SEROLOGICAL PREDICTIONS OF GENETIC RELATIONSHIPS AMONG OAT VARIETIES AND CORN INBREDS

by

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INTRODUCTION

One of the foremost problems in any plant breeding program in which hybridization is employed is the task of predicting the most desirable crosses to make. For example, the small grain breeder wants the cross that will produce the maximum frequency of superior segregates, and the corn breeder wants to know which two inbreds when crossed will produce the single cross with maximum vigor. At present the only means of securing this information is to make a series of crosses and in the case of small grains, to study the magnitude of variability in each for one or more segregating generations, and for corn, to assess the vigor of the single crosses. Any method for efficiently predicting such information prior to the making of a series of crosses would be of inestimable value to plant breeders in (a) saving time and financial resources spent on assaying worthless crosses, (b) allowing more rapid utilization of desirable crosses, and (c) permitting the assaying of more germplasm for usefulness.

In general, it is considered that the magnitude of segregation in small grain crosses and vigor in corn single crosses is directly related to the degree of genetic diversity of the parents. Obviously, a direct chemical analysis of the genetic material, desoxyribonucleic acid (DNA), is not possible at present, and even if it were, the information might not

be of much practical value because of the complex interaction and pleiotropic effects of genes expressed in quantitative characters. However, because of the unique and proximate sequence of reactions leading from DNA to protein synthesis there should exist a crude index for the genetic information in the protein complex produced by a particular genotype.

There are numerous methods for qualitatively studying individual proteins, but serology is probably the best single method for tagging a complex of proteins and assigning each a unique identity. The study of antigen-antibody reactions constitutes the methods of serology. An antigen is a unique compound (such as a protein) characterized by large molecules, specificity, and capacity to stimulate the production of a modified form of serum globulin when injected into an animal. The modified globulin is called the antibody which is in turn specific in reactivity for the antigen which caused its production. The relation of an antigen to its homologous antibody is determined by the stereochemical configuration of each. When placed together in an electrolytic solution they unite in what has been pictured as a "lock and key" arrangement and precipitate. This is termed the precipitin reaction. Certain molecular and reaction peculiarities tend to make the serological technique complex and eccentric. A protein molecule may have several antigenic sites some of which are specific and some of which are common to several protein types; thus anti-

bodies may react with antigens which are not responsible for their production. This cross reaction leads to errors of estimation. In addition, there is in each system an optimal-proportion phenomenon which yields maximum precipitate only when the proper antigen and antibody concentrations are mixed. This is called the optimal proportion point or equivalence zone.

Serology, based on these principles, has been used sporadically for about 60 years in plant systematics. It is conceivable that the techniques and principles used for intra-family or -generic studies in plant systematics, if refined somewhat, could be used to distinguish between genotypes within a species. Success of this idea could provide the plant breeder with a technique or "tool" with which to estimate the genetic relationship between varieties of small grains or inbreds of corn; thus making it possible to predict the most desirable crosses.

The specific objectives of this study were:

1. To predict with the use of serological techniques the degree of genetic relationship among seven oat varieties and among four corn inbreds and to correlate this information with indices of variability and vigor for agronomic characters in the oat and corn crosses, respectively.
2. To study serologically the qualitative differences

of the proteins among the oat varieties and among the corn inbred lines.

REVIEW OF LITERATURE

Relation of Protein to Genotype

Genetic control of production of a specific enzyme (protein) was first conceived in 1909 by Garrod (1909), according to Srb and Owen (1957), in an explanation of the genetics of the disease, alcaptonuria. However, proof of this idea was not presented until 1941 when Beadle and Tatum (1941) used their data from Neurospora crassa studies to compound the "one gene - one enzyme" theory.

Although now modified, the "one gene - one enzyme" theory generally connotes that a single gene controls the production and presence of a specific enzyme. The theory has been supported by data from many studies including some on fungi (Bonner (1946); Suskind et al. (1955)), bacteria (Umbarger and Mueller (1951)), higher plants (Langridge and Brock (1961); Schwartz (1960)), and animals (Ingram (1957); Anfinsen (1959)). Most of these studies have related mutations or genetic changes (usually at a single locus) to a resultant "mutant" or altered protein. Anfinsen (1959) presented a partial list of altered proteins attributable to mutations. The alterations noted were based on differences between the original and mutant type proteins in electrophoretic mobility, heat stability, and antigenic specificity. However, as Anfinsen (1959) pointed out, even though charge and antigenicity are specific

they may be influenced by small organic molecules tightly bound to the protein and polysaccharides or haptenes, respectively. Hemoglobin provides an example where an unequivocal difference in chemical composition between the original and mutant forms is known to exist. Of course, many proteins are not easily analyzed for intra-molecular sequence either because of their complexity or the extremely small quantities in which they occur, e.g., proteins from microbial sources.

Ingram (1957) demonstrated that the difference between normal-adult and sickle-cell hemoglobin was due to the substitution of a single amino acid which was under the control of a single gene locus. Recently, additional hemoglobins have been discovered which also are monogenically inherited and have one or several substituted amino acids (Ingram (1961)). From the fact that the protein portion of a hemoglobin molecule contains two α and two β polypeptides (the two polypeptides of normal-adult hemoglobin), each controlled by one gene, Ingram restated the "one gene - one enzyme" theory as "one gene - one polypeptide". Earlier, Benzer (1957), who had done much to elucidate the chemical nature of the gene, suggested that one gene (one functional unit) may control all the steps leading to one end product, or simply the synthesis of one enzyme, one peptide, or position of one amino acid in a protein.

Much of the research on protein synthesis supports the

concept that proteins are representative of the genotype. Zamecnik (1960) has presented an extensive discussion of the intermediary metabolism of protein synthesis. The current concept proposes that the DNA acts as a template for producing a complementary RNA (ribonucleic acid) found in the microsome. The complementary RNA acts as a template upon which transfer RNA molecules may orient themselves in the proper sequence. Each type of transfer RNA is specific for a certain amino acid so the sequential orientation on the template gives a coded linear sequence to the amino acids which then form a specific protein.

Of course, a mutation which results in a changed protein due to an amino acid substitution may not alter the antigenic specificity of the protein, since the site with antigenic capacity may not correspond to the site of amino acid substitution. In such a case the change would not be detected serologically.

Serology in Plant Systematics

Most of the studies in the application of serology to plant systematics were based upon the precipitin reaction first discovered and reported by Kraus (1897), according to Carpenter (1956). Nuttall (1901) employed the precipitin reaction in systematics to demonstrate a relationship between man and monkey, and ox and sheep using serum antigens. (Ac-

cording to Boyden (1942), Bordet (1899) first proposed the use of serology in systematics, but with the agglutination reaction.)

Chester (1937) presents an excellent review of systematic plant serology encompassing the period up to the date of his publication. Consequently, an extensive review of this material will not be considered here. Chester (1937) credits Kowarski (1901) with the first work in plant sero-systematics. The latter investigator found that antisera to heat-resistant wheat albumoses (polypeptides) reacted strongest with wheat albumose and only to a limited extent with albumoses of rye, barley, oats, and peas.

Nelson and Birkeland (1929) were the first workers to attempt to differentiate between varieties within a species. Their serologic ranking of the varieties paralleled somewhat the genetic relationship based upon parentage. It is of significance that their stated purpose in ranking the wheat varieties was to aid plant breeders.

Interest in plant sero-systematics lagged from Chester's review (1937) until Hammond (1952; 1955) reported on work done in classifying genera within the Ranunculaceae and Solanaceae families, respectively. Johnson (1954) and Baum (1954) also published on a serological breakdown of the Magnoliaceae and Cucurbitaceae, respectively.

Urano (1955), working with inbred lines of corn, demon-

strated a stronger serological reaction between two lines separated in the S_{10} generation than between either of these lines and a third one separated from the same source in the S_3 to S_5. Similar results were presented by Yamasaki et al. (1957). In addition, Yamasaki et al. (1957) made single crosses between inbred lines of corn derived from Japanese flint and Corn-Belt dent types. Heterosis indices and phylogenetic relationship indices were calculated from the grain yield and the number of hetero-pairs of chromosome knobs, respectively, in the single crosses. These indices were correlated with the amount of precipitin between parents used in making the single crosses. A high negative correlation was found between the heterosis indices and quantities of precipitin and between numbers of hetero-pairs of chromosome knobs and quantities of precipitin. A high positive correlation existed between the heterosis indices and numbers of hetero-pairs of chromosome knobs. When they used races of corn as parents the corresponding correlations were not significant.

In a later study Urano (1959) also found a high negative correlation between heterosis and the amount of precipitin reaction between the parental inbred lines. There was also a strong precipitin reaction between the single cross and either parent even in cases where the reaction between the parents was weak. Also, the backcross and the recurrent parent gave a stronger reaction than did the single cross and the same

parent. Based on his research experience Urano suggested that the precipitin reaction should make it possible to predict the degree of hybrid vigor in a single cross of two corn inbreds without making the cross. Davidson and Thompson (1956) found a good correlation between serological data and genealogy of lines of dent corn. However, the serological relation of dent and pop corn was inconsistent with the genealogy.

Urano (1959) found that the antigenicity of corn pollen was manifested primarily in the albumin and β- and γ-globulins. However, only the albumins and α-globulins varied in quantity between lines suggesting that the phylogenetic specificity was restricted to the albumins.

Gell et al. (1960) were able to separate species within the genus Solanum using immunoelectrophoresis. Hall (1959), using the same technique, compared the proteins of rye, wheat, and their allopolyploid hybrid, ryewheat. The wheat and ryewheat were indistinguishable, but the latter was deficient in some of the rye-specific proteins. No new proteins peculiar only to the ryewheat were found.

MATERIALS AND METHODS

Materials

The biological materials used for this study consisted of (a) 7 oat varieties and certain crosses among them, and (b) 4 corn inbreds and all possible single crosses among them. Six oat varieties, Bonham, Andrew, Nemaha, Richland, Minland, and Mo. 0-205, were crossed to Cherokee during the winter seasons of 1953 to 1955. These 6 varieties, in the order named, represented descending degrees of genetic relationship to Cherokee variety based upon the common parentage between each of them and Cherokee. Of course, the exact degree of relationship of any particular variety may be more or less closely related to Cherokee than expected, depending upon the sample of genes each variety encompassed. However, this series was expected to give a range of relationships whether in the predicted order or not. In 1956, a sample of F_2 seeds from each cross was space-planted in the field. The seeds used in each cross were taken from a single F_1 plant. At maturity, seed was harvested from each of 90 random F_2 plants in each cross. In 1957, 60-70 seeds from each F_3 progeny were sown in a progeny row, and at maturity the whole row of each line was harvested and threshed separately. In addition, 30 lines from Cherokee and 5 lines from each of the other parent varieties were treated in the same way as the segregates from

the crosses.

In 1958, the 540 F_4 generation lines and the 60 parental lines were grown in an experiment at Ames in a randomized block design with 4 replications. Each plot was a hill sown with 30 seeds, which approximated a seeding rate of 3 bushels per acre. The hills were spaced one foot apart in perpendicular directions and the whole experiment was bordered with 3 rows of hills.

The data recorded from this experiment were for the attributes, heading date, height, and yield. Heading date was recorded on a plot when panicles on half of the culms had emerged from the sheath. Heights were recorded prior to harvest in inches from the ground surface to panicle tips. At maturity the plots were harvested and threshed, and the grain yield per plot was recorded in grams. All of these data were transcribed onto punch cards, and the analyses of variance used in the estimation of variance components were calculated on I.B.M. equipment.

For the other portion of the study 2 sets of 2 sib inbred lines of corn were obtained from Dr. W. A. Russell, Ames. Lines 1 and 2 were derived from (N32 x B14) B14. Two selfing generations followed the BC_1 generation at which time inbreds 1 and 2 were isolated, and each was then selfed an additional generation. Lines 3 and 4 were separated after one generation of selfing in Pennsylvania Late Synthetic. Before being used

in this study each was selfed 3 more generations.

In 1960 all possible single crosses including reciprocals among these 4 inbred lines were made, and in 1962 these 12 crosses were grown in a randomized block design at Ames utilizing 10 replications. A plot consisted of 3 rows 40 inches apart with 10 plants spaced 20 inches apart in the row. This approximated 8000 plants per acre.

Only the center row of each plot was harvested. The border rows were necessary because of anticipated differential border effects due to differences in height, general vigor, and maturity between crosses. Some plants had to be transplanted into the center rows to maintain the desired population. These plants were considered competitive but were not harvested for yield determinations: thus there were unequal numbers of plants per plot.

At maturity the plants in a plot were cut at ground level, put into a burlap bag, and dried to constant moisture. The total plant weight and ear weight per plot were recorded and adjusted to a per-plant basis to be used as an index of the hybrid vigor. The data from reciprocals of each cross were averaged for the purpose of analysis.

The antigen source for the corn and oat materials was pollen. The pollen was collected from field plantings and after drying at room temperature was stored in desiccators for later use. Collecting sizeable quantities (25 to 100 g) of corn

pollen was easy, but the quantity of oat pollen collected from each variety ranged from 3 to 8 g.

The oat pollen was collected from drill-widths 150 feet long. Two methods of collection were used: (a) During anthesis several culms were grasped below the panicle, carefully bent over and shaken into paper bags; (b) In the forenoon bundles of culms were covered with eighth-barrel paper bags and loosely tied. After anthesis each bundle of culms was bent over and shaken vigorously to insure maximum pollen collection after which the bag was carefully removed. Factors affecting pollen collection were the general stage of anthesis for the variety, temperature, humidity, and wind. Hot, humid days with very little breeze were best suited for pollen collection. Any significant air movement precluded pollen collection by method (a).

Methods

Production of antisera

Six pairs of the Hartley Strain of guinea pigs were obtained from the Public Health Service in Bethesda, Maryland. Offspring from these were used for the production of all oat antisera and for two antisera samples of each of the 4 corn inbreds. Protein extracts of pollen were used for both the oat and corn antigen. The antigen for injection was prepared according to the incomplete adjuvant technique (water-in-oil

emulsion, Freund (1948)) giving an emulsion with a final concentration of 1% protein. The pollen was ground first dry and then in 0.1 M phosphate-buffered physiologic saline solution (pH 6.7), followed by incorporation into the emulsifier, Falba (Pfaltz and Bauer, Inc. New York), and paraffin oil.

Healthy guinea pigs ranging in weight from 300 to 600 g were given simultaneous intra-muscular injections in each thigh with 0.25 ml of the antigen-adjuvant mixture. Just prior to the injection, 5 ml of blood were drawn from the heart of each guinea pig to serve as a control. One month after injection 6-8 ml of blood were taken from each guinea pig, and subsequent drawings were taken at weekly intervals. The blood was refrigerated overnight in a capped test tube coated with stopcock grease to facilitate the clot to pull away from the sides of the tube. The serum was then decanted, spun at 20,000 x g for 30 minutes in a refrigerated centrifuge, and stored until used at -17° C.

Animals which did not produce a satisfactory titre after the first injection were given a second identical injection. If the second injection did not raise the titre adequately, the animal was discarded. In guinea pigs which produced good titre, booster injections were given as needed to maintain good antisera production. Finally, antisera were obtained from two guinea pigs for each oat variety and one guinea pig for each corn inbred.

To determine the specificity of antiserum taken after a short immunization period, antigens from each corn inbred were injected into 2 guinea pigs (250 g each) using the procedure described above. A second injection was given to each pig after 2 weeks. Blood was taken 3 weeks after the initial injection, and the animals were sacrificed after one month. The serum from both lettings from each guinea pig was pooled except for inbred 1 where one of the guinea pigs died.

Chickens from the Hyline Poultry Farms in Johnston, Iowa were used for production of one antiserum to each of the 4 corn inbreds. For each corn inbred one hybrid rooster was injected with 125 mg of ca. 20% protein in 1 ml of buffered saline. The protein had been extracted previously in buffered saline and concentrated by lyophilization. Blood was taken from the heart 8 days after injection. The roosters were bled at 9:00 a.m. and the test tubes of blood were placed in a water bath maintained at 37-40° C. The clots were broken as soon as they formed with a metal rod. The blood was kept in the water bath till 4:00 p.m. when the serum was decanted and stored in the refrigerator overnight. Additional serum squeezed from the clot during the night also was saved. Subsequently the serum was centrifuged, bottled, and stored at -17° C. As with the guinea pigs, control serum was drawn from each rooster before injection.

Protein extraction for precipitin tests

The antigen for *in vitro* testing was prepared by defatting 700 mg of whole pollen twice in 10 ml of ether for 2 hours at 11° C. The defatted pollen was air dried and ground with a mortar and pestle until at least 95% of the pollen grains were ruptured. Six ml of 0.1 M phosphate-buffered physiologic saline, pH 6.7, prechilled to 6° C were added to the ground pollen and mixed until the system was homogeneous. To prevent microbial growth, 0.1% sodium azide was added to the buffer. The protein suspension was refrigerated at 4° C overnight and then centrifuged at 20,000 x g at 1° C for one hour. This yielded approximately 5 ml of 1.5% protein solution.

Quantitative serological tests

The method outlined by Boyden and DeFalco (1943) with several modifications was used for the quantitative serological determinations. The entire range of antigen-antiserum reaction from antigen excess to antibody excess was assayed by a series of doubling antigen dilutions and a constant amount of antiserum. The antiserum level was predetermined by finding the concentration that would give no reaction with an antigen excess near a 1:500 (mg protein:ml diluent) antigen dilution. One-half ml of the proper antiserum dilution was added to 1.0 ml of each antigen dilution.

The precipitin reaction was measured by the apparent turbidity of the antigen-antibody system with a Coleman Model 9 Nepho-Colorimeter. The cuvette immersion well was modified to hold the 10 x 75 mm culture tube in which the reaction occurred.

Insofar as possible, all serological comparisons were made between antisera which were drawn the same number of days after injection and of comparable titre.

The diluent used for the chicken antiserum was a 0.15 M phosphate buffer, pH 7.6, containing 8.0% sodium chloride and 0.05% sodium azide. Because the chicken antigen-antiserum reaction was extremely rapid, it was impossible to incubate all tubes for a constant period of time. Instead, the reactants were mixed rapidly, and the reaction in each tube was followed until maximum turbidity was reached, flocculation was begun, and the reflected light reading decreased. The amount of reaction was recorded as the difference between the initial and maximum turbidity. Most tubes reached maximum turbidity within one minute, but some reacted as quickly as 20-25 seconds, and others required 3-4 minutes. Care was taken not to invert the tube too rapidly when mixing antigen and antiserum since air bubbles introduced by rapid mixing could cause an erroneous reading. The reaction was performed at room temperature, and the water in the immersion well was changed frequently to avoid excess heating from the exciter lamp.

The diluent for assaying the guinea pig serum was a 0.05 M phosphate buffer, pH 6.7, containing 0.5% NaCl and 0.05% sodium azide. Tests involving guinea pig serum were incubated at 37° C for 2 hours. The antigen and antiserum were mixed in the culture tube and the turbidity then recorded. After incubation the turbidity was recorded, and the difference between the two readings was taken as due to the precipitate reaction. No problem ensued due to imperfections in the tubes since an individual reaction was based on the turbidity difference in the same tube.

For each antiserum or antigen source, control tubes of each dilution were assayed for turbidity, and these values were subtracted from the proper reaction tube readings.

Determination of relation between turbidity and precipitate nitrogen

The relationship between turbidity and precipitate nitrogen (converted to protein) was determined for chicken antiserum for corn inbred 3 and its homologous antigens. Duplicate tubes of doubling dilutions of antigen from 1:1000 to 1:16,000 were used. Because a relatively large amount of precipitate was needed for the micro-Kjeldahl determination, 3.5 ml of antigen and 2.0 ml of antiserum were mixed in an 18 x 150 mm test tube. The turbidity was measured after which the reaction tubes were refrigerated overnight. The precipi-

tate was washed twice with saline solution, and the resultant pellet was transferred to a 30 ml Kjeldahl flask. The precipitate which adhered to the centrifuge tube was resuspended in ammonia-free water, and the suspension was transferred to the Kjeldahl flask. This procedure was repeated several times to insure complete transfer of all precipitate.

Calibration of Nepho-Colorimeter

The Coleman Model 9 Nepho-Colorimeter was calibrated with barium sulfate suspended in sulfuric acid, as described by McFarland (1907), at concentrations which fell within the range of the instrument. The barium sulfate was ground with a mortar and pestle and then added to 1% sulfuric acid to make a 200-µg-per-ml stock suspension after which the suspension was stirred with a magnetic stirrer for one hour. Samples of the stock were then diluted to give 10-µg-per-ml increments over the range of 10 µg to 150 µg. Duplicate samples were prepared starting with a new stock suspension.

Immunodifusion in agar

The Ouchterlony agar plate technique employed was essentially the same as that described by Feinberg (1956). One percent Oxoid Ionagar No. 2 (Consolidated Laboratories, Inc. Chicago Heights, Illinois), a clarified agar made especially for diffusion studies, was dissolved in 0.1 M phosphate buffer,

pH 6.6, with 1.0% sodium azide. One and eight percent sodium chloride was dissolved in the buffer for analysis of the guinea pig and chicken antisera, respectively.

The Ouchterlony determinations were conducted in disposable plastic petri dishes (5 cm diameter) coated with a thin film of stopcock grease to prevent under-running of the reactants. Five ml of agar were poured into each dish. This gave an agar depth of 2.5 mm. After the agar in a dish had gelled, 6 wells, each 1/8" in diameter surrounding a central well of 15/32" diameter, were cut using a No. 1812 Agar Gel Cutter (Consolidated Laboratories, Inc. Chicago Heights, Illinois). The agar cores were removed from the wells by suction on a drawn glass tube. Distance from the proximal edges of the center and peripheral wells of the cutter was 5.78 ± 0.06 mm.

The quantities of antiserum added to the central well ranged from 0.10 to 0.13 ml depending upon the relative antiserum strengths, and the quantity of antigen added to each peripheral well ranged from 0.01 to 0.02 ml of solutions containing from 0.5 to 2.0% protein. Dishes with guinea pig antiserum were incubated at 37° C for 30 hours, and those with chicken antiserum were incubated at 24° C for 72 hours. The antigen and antibody diffused toward each other, and where homologous systems were involved a line of precipitate developed. The number of visible lines represented the minimum

number of antigen-antibody systems present. Representative dishes of each oat and corn reaction system were photographed.

Immunoelectrophoresis

Immunoelectrophoresis was conducted on 5 x 12 cm cellulose acetate strips in a horizontal tank using a Shandon Universal Electrophoresis Apparatus (Consolidated Laboratories, Inc. Chicago Heights, Illinois), according to Kohn (1960). All runs were made for one hour with a constant current of 0.8 mA per cm of strip width across a bridge gap of 9 cm. The strip was maintained at pH 8.6 with Oxoid Barbitone Buffer (Consolidated Laboratories, Inc. Chicago Heights, Illinois). The immunoelectrophoretic procedure was as follows: two different antigen samples of 10 µl of 1.0% protein were spotted 3 cm from the cathode end of the strip and 1.2 cm from each edge. After the antigen components were distributed by the electrophoretic run, about 25 µl of antiserum were applied to a 4.0 x 0.4 cm filter paper strip placed laterally 1.1 cm from and midway between the antigens. The amount of antiserum solution applied varied according to relative strength but was constant for a given antiserum. The arrangement of the 2 antigens and the antiserum on the strip permitted a direct comparison of homologous and heterologous systems. The antiserum was allowed to soak into the cellulose acetate strip for 1 hour while still in the enclosed electrophoresis chamber.

The strip then was submerged in cottonseed oil to maintain constant humidity for 3 days at 24° C, after which the excess oil was removed by wiping and dissolving in ether. Unprecipitated reactants were removed by soaking in saline solution for 3 hours. The membrane was then dried for 10 minutes at 100° C and stained with 0.2% Ponceau S in 3.0% trichloroacetic acid. Excess stain was removed by washing in 5% acetic acid until the membrane background was completely white. The strip was then blotted with filter paper and dried under weights to avoid curling. The immunoelectrophoretic procedure allowed further resolution of reaction lines observed by the Ouchterlony method, since 2 superimposed lines on the agar would be separated if the antigens had different electrophoretic mobilities.

Immunodiffusion on cellulose acetate

Immunodiffusion with cellulose acetate was performed according to the method of Consden and Kohn (1959). Five µl of antigen and antiserum were spotted 1 cm apart and allowed to soak in, after which the membrane was immersed in cottonseed oil. The remainder of the procedure was identical to the immunoelectrophoresis. The reactants were added rapidly to prevent the cellulose acetate from drying out.

EXPERIMENTAL RESULTS

Turbidity

Before using relative turbidity measurements for quantitative determinations it was necessary to: (a) Determine whether the relationship between turbidity readings and suspension concentrations was linear or curvilinear, and (b) Establish the precision of the nepho-colorimeter. The regression of turbidity on barium sulfate standards was obviously linear (Figure 1). The deviations from regression were significant, but the ratio of mean squares for linearity to deviations was of the order of 10,000 to 1; therefore, the relationship within the working range of the machine was linear. The same situation was found for the regression of turbidity on µg of precipitated protein (Figure 2). The correlation between turbidity and precipitate protein was 0.976 showing that this relationship was also linear. The regression line failed to pass through the origin, but with a linear response this would be inconsequential since all determinations are relative. It was impossible to determine more points on this graph with either guinea pig or other chicken antiserum because such a large quantity of antiserum was required for each analysis.

The precision or repeatability of the nepho-colorimeter was good as evidenced by the extremely high correlation be-

Figure 1. Regression of turbidity (in galvanometer units) measured by the Coleman Model 9 Nepho-Colorimeter upon barium sulfate standards

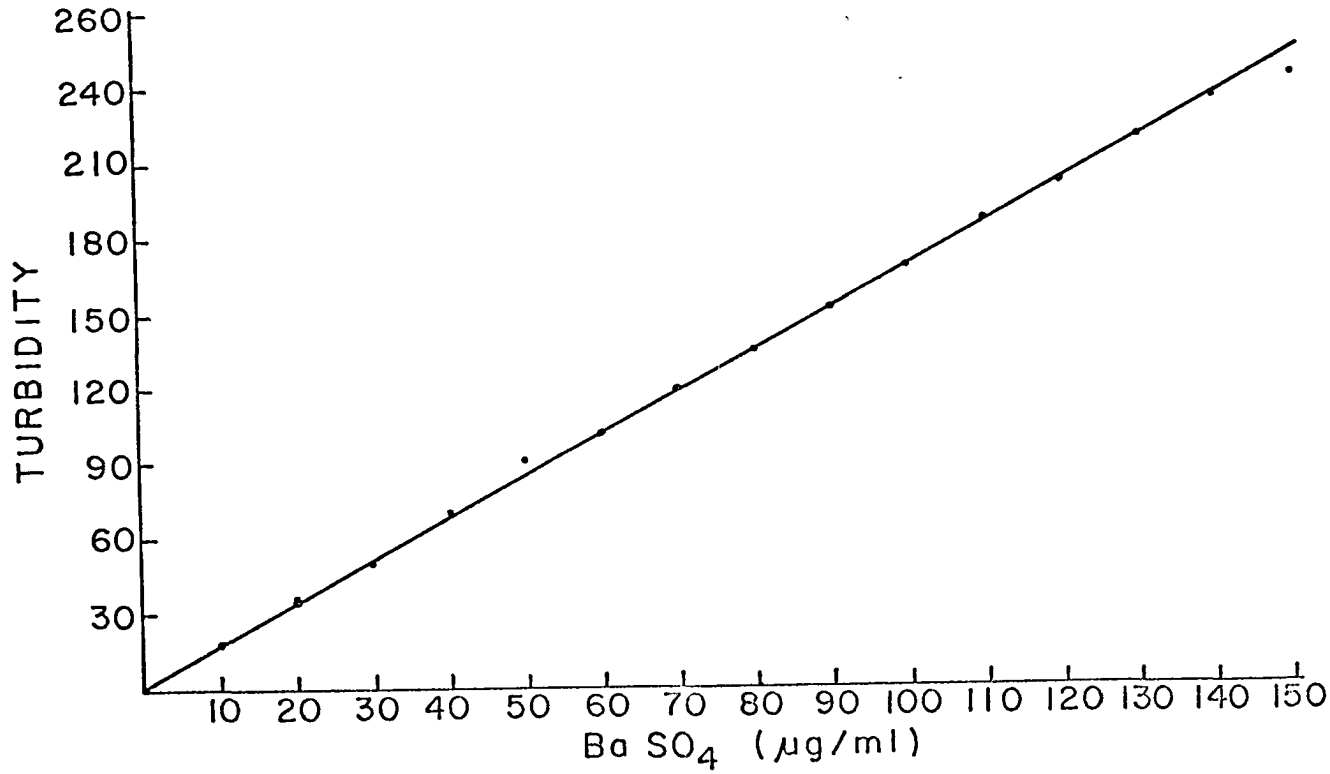
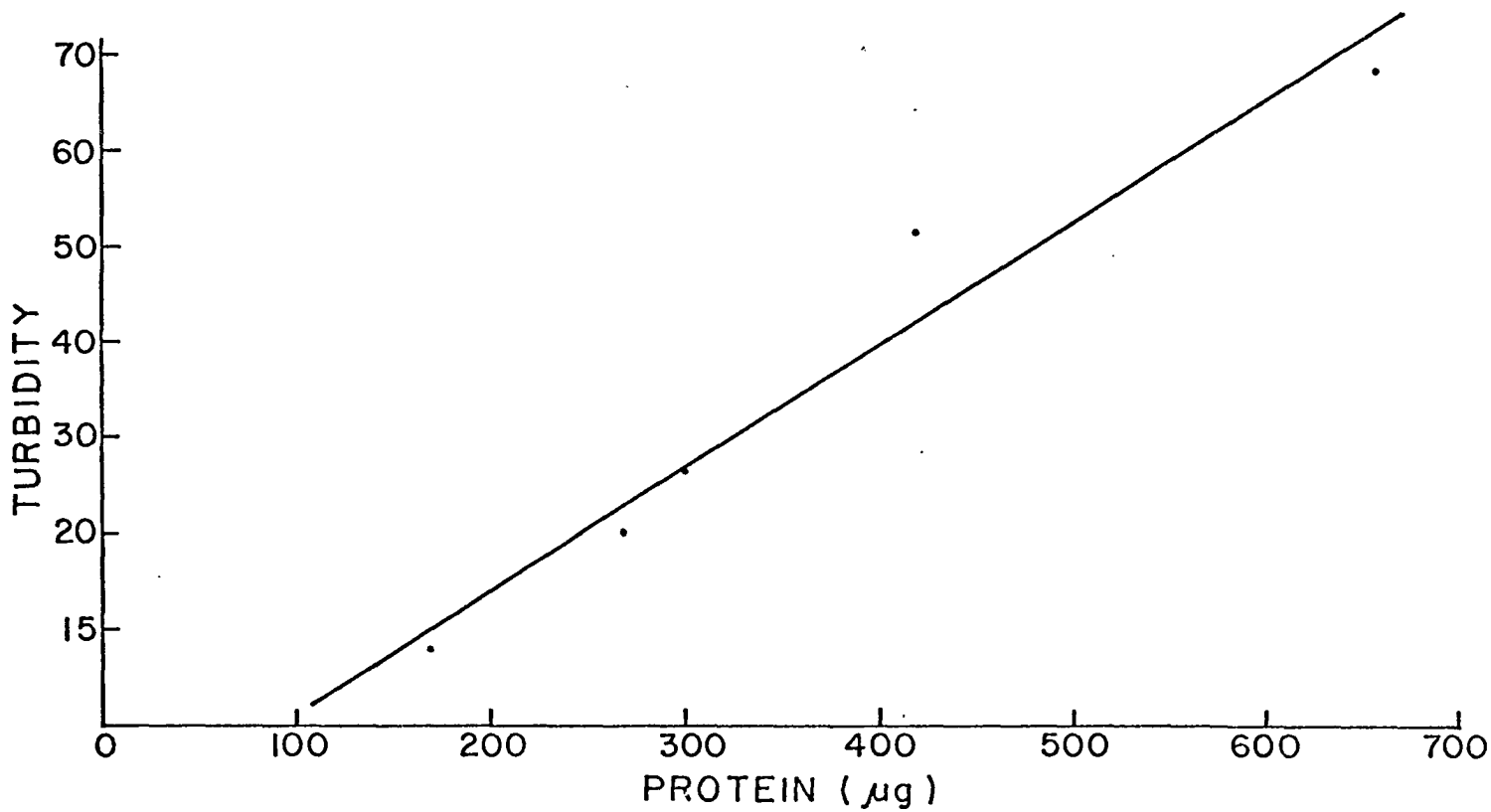

260
240
210
180
150
120
90
60
30
TURBIDITY
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150
Ba SO4 (µg/ml)

Figure 2. Regression of turbidity (in galvanometer units) upon μg of protein precipitated as determined by micro-Kjehdahl


70
60
50
40
30
20
15
TURBIDITY
0
100
200
300
400
500
600
700
PROTEIN (μg)

tween duplicates of barium sulfate standards (r = 0.9994).

The total amount of antigen-antibody reaction in a tube increased to a maximum over a period of time. With chicken antisera the maximum reaction usually occurred within 1 to 3 minutes, whereas with guinea pig antisera the reaction progressed more slowly.

The rapidity of reaction over the period of 1 hour and 45 minutes for 3 typical antigen-antibody systems using guinea pig antisera is shown in Figure 3. The reactions for all 3 tubes were completed at 1 hour. Readings taken after 6 hours were identical to those at 1 hour indicating a plateau was reached and maintained after 1 hour. The incubation period established for the guinea pig antisera reactions was 2 hours since there was some variability, but all antisera seemed to reach a plateau during that interval. Leaving tubes for extended periods beyond 2 hours might have given lower readings due to subsequent flocculation of the precipitate particles. Throughout the study random tubes were periodically re-read after 3-6 hours to confirm that a maximum reading had been taken at 2 hours.

The serological relationship of Cherokee oat variety to each of the other varieties is given in Table 1. Homologous reactions of 2 antisera to the same antigen were not necessarily the same in total amount of turbidity (see Table 4 in the Appendix), so the relationships are expressed in percent

Figure 3. Development of reaction (turbidity) over time

(3) Homologous reaction of corn inbred 3 at 1:1 antiserum and 1:1000 antigen dilution

($Mo_{(a)}$) Homologous reaction of Mo. 0-205 at 1:0.5 antiserum and 1:1000 antigen dilution

($Mo_{(b)}$) Duplicate determination of $Mo_{(a)}$

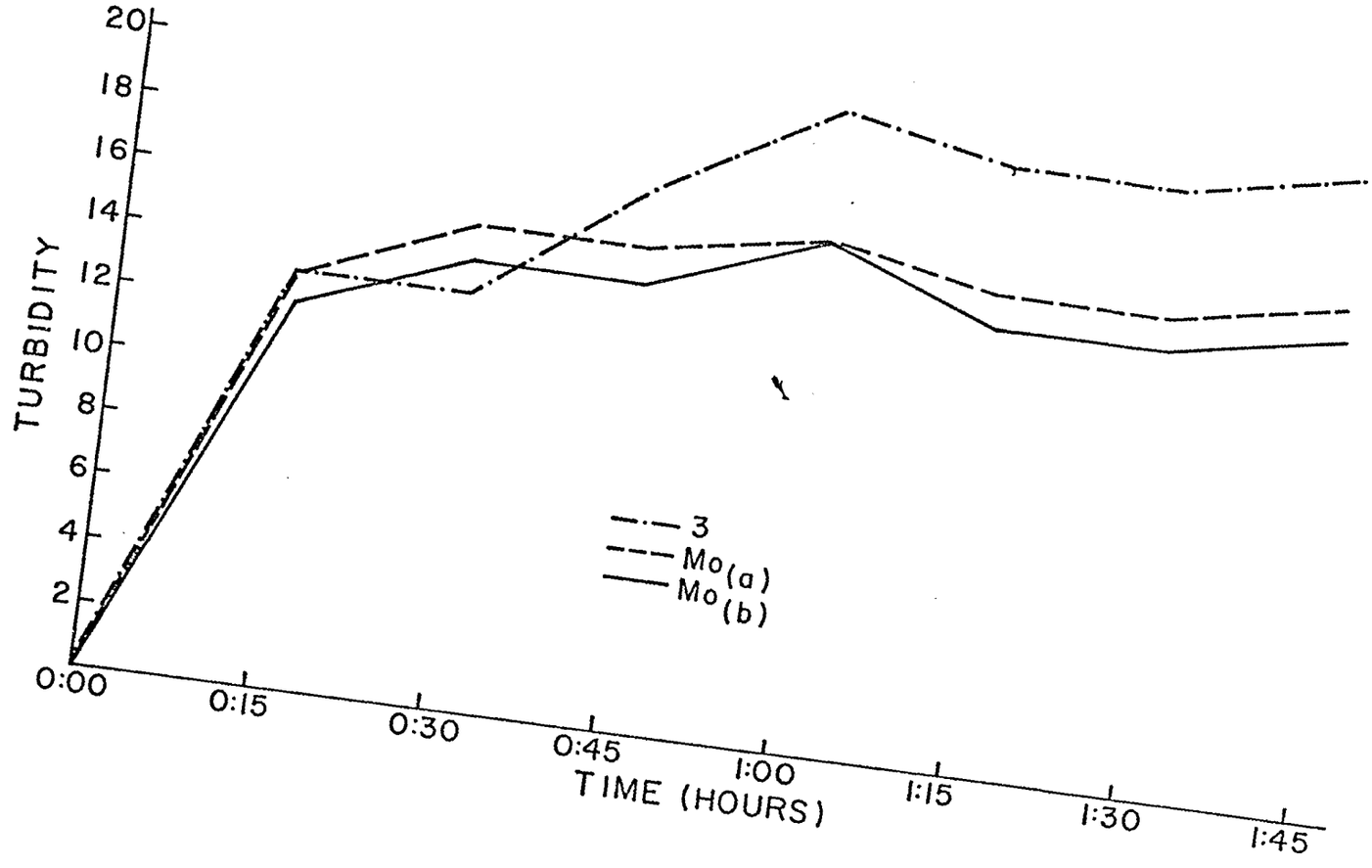
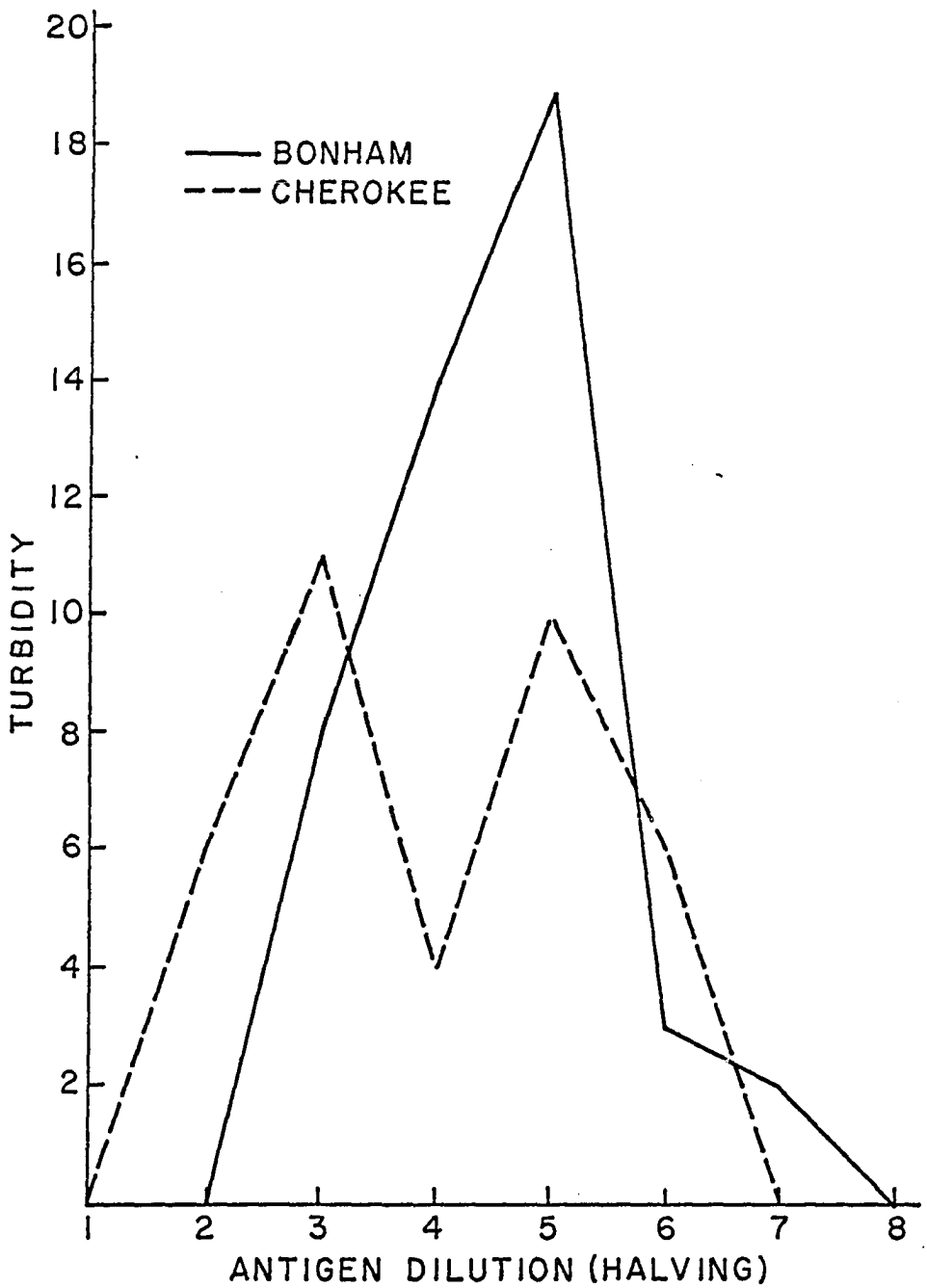

20
18
16
14
12
10
8
6
4
2
TURBIDITY
0:00
0:15
0:30
0:45
1:00
1:15
1:30
1:45
TIME (HOURS)
3
Mo(a)
Mo(b)

Table 1. Serological relationship of Cherokee to six selected oat varieties expressed as (heterologous turbidity ÷ homologous turbidity) x 100

Antiserum	Antigen	Guinea pig 1	Guinea pig 2	Mean
Andrew	Cherokee	61	88	74.5
Bonham	Cherokee	111	80	95.5
Minland	Cherokee	72	44	58.0
Mo. 0-205	Cherokee	62	34	48.0
Nemaha	Cherokee	96	118	107.0
Richland	Cherokee	105	55	80.0
Cherokee	Andrew	118	108	113.0
Cherokee	Bonham	148	112	130.0
Cherokee	Minland	128	98	113.0
Cherokee	Mo. 0-205	252	100	176.0
Cherokee	Nemaha	111	67	89.0
Cherokee	Richland	82	92	87.0

ratios of total turbidity for the heterologous reaction to total turbidity for the homologous reaction. A typical pattern for the amount of reaction over the range from antigen excess to antibody excess is given in Figure 4. The percentage reactions of Cherokee antigen to antisera produced against the other oat varieties were all within reason and ranged somewhat as expected. Three readings over 100% were considered as an estimation of 100%. However, the reaction percentage of antigens from the other 6 varieties to Cherokee antisera did not follow a similar pattern, and in some cases they were completely unreasonable, e.g., 252% for Mo. 0-205 antigen. Since

Figure 4. Titration of Bonham and Cherokee antigens with Bonham antiserum at 1:3 dilution. Intial antigen dilution was 1:250. Percent relationship was 80


20
18
16
14
12
10
8
6
4
2
TURBIDITY
BONHAM
CHEROKEE
1
2
3
4
5
6
7
8
ANTIGEN DILUTION (HALVING)

the Cherokee antisera were taken over a longer period of time than the other 12 antisera, their specificity may have been lost. As a result no confidence is placed in these values, and they have not been considered in any of the subsequent comparisons. Even though the possibility of loss of specificity was realized, it was nevertheless necessary to collect blood over an extended period in order to obtain enough antiserum for the serological tests.

In Table 2 the percent of serological relationship derived from the reaction of Cherokee antigen to the antisera of the other 6 oat varieties has been paired with the coefficient of relation between Cherokee and the other varieties and the variance components among lines within crosses for heading date, plant height, and grain yield. The variance components within crosses were considered to be the base or true indices of the degree of genetic relationship between 2 varieties since crosses between parents of diverse germ plasm would be expected to show more variability in the segregating generations than would crosses involving parents of close genetic relationship. The mean serological relationships closely paralleled the variance components for yield. Of course the relationship was negative because close genetic relationship between 2 varieties results in a high percent reading for serology and a low variance component for segregation within a cross. The variance components for plant height and heading

Table 2. Within cross variance components for 3 quantitative characters, serological relationship and the coefficient of relation of parents of six oat crosses

Cross	d.f.	Characters: Plant height	Characters: Heading date	Characters: Grain yield	Mean serological relationship of parents (%)[a]	Coefficient of relation of parents
Cherokee x Nemaha	89	0.12	0.00[b]	0.25	107.0	0.25
Cherokee x Bonham	89	0.08	0.06	3.12**	95.5	0.50
Cherokee x Richland	89	2.32**	5.65**	5.05**	80.0	0.25
Cherokee x Andrew	89	0.52**	0.77**	14.22**	74.5	0.375
Cherokee x Minland	89	1.64**	1.09**	18.92**	58.0	0.0625
Cherokee x Mo. 0-205	89	4.74**	4.12**	18.75**	48.0	0.0625
Error	267					

[a]Vertical lines indicate serological relationships not significantly different at the 10% level using Duncan's multiple range test.

[b]Negative variance assumed to be 0.00.

**Exceeds the 1% level.

date for crosses involving Bonham and Nemaha fit the serological data, but the variance components for the remaining crosses did not. The coefficients of relation agreed well with the serological data and yield variance components for all crosses except for Nemaha x Cherokee. According to the supposed parentage of these 2 varieties, they have a coefficient of relation of 0.25. However, oat breeders of the Midwest have long suspected that Cherokee and Nemaha are of the same parentage and perhaps even of the same genotype. It is conceivable that a mistake was made in record keeping and that Nemaha and Cherokee merely represent samples from the same bag of seed. All avenues of evidence, i.e., agronomic comparisons, serological relationships, and variances within the cross between Cherokee and Nemaha, support the suspicion that these 2 varieties are of the same genotype and probably come from the same cross. According to Duncan's multiple range test the serological relationships of Cherokee to Minland and Mo. 0-205 were significantly different from those of Cherokee to Nemaha at the 10% level. Also, the relationship of Cherokee to Mo. 0-205 was significantly different from Cherokee to Bonham.

Significant variability among strains within varieties was found for both heading date and plant height in Minland and Mo. 0-205 and for yield in Cherokee. Of course, significant variability among parental lines did not contribute to variability within crosses because all of the strains used

from a particular cross were derived from a single F_1 progeny.

Even though the serological data for the oat variety comparisons reported herein are meagre, and experimental expediency (i.e., collecting antiserum from guinea pigs injected with Cherokee antigen for too long a period) may have negated certain data, the results are encouraging. In a general way it appears that the degree of genetic relationship between 2 oat varieties can be predicted by serological tests.

The serologic relationship among the 4 corn inbreds together with the mean plant weight and ear-weight per plant are given in Table 3. Total turbidities of homologous and heterologous reactions are given in Table 5 in the Appendix. The comparative magnitudes of plant or ear vigor in the single crosses between corn inbreds are commonly accepted as indices of the genetic relationship between the inbreds (Hayes and Johnson (1939)). Neither mean plant weight nor ear-weights per plant in the crosses inbred 1 x inbred 2 and inbred 3 x inbred 4 were significantly different from each other, but both differed significantly from the other 4 single crosses at the 1% level. None of the crosses in the latter group differed significantly from each other in mean plant weight. Mean ear-weight per plant in the cross, inbred 1 x inbred 4, differed significantly from those of inbred 2 x inbred 3 and inbred 2 x inbred 4, but not from the cross, inbred 1 x inbred 3. However, the latter cross was not significantly different

Table 3. Serological relationship of parents and plant weight and ear weight of single crosses among 4 corn inbreds

Cross or Antiserum x Antigen	Serologic relationship (%) Guinea pig antiserum	Mean of reciprocals	Chicken antiserum	Mean of reciprocals	Mean weight of reciprocals (lbs.) Wt/plant	Ave. ear wt/plant
1 x 2	115	104.5	105	100.5	1.02	0.54
2 x 1	94		96			
1 x 3	121	109.0	78	92.0	1.62	0.85
3 x 1	97		106			
1 x 4	107	99.5	97	101.5	1.52	0.78
4 x 1	92		106			
2 x 3	92	82.5	89	92.5	1.50	0.93
3 x 2	73		96			
2 x 4	84	93.0	60	80.5	1.56	0.89
4 x 2	102		101			
3 x 4	113	89.0	87	94.0	1.10	0.62
4 x 3	65		101			

from the crosses, inbred 2 x inbred 3 or inbred 2 x inbred 4.

The only serological comparison from corn inbreds that showed a definite trend was that with chicken antiserum to the antigen from inbred 2. With this inbred the serological data paralleled (negatively) the ranking of plant weights for single crosses. There was a somewhat similar but slight trend in guinea pig antiserum to the antigen of inbred 2. None of the serological measurements fit the pattern of mean ear-weight per plant for the single crosses. As with the guinea pigs injected with Cherokee antigen, those injected with the corn inbred antigens were bled at regular intervals over an extended period after the injection. Since loss of antibody specificity has been demonstrated to occur under these circumstances (Leone 1952), it was decided to inject another set of guinea pigs with corn antigens and use only the antisera from the first 2 blood drawings. The results from this test were very erratic and showed no definite trends. Unfortunately, the only guinea pigs available for these injections were probably too small to withstand the shock from the antigen. After the injections their body weights decreased and arthritis was quite severe. Consequently, this test was not considered definitive either. Additional experiments could not be conducted because the pollen and guinea pig supplies were exhausted.

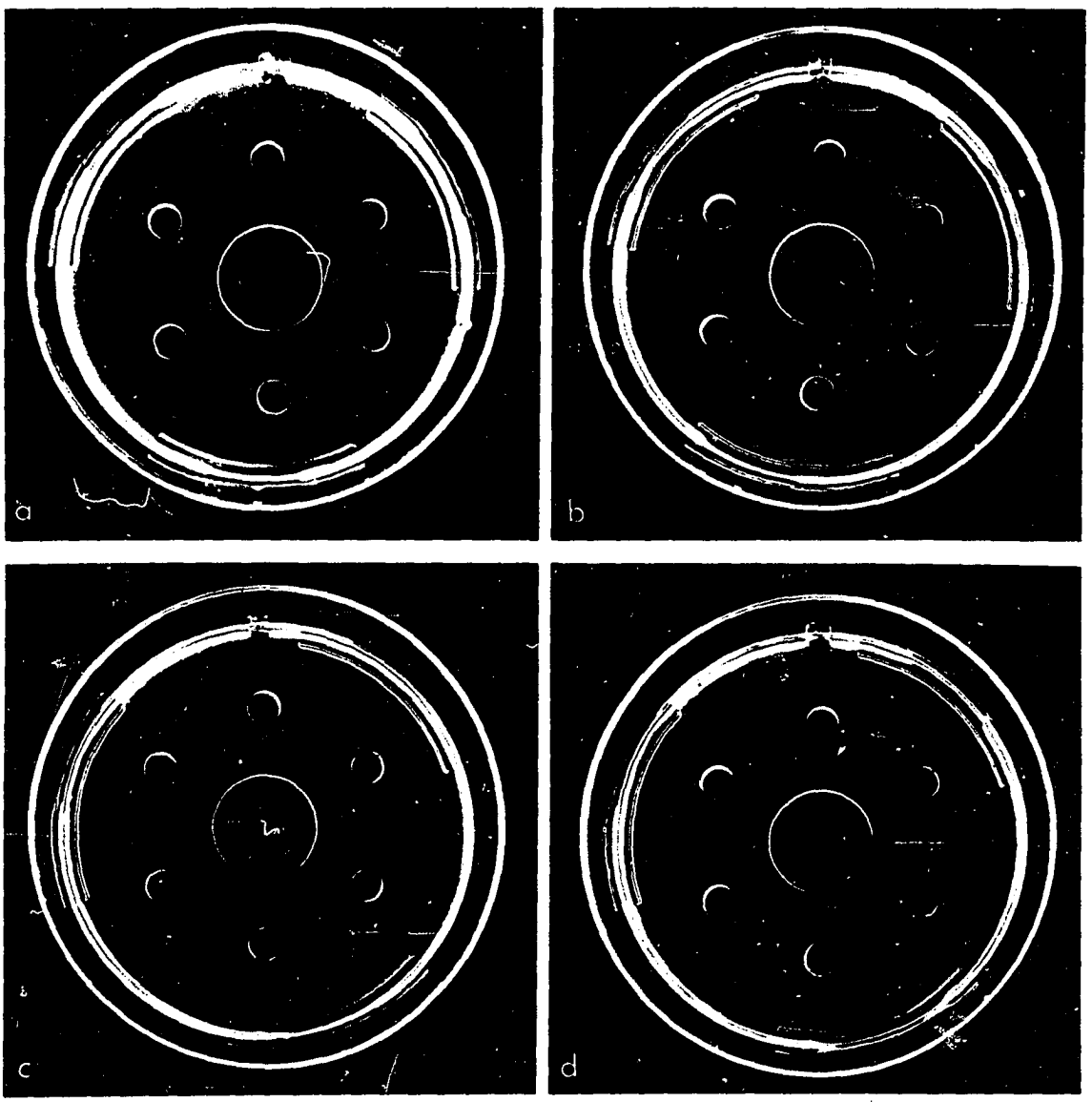
Agar diffusion

The agar diffusion technique when used on the oat variety antigen-antibody reaction systems always differentiated a single band, and in some plates a second band nearer to the antiserum was evident (Figure 5). Plates a and b in Figure 5 both contained Mo. 0-205 antiserum, but a contained 0.06 mg of antigen per well and b contained 0.12 mg per well in the peripheral wells. The b plate developed two bands at the third and sixth wells, whereas only one band developed at similar wells in plate a. However, in a similar experiment with Nemaha antiserum, (plates c and d), no second line was visible in either plate.

Another factor which may contribute to variability in the number of bands which develop in a reaction plate is the antiserum source. For example, antiserum to Mo. 0-205 from one guinea pig developed 2 bands when reacted with antigens from the 7 varieties, whereas a comparable antiserum from a second guinea pig produced only one band. The same situation existed for 2 sources of Bonham antisera, and similar differences but to a lesser degree were apparent for the Andrew, Cherokee, and Richland antisera. Apparently there were quantitative differences among oat varieties for antigen-antibody reactions, but these differences were difficult to detect consistently.

For the antigen-antibody reaction of corn inbred 1, using guinea pig antiserum, two bands developed to antigens from

Figure 5. Agar diffusion plates of guinea pig antisera to Mo. 0-205 (a and b) and Nemaha (c and d). Clockwise order of antigens starting at the top was Andrew, Cherokee, Minland, Mo. 0-205, Nemaha, and Richland. Plates a and c contained 0.06 mg of antigen and plates b and d contained 0.12 mg of antigen.


a
b
c
d

inbreds 1 and 2 and 3 bands to antigens from inbreds 3 and 4 (Figure 6a). In this case the 2 inner bands near wells 3 and 4 simply coalesced at wells 1 and 2 due to a higher concentration of those particular antigen-antibody systems. In plates b, c, and d (antiserum to inbreds 2, 3, and 4, respectively) there are only 2 bands present at all 4 wells even though the inner band at d did not develop in the photograph. No qualitative differences were observed in any of the guinea pig antisera to the 4 corn inbreds.

No qualitative or quantitative differences could be detected among any of the antigen-antiserum comparisons for corn inbreds when chicken antisera were used in the agar diffusion plates (Figure 7). Antiserum to inbred 1 developed 4 bands of precipitate, antiserum to inbred 2 developed 3 bands, and antisera to inbreds 3 and 4 developed 2 bands each.

The chicken and guinea pig antisera, when tested with the antigens from the single crosses, gave results similar to those when the inbred antigens were used. Incidently, the plates shown in Figures 6 and 7 were selected to show the optimum resolution of the systems and to illustrate points of discussion: thus all did not contain the same antigen concentration.

In all the agar diffusion studies, the data was collected from plates where the amount of antiserum added was that which gave the greatest resolution.

Figure 6. Agar diffusion plates of guinea pig antisera to the 4 corn inbreds. Plates a, b, c, and d contain antiserum to inbreds 1, 2, 3, and 4, respectively. The antigens appear in numerical order starting at the top.

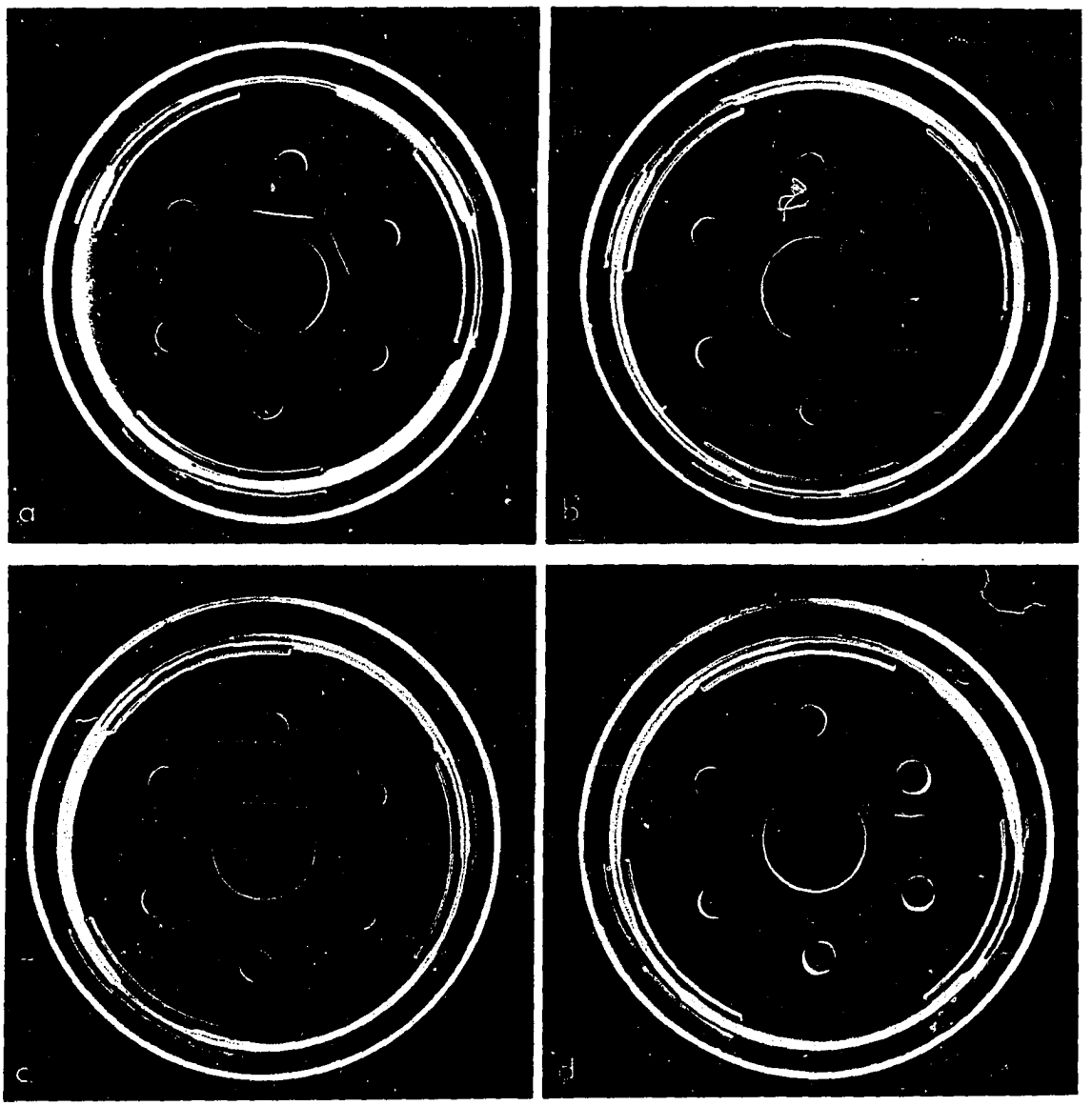
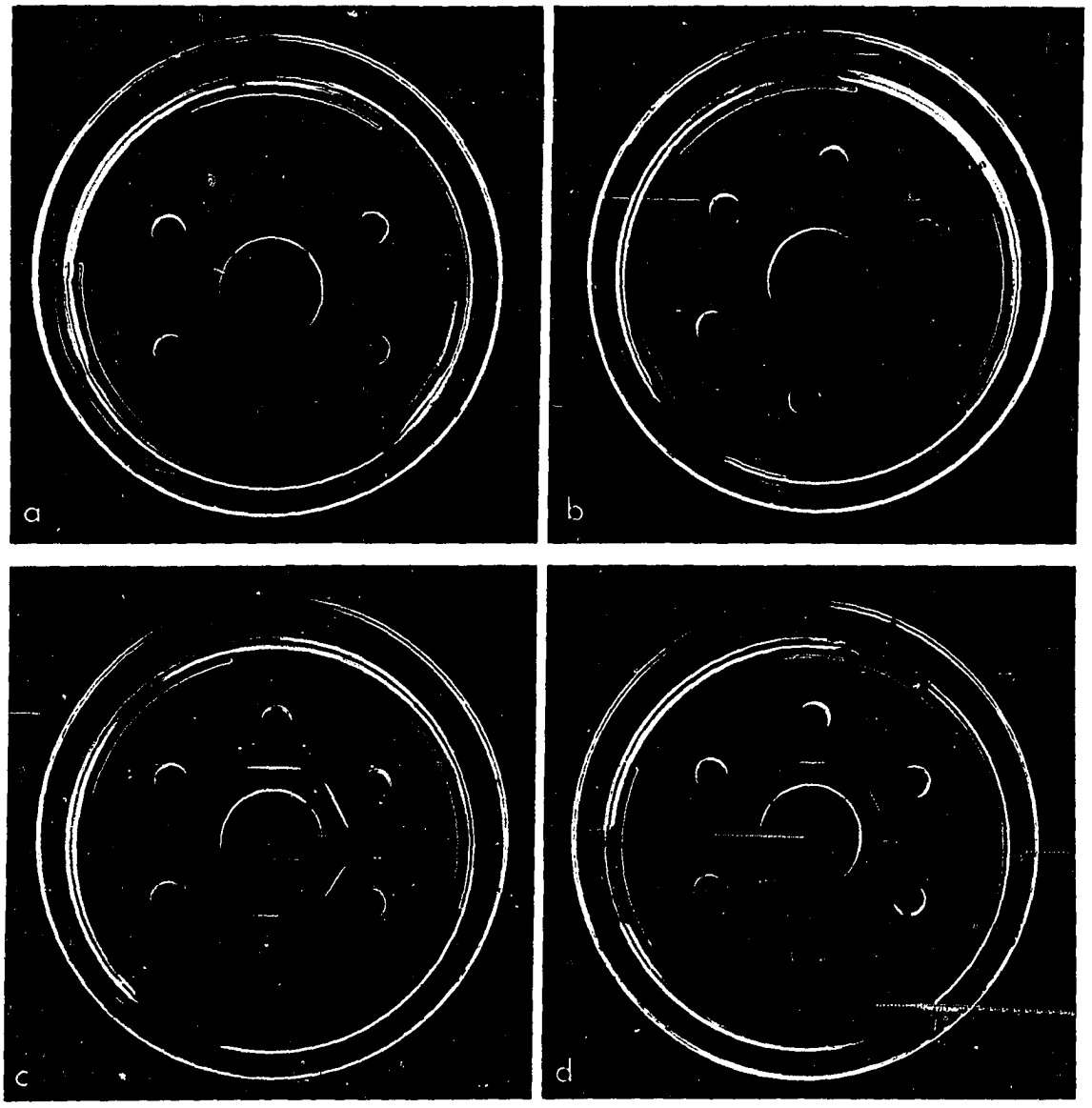

a
b
c
d

Figure 7. Agar diffusion plates of chicken antisera to the 4 corn inbreds. Plates a, b, c, and d contain antiserum to inbreds 1, 2, 3, and 4, respectively. The antigens appear in numerical order starting at the top.


a
b
c
d

Immunoelectrophoresis resulted in the development of the same number of reaction bands as did the agar diffusion method except for guinea pig antiserum to inbred 2. In this case a third band was resolved and found in all 4 corn inbreds. This may represent the same band as the third one found by agar diffusion for guinea pig antiserum to inbred 1. Even though varying amounts of antigen and antiserum were used, only one band of precipitate was found in the immunoelectrophoreses tests for oats. Immunoelectrophoreses with chicken antisera were without success.

Immunodiffusion on cellulose acetate gave excellent bands of precipitate, but results were extremely erratic.

DISCUSSION

The majority of the studies on plant sero-systematics have been conducted by measuring only the end point (the greatest antigen dilution yielding a positive result) or the amount of precipitate at the equivalence zone. However, as Boyden (1942) pointed out, the end point determination indicates only sensitivity and is not necessarily a quantitative estimate of serological relationship. In addition, the equivalence zone of a particular antiserum is not always at the same antigen concentration for all antigens. Since the equivalence zone is usually determined with the homologous antigen, the errors resulting from this test with heterologous antigens are obvious. So, in order to properly determine the total reactivity of an antigen to a particular antiserum, the antigen must be tested over its entire range of reactivity to a constant amount of antiserum. Bolton (1947) demonstrated that the end point and optimal proportions information are not only fragmentary but many times misleading when compared to an antigen titration test. Boyden (1958) has presented a comprehensive discussion on the use of this technique in systematics.

The photoelectric measurement of the serological reaction using antigen titration was developed by Boyden (1942; 1954). The utility of the photoelectric method is dependent upon the

precision in measuring turbidity and the type of relationship between turbidity and amount of antigen-antibody precipitated. It was obvious from the barium sulfate standard curve that the Nepho-colorimeter used in this study afforded excellent precision and a linear relationship in measuring turbidity, so readings from different points could be compared directly throughout the entire range of the instrument. The relation of turbidity and precipitate nitrogen was also linear. Boyden et al. (1947) and Goodman et al. (1951) working with rabbit and chicken antisera, respectively, also obtained good correlations between turbidity and precipitate nitrogen.

The loss of specificity of the Cherokee antisera and all of the guinea pig and chicken antisera to the corn inbreds, except possibly inbred 2, was an acute problem. Leone (1952) demonstrated a decrease in specificity as the period of immunization was lengthened. This leads to the conclusion that the titre of the antiserum should be raised rapidly and the blood drawn immediately. However, all the chicken antisera were produced in 8 days, and antisera from the second group of guinea pigs were taken after only 4 weeks. Yet in both cases the desired specificity was already non-existent. In the latter case the dosage of protein injected was at least twice as large as recommendations based on body weight. Five times more protein was injected into each chicken than into a guinea pig, but, of course, the chicken was considerably

larger. Nevertheless, the chicken injections were intravenous which introduced a large quantity of antigen into the bloodstream immediately. Perhaps not only period but also intensity of immunization are important variables which must be controlled to give antisera of comparable specificity. Use of animals larger than guinea pigs, e.g. rabbits, would shorten the immunization period because a larger quantity of antiserum could be obtained at the first drawing.

Another factor which may influence the readings of the degree of relationship of 2 antigen sources is the dilution of the antiserum. Boyden and DeFalco (1943) reported that the specificity of the antiserum increases upon dilution, but Leone (1952) did not find this phenomenon in all antisera.

Even though there was considerable variability in the duplicate determinations of antisera to the 6 oat varieties, the mean values for the variety comparisons agreed remarkably well with the within-cross variance components for yield. The lack of good relationship of the serologic readings to the variance components for heading date and height is not surprising since these characters probably are more simply inherited than is grain yield. Grain yield may be considered as a sum total of all genetically controlled biochemical processes throughout the life of the plant. Since the serological relationship is based upon the total salt soluble protein complex of the pollen grains, it should be more closely corre-

lated with data from an attribute which samples the effects of the total germplasm, which in this case is probably grain yield.

The coefficient of relation of two genotypes is based on the random distribution of genes from common parents. Full sibs would have an average relation of 0.50 since by chance they could be as unrelated as the parents or have identical genotypes. As a result heterozygosity in the parents and sampling of genes in segregating generations may give actual genetic relations other than predicted. This would account for the coefficients of relation of Cherokee to Andrew and Richland reversed in rank when compared to the within-cross yield variances and the serological data.

In this study 2 different statistical methods were used to assay the degree of genetic relationship between 2 parents of a cross: (a) Within-cross variability in segregating generations for oats and (b) Magnitude of hybrid vigor in the F_1 generation of corn single crosses. In the first case genetic variances are compared, and for corn the means are compared. It may be that the serological comparisons will more closely parallel the genetic relationship established through genetic variances within crosses than through magnitude of hybrid vigor. In both situations, e.g., corn and oats, the proteins were extracted from pollen of "pure lines". The primary type of gene action which will be measured in the

variance data for oats is additive, and this can be measured directly from the parent varieties. However, in corn the mean vigor of the hybrids will be strongly influenced by non-additive gene action which cannot be measured directly from the parent inbreds. This may be part of the reason why the work with the corn inbreds was not positive, especially with the chicken antisera. The agreement between the serology data and hybrid vigor associated with inbred 2 could result from the fact that this inbred contributes primarily additive gene action to its cross. Thus, the serological prediction would be more useful in oats and other self-pollinated crops where segregates are superior due to additive gene action, than in corn or other similar crop species where first generation hybrids are utilized commercially.

Pollen was used as the antigen source since it was reasoned that the proteins specific to the genotype would be found therein without an excess of secondary or storage proteins. Storage proteins are often not variety specific but may be only species or genus specific and thus might conceivably cover up real intra-species genetic differences. A number of plant parts as well as the entire plant have been used as antigen sources, e.g., pollen, seeds, tubers, leaves, cotyledons, and subcotyledonous parts of seedlings. Although the pollen appeared to be a satisfactory antigen source for the oats, there was considerable difficulty in collecting enough

pollen for the required tests (ca. 3 g). Because of this, another plant part will have to be used if the technique is to be employed on an extensive basis.

It was not possible to determine the utility of the corn pollen as a genotype-specific antigen source. This factor was confounded by an apparent loss of specificity of the antisera due to prolonged immunization and as has been suggested, perhaps type of gene action.

Even though presence or absence of the development of a second band on the agar plates appeared to be associated with certain oat varieties, no definite conclusion can be made because there was variability between different antisera sources to the same variety. Munoz (1954) used 18 different rabbits and found that 1 rabbit gave no precipitin bands, 12 gave 1 band, 2 gave 2 bands, and 3 gave 3 bands. For greatest resolution with the agar-plate technique it is good and proper practice to inject several animals with the same antigen and then select the antisera which produce the maximum number of bands. Incidently, the agar-plate technique is treated extensively by Crowle (1961), Grabar (1958), and Korngold (1958).

It was possible through immunoelectrophoresis to resolve 2 bands from 1 in the guinea pig antiserum to corn inbred 2. The identification of a single protein difference controlled by one gene has been demonstrated using this technique (Schwartz (1960)), but examples of this nature are very rare.

There are many variables which must be considered before plant serology can be verified as a practical and routine tool to plant breeding. The intensity and length of immunization must be studied to learn more about obtaining sufficiently specific antisera. The plant organ which provides both the most convenient and best antigen source in each species must be determined. The number of replications of antisera to the same antigen that is required to give satisfactory precision must be estimated for both the plant species and the laboratory animal being used.

Notwithstanding the difficulties encountered in this study there was sufficient positive evidence herein and in other reported studies to warrant further investigation of this technique as a means of estimating genetic relationship and from this predicting superior crosses.

SUMMARY

The serological relationships of oat varieties used as parents for six oat crosses involving a common parent, and 4 corn inbreds used in single crosses were determined quantitatively. All homologous and heterologous antigens were tested for reactivity to all antisera by measuring photoelectrically the turbidity from each antigen-antibody system. The relationship of the parents of each cross was established by the ratio of total turbidity of heterologous to homologous reactions.

The serological relationships of the parents in the oat crosses closely paralleled the within-cross variance components for grain yield. The variance components for heading date and height and the coefficient of relation of the parents agreed with the serological data in isolated cases only. The agreement of the serological data and the yield variance components was attributed to the fact that both represented a measurement of the total expression of germ plasm of the genotype.

All but one of the corn antisera and both antisera to the common oat parent, Cherokee, lacked specificity. This may have resulted from 2 causes: (a) Where guinea pigs were used as antibody sources for these genotypes a long period of immunization occurred and (b) A different type of gene action

may have been measured statistically in the corn than in the oats. It is suggested that non-additive gene action which is apt to be important in the expression of vigor in corn hybrids may not be measured by serological technique because serological measurement was based on proteins obtained from homozygous lines where non-additive gene action would be nearly inoperative.

The agreement between the oat serological and grain yield variance data was positive enough to encourage further investigation of the technique as a means of predicting superior crosses.

Agar diffusion studies revealed only limited quantitative differences and no qualitative differences in oat varieties or corn inbreds.

Immunoelectrophoresis separated only one antigen-antibody system more than was already separated with the agar plate method, while immunodiffusion on cellulose acetate did not provide sufficient precision to permit its use.

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APPENDIX

Table 4. Total turbidity (in galvanometer units) of homologous and heterologous serological reactions of 7 oat varieties

Antiserum	Antigen	Guinea pig antiserum 1	Guinea pig antiserum 2
Andrew	Andrew	61	32
Andrew	Cherokee	37	28
Bonham	Bonham	70	46
Bonham	Cherokee	78	37
Cherokee	Cherokee	54	51
Cherokee	Andrew	64	55
Cherokee	Bonham	80	57
Cherokee	Minland	69	50
Cherokee	Mo. 0-205	136	51
Cherokee	Nemaha	60	34
Cherokee	Richland	44	47
Minland	Minland	39	46
Minland	Cherokee	28	20
Mo. 0-205	Mo. 0-205	50	44
Mo. 0-205	Cherokee	31	15
Nemaha	Nemaha	51	56
Nemaha	Cherokee	49	66
Richland	Richland	39	40
Richland	Cherokee	41	22

Table 5. Total turbidity (in galvanometer units) of homologous and heterologous serological reactions of 4 corn inbreds

Antiserum inbred no.	Antigen inbred no.	Guinea pig antiserum	Chicken antiserum
1	1	115	238
1	2	132	298
1	3	139	222
1	4	123	274
2	1	149	195
2	2	158	202
2	3	146	179
2	4	133	122
3	1	87	263
3	2	66	238
3	3	90	249
3	4	102	217
4	1	47	237
4	2	52	225
4	3	33	225
4	4	51	223