antigenic constituent, g.

Constituent g is the only protein present in poly-*dl*-alanine gliadin. It is the most important constituent in all other preparations.

Immunochemical identity reactions prove that protein constituents appearing in electrophoresis with a mobility different from that of g, with the exception of component 1, nevertheless have a structural resemblance to g.

Comparative Studies on Gliadin and Glutenin. Figure 14 shows comparative electrophoretic and IE analyses of an alcohol-purified preparation of gliadin and of a glutenin preparation. Simple electrophoresis (left side of Figure 14) shows that the gliadin is rich in the g component and relatively poor in the R component, whereas for the glutenin preparation the proportions are reversed.

Chromatography on carboxymethylcellulose separates this gliadin into two subfractions which appear in the first and second eluates. With the glutenin also we obtained two subfractions, but they are eluted with the second and third eluents.

The IEA (right side of Figure 14) shows a protracted line of precipitation for both preparations, naturally more pronounced in the case of gliadin. When these two preparations are compared by double diffusion, they give a reaction of antigenic identity.

If the electrophoresis is done not at pH 8.2, as previously, but at pH 6.5 and always with $3M$ urea, the aspect is somewhat different in simple electrophoresis (Figure 15, left), but in IEA again we see one main protracted line with the gliadin, whereas the glutenin seems to present an additional factor, migrating as a short line (Figure 15, right). But as this analysis has been made with an antigliadin immune serum, both precipitin lines given by the glutenin must correspond to constituents present in the gliadin. Some preliminary experiments, in which fractions obtained by preparative electrophoresis from gliadin and from glutenin have been compared by double diffusion in agar gel, have shown that they are immunochemically identical. Thus again, we may say that these preparations contain proteins possessing some physical-chemical differences, such as solubility and electrophoretic mobility, but they have the same antigenic structures.

Acknowledgment

The authors are indebted to Jocelyne Cordier and Anne-Marie Racine for skilled technical assistance.

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Received for review November 16, 1964. Accepted July 12, 1965. Division of Agricultural and Food Chemistry, 148th Meeting, ACS, Chicago, Ill., September 1964. Work supported in part by grants from the Foreign Research and Technical Programs Division, U. S. Department of Agriculture, and the Syndicat de la Brasserie Française.