Genetic determinations in winter wheat Triticum aestiyum L. em. Thell. aneuploids using serological techniques
by Raymond Bradford Volin

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Agronomy
Montana State University

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Antibodies were produced in rabbits by injecting them with leaf extract antigens from 2 sources: (1) a disomic which was derived from segregating monosomic and disomic lines of the variety Kharkoff MC22 and (2) a single plant disomic selection. Two lots of crude sap and two lots treated with ammonium sulfate saturation methods were used.

Ouchterlony's gel diffusion techniques revealed no differences between the different inject antigens or between the monosomic and disomic antigens when reacted against any one antiserum. Precipitate lines of partial or nonidentity were not present. All antigens were absorbed by any one of the 4 antisera sources. A soluble precipitate complex occurred when crude antigen or absorbed antisera was reacted against the purified antigen but was not antigenically important.

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Agronomy

Approved:

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MONTANA STATE UNIVERSITY
Bozeman, Montana

March, 1968

ACKNOWLEDGMENT

The author wishes to express his most sincere appreciation to Dr. J. R. Welsh for his advice and constructive criticism and encouragement during this study and manuscript preparation.

A debt of gratitude is also due Dr. F. H. McNeal and Dr. Stephen R. Chapman for their help in manuscript preparation. I offer thanks to Dr. Chapman and Dr. G. A. Strobel for the use of their research facilities.

I am obliged to Mr. John Ericson for his invaluable advice offered during this investigation.

Special gratitude is extended to Betty Volin for her encouragement and patience throughout this study.

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ABSTRACT

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INTRODUCTION

Plant breeders and cytogeneticists have long searched for workable methods to economically improve cereal crop varieties genetically.

With the development of monosomic (2n-1) and nulisomic (2n-2) lines in hexaploid wheat, a new technique of genetic improvement became available. Through use of this technique, complete chromosomes containing genes of desirable cereal quality or disease resistance may be substituted into an otherwise acceptable genetic complex. The resultant goal is a variety engineered to meet the plant breeder's specifications.

A major problem in this technique lies in positive cytological identification of the aneuploid condition. While microscopic analysis reveals the monosomic condition, it may be seasonally untimely, as well as laborious. An accurate, less time consuming, method is needed.

Serology, the study of blood serum, is used as a method in biosystematic and plant taxonomic comparisons. By reacting immune sera with homogenous antigenic material, and relying upon the specificity of the immune sera, corresponding relationships may be quantitated and evaluated.

The nature of the biochemical components of the plant are determined by chromosomes. These units of heredity direct processes including protein synthesis; the composition of such proteins being determined by the nucleotide "code" inherent within the chromosomes.

Welsh and Hehn (1964) launched a study to determine, through the use of monosomic (2n-1) lines, the chromosome or chromosomes which carry factors influencing bread flour quality. Tests indicated that chromosome 1D (XVII) was very important in the determination of bread flour charac-

teristics. The monosomic condition of this chromosome resulted in drastic reduction in gluten strength of the flour. It was postulated that some structural change in protein had occurred allowing for the alteration of various physical properties of the endosperm.

The objective of this investigation was to determine if there are detectable serological differences between a disomic (2n) line of hard red winter wheat Triticum aestivum L. em. Thell variety Kharkof MC22 and several derived monosomic (2n-1) lines of the same variety. Meaningful differences could then be useful in identifying monosomic individuals.

REVIEW OF LITERATURE

I. Serology

Bordet (Boyden 1942) initiated interest in the use of serology for studying organism relationships in 1895 when he recognized the agglutinin reaction as a "distinct phenomenon of immune serum". Bordet indicated by further study that an injected animal will not respond to the injections of proteins from closely related species and that this inability to respond may serve as a guide to close relationships.

The discovery of the precipitin reaction is generally accredited to Kraus (Boyden 1942). In 1897 he mixed antiserum obtained from goats with filtrates used for injection and observed the presence of a precipitate.

Nuttall (1901) pioneered in zoosystematics by publishing on the phylogenetic relationships of many animals using the precipitin reaction. The intensity of the antiserum-antigen reaction was found to parallel the systematic position of the species tested. The application of these same techniques to plant systematics was undertaken in the same year by Kowarski (Chester 1937). Kowarski injected heat-resistant wheat albuminose into rabbits and found that the induced precipitins reacted strongly with wheat albumin extracts and weakly, or not at all, with the extracts of rye, barley, oats or peas.

One year later, Bertarelli (Chester 1937) successfully applied the precipitin method in the detection of wheat flour contamination by Vicia sativa.

Ballner and Burow (Chester 1937) working with grains in 1911 showed that rye is serologically more closely related to wheat than to barley and oats. They also found that rice and maize were still more distantly related to rye. In 1911 Relander (Chester 1937), after working with varieties of barley, oats, Vicia, clover and lupine, succeeded in serologically separating the various species and, in some cases, the varieties of a single species.

Applying serology to the study of grape hybrids Rives (Baldwin et al., 1927) found in 1923 that closely related grape hybrids could be grafted upon each other. Those which did not show close serological relationships could not be grafted together. Green (1926) used similar techniques with Rutaceae, Rosaceae, and Solanaceae and came to the same conclusion as Rives.

Baldwin, Fred, and Hastings (1927) made a serological comparison of cultivated legumes. Their results showed, "all members of any cross-inoculation group are closely related with respect to the protein characteristic of their seeds and, in the majority of cases, all legumes which possess closely related seed protein complexes cross inoculate".

Early application of serological methods was undertaken by Nelson and Birkeland (1929) to associate serological correspondence and stem rust resistance. Mindum durum (2n=28) and four common wheat varieties (2n=42) - Marquis, Hard Federation, North Dakota Selection 1656-81, and Hope - were used. Generally, it was found that the varieties of wheat possessing the greatest number of genetic factors

in common showed the closest serological relationship. For example, close relationship was shown between Hope, selection 1656-81, and the common parent, Marquis. In conclusion it was stated "since the genetic composition of the variety is responsible for its morphological as well as physiological characteristics, the serological reactions may be used, within limits, to ascertain these characteristics. The apparent parallelism in serological relationship and rust resistance seems to offer data more hopeful for study than any that genetic or cytological research such as chromosome determination has so far produced".

During the period 1920-1930 many botanical and zoological investigators felt that the serological technique would provide an objective approach to systematics. A noted group of plant serologists emerged at Königsberg, Germany headed by Gohlke in 1913 and later by Mez (Chester 1937). After extensive research Mez and Ziegenspeck in 1926 (Chester 1937) published the book "Serodiagnostiche Stammbaum" showing a phylogenetic tree derived almost entirely from comparative serological investigations of the plant kingdom. Much criticism of the system came from classical morphological systematists whose data showed little correlation. Gilg and Schurhoff (1927), serological investigators in Berlin, attempted to duplicate results of the Stammbaum using modified techniques. Being relatively unsuccessful they stated, "The serodiagnostic method is, for investigation of plant relationships, completely useless". Others, however, felt that serological techniques could be used if supplemented by morphologic

and cytologic data.

Serological results and techniques of the many zoological investigations have been helpful to certain aspects of botanical serology. Levit et al. (1936) after applying serological techniques in a genetic study of Drosophila melanogaster concluded that such methods could be used to detect the presence of the Y chromosome in males and the attached X chromosome in females. Cumley (1940) in evaluation of Levit's work felt it possible to apply immunological techniques to the study of the expression of individual chromosomes or even genes. Irwin and Cumley (1943), working with several species of doves, their hybrids and backcrosses, concluded that the species specific antigens of the serum are controlled by genes. Thus, a genetic basis of classification is given the systematist who uses serology in his work.

Hyun in 1949 investigated the serological relationship of different species of the genus Quercus. Proteins were extracted from seeds in the first stage of germination and injected into rabbits to stimulate antibody production. He found that his results agreed with the treatment of systematic botanists.

Recent botanical investigations in systematic serology at Rutgers University began with the work of Johnson (1954) on the Magnoliaceae. Several genera were compared with Magnolia and then several species of Magnolia were compared to establish intrageneric, serological relationships. Intergeneric serological comparisons confirmed the classical taxonomy groupings. Other data presented by Johnson

indicated that inter-specific differences in Magnolia surpass the inter-generic differences in certain cases. No explanation of this apparent paradox is presented.

Lewis (1952) applied the precipitin ring test in an attempt to determine the nature of certain competitive interactions which occur between incompatibility alleles of Oenothera organensis and also to give a clue to the nature of the incompatibility system. Pollen extracts of four different incompatibility genotypes were used as antisera sources. The results of twenty-five different combination tests, with the exception of two cross reactions, were found to agree with the theory that the "S" alleles produce specific substances in the pollen each of which is antigenically different. It was also shown that the stylar incompatibility substance is preformed and is not the result of an antigenic stimulus from the pollen tubes.

Hammond (1955) compared a number of genera in the Ranunculaceae on the basis of their serological interactions and this criterion, together with cytological and morphological data, was used to produce a new systematic treatment of the genera.

Considerable disagreement arose among earlier investigators as to whether individual plants were serologically homogenous or whether different organs or tissues from the same plant had different antigenic complements. Mez believed that plants were homogenous but the Berlin group disagreed. Chester (1937) noted, however, that comparisons between seed proteins and proteins from other plant parts were distinctively different.

Gell et al. (1960) with the application of immunological methods studied the taxonomy of 15 Mexican species and 22 South American species of the genus Solanum. Extracts were prepared from the tubers and the crude juices were adjusted to yield a protein concentration of 0.5 per cent. The gell-diffusion technique developed by Elek (1948) and Ouchterlony (1948, 1958, 1964) was used as modified by immuno-electrophoresis developed by Grabar and Williams (1953). Their scheme of relationships between species of the Mexican species was similar to that already postulated on the basis of morphological and cytogenetical studies. The technique was ineffective in separating the South American species. The researchers maintained that the serological method regardless of its limitations in this study could be used, together with other data, in taxonomical or evolutionary appraisals.

Wright (1960) has refined further investigation of organ specific antigens. By combining ultracentrifugation and immunodiffusion he was able to demonstrate an antigen in the microsome fraction of three-day old coleoptile tissue of wheat. In order to exclude non-microsomal antigens, the antiserum was first absorbed with the supernatant of the microsome fraction. The precipitin band associated with the microsome fraction of the three-day old coleoptile tissue was absent from coleoptile tissue of a younger age and from root and leaf tissue. Wright indicated that "a non-organ specific meristematic pattern of antigens has superimposed upon it, during

differentiation, a combination of proteins characteristic of differentiated cells".

The serological distinctness of different organs of a single plant was proven by Kloz et al. (1960). By comparing the antigenic substances from cotyledons, seedling roots, and mature leaves of Phaseolus, it was found that differences in organ reactions often exceeded differences in species reactions. As an example, a serological comparison of the individual organs of the same species (P. vulgaris) as against different species reactions showed the following precipitin results:

(1) Organ interactions

(Antiserum against cotyledons tested against sera from the following sources):

Source	Reaction (Per cent)
Cotyledons	100.0
Seedling roots	8.9
Mature leaves	5.2

(2) Species interactions

Phaseolus vulgaris (Antiserum of leaves against sera from leaves of following species):

P. vulgaris	100.0
P. coccineus	89.7
Glycine soja	41.7
Vicia faba	19.9

Tucker (1963) working with two subspecies of Marchantia polymorpha was able to show a serological difference between them using double-diffusion techniques where antiserum-antigen and absorbed

antiserum-antigen methods both were used. He concluded that "results show antigenic proteins may be extracted from green tissues and used in study of plant relationships".

Serological studies were conducted within families Cornaceae and Nyssaceae by Fairbrothers and Johnson (1964). Seeds were ground and the colloidal precipitate after protein extraction was injected into rabbits. The photronreflectometer and Ouchterlony methods were used and it was generally concluded that serological groupings obtained for the taxa studied were in agreement with those presently employed by some taxonomists.

Creel (1964)[1/] compared the species of Agropyron serologically and suggested that not all four sections of the genus Agropyron (Goulardia H., Holopyron H., Agropyron and Eremopyrum), as proposed by Holmberg (1926), were valid.

Serological relationships were determined by Kleese and Frey (1964) between Avena sativa var. Cherokee and six other oat varieties used as parents in crosses. Relationships were also studied between 4 corn inbreds used in single crosses. Reactivity was tested quantitatively by photoelectrically measuring the turbidity (after Boyden and Defalco, 1943) from each antigen-antibody system. The researchers concluded that the serological relationships of the parents used in the oat crosses agreed with the within-cross variance components for

1/ Creel, G.C. 1964. Biosystematic comparison of species in the genus Agropyron Gaertn. with particular emphasis on serological studies. Ph.D. Thesis. Montana State University.

grain yield whereas comparisons to heading date and height corresponded only in isolated cases. Similar methods used in comparison and predictions of the corn inbreds yielded less conclusive results.

Vaughn et al. (1965) used immunodiffusion, double-diffusion (method by Ouchterlony) and electrophoretic techniques in taxonomic analysis of three species of the genera Brassica. Evidence based on seed proteins supported the classification of the three plants into three taxa and also supports classification by Schultz (1919) on morphological grounds.

Lester et al. (1965) compared 14 species of Baptisia by means of double-diffusion and immunoelectrophoretic techniques. A few definite and repeatable differences could be detected; however, in general, the serological differences appeared "conservative" when compared to morphological criteria. The investigators felt that "serological data should be regarded as adjuncts to other systematic knowledge only on the basis of empirical manifestations of their utility. There is no clear justification for regarding serological data as intrinsically either superior or inferior to other systematic criteria".

The Ouchterlony double-diffusion plate method was used by Esposito et al. (1966) for the successful identification and differentiation of nine Medicago sativa L varieties. Specific serological tests were reported as "sufficiently definite" for the identification of seed protein phenotypes. The results indicated that it is possible

to identify the nine varieties and to distinguish one from another. The researchers indicated, however, that a combination of genetic, as well as environmental components, may have influenced seed protein content; thus, either or both components may have been responsible for varietal diversifications displayed.

More recently, Damirgi et al. (1967) reported applying a serological technique in a study which was conducted to determine the distribution of rhizobial strains of Rhizobium japonicum in nodules of soybeans grown on certain soils. Homogenized nodule suspensions were used as antigens and the agglutination test was used to compare the groups serologically. The results were then used to distinguish specific strains of rhizobia found in different areas and in various soils.

Rewarding contributions in the research of hybrids has been brought about by serological determinations. One of the classical papers on the serological study of plant hybrids was that of Zade (Alston and Turner 1963) who published a study including a serological comparison of three species of clover: Trifolium repens, T. pratense, and T. hybridum. At that time, T. hybridum was regarded as a hybrid of T. pratense and T. repens (this is questioned by recent investigators). Zade reported that T. hybridum serum reacted more strongly with the supposed parents than did reciprocal tests with serum from the parents. Chester (1937) who discussed this work mentioned "Zade with the precipitin test showed that Trifolium pratense and T. repens are related but serologically distinct. Their hybrid T. hybridum

reacts so strongly with both as to demonstrate its hybrid nature". Had there been no previous assumption of the hybrid nature of T. hybridum it may have been concluded that this species was the closest of the three to some primitive Trifolium stock. The results are open to various interpretations.

Hall (1959) when serologically investigating rye-wheat crosses and parents conclusively indicated all proteins immunoelectrophoretically identified in the wheat could be traced in the rye-wheat hybrid and specific rye-wheat proteins could not be detected.

Irwin and Cumley (1943) studied serological relations in the Pearlneck dove, the ring dove, and their hybrids. They found in the hybrids all the antigens shared by the parental species and most of the characters specific to each parent. In contrast to Hall's report, all of the hybrids possessed a "hybrid substance not found in either parent".

Commenting on techniques to disclose hybrids through serological methods, Alston and Turner (1963) wrote: "Suppose, for example, that species A contains antigen complement a+b and species B contains antigen complement b+c. Thus, b represents the common antigenic substances. The hybrid should, therefore, possess a complement a+b+c, and a hybrid antiserum, if absorbed with serum type A and then with serum type B, should be completely neutralized. It should then give a negative response to hybrid serum. Presumably then, if a residual activity remained in the antiserum after absorption with

sera A and B, one of three explanations might hold:

(1) The plant was not a hybrid.

(2) New "hybrid-type" antigenic substances were present.

(3) Genetic heterozygosity in one or both parents led to individual differences in antigenic complement.

However, if serum of the "hybrid" completely neutralized antisera of type A and type B, this would offer strong support for the true hybrid nature of the plant in question.

II. Cytogenetics - Aneuploidy

The first extensive aneuploid genetic program in wheat was initiated by Sears (1939) when he observed two haploid plants in a field culture of 105 plants grown from seed of Triticum vulgare variety Chinese Spring (n=21). Both of the haploids were completely male sterile. One was female sterile but the other set 14 seeds from florets pollinated by normal wheat pollen. The 14 seeds yielded 13 mature plants which ranged from 40-42 in somatic chromosome numbers.

Each of these plants was investigated cytologically at meiosis. Five of the 41 chromosome plants showed 1 univalent and 20 bivalents, and 2 showed 2 univalents and 1 trivalent each. Of the two 40 chromosome individuals one had 17 bivalents, 2 univalents and 1 ring of four and the other had 19 bivalents and 2 univalents. Of the four 42 chromosome plants, 2 were normal with 21 bivalents each, 1 had 15 bivalents, 2 univalents, 2 trivalents, and a ring of four, and the other had 19 bivalents, 1 trivalent and 1 univalent. Sears hypothesized that the different meiotic configurations in the progeny

were the result of abnormal division or segregation of the 21 univalent chromosomes at meiosis of the original haploid plant. The resulting gametes which carried duplications or deficiences for one or more of the chromosomes when combined with the normal male gametes, would result in progeny which were abnormal in their chromosome numbers and pairing configurations. Sears proposed that the occurrence of the rings of four was the result of segmental interchanges which occurred in the haploid as a result of crossovers between paired homologous chromosomes. The tetrasomic condition was an unlikely contribution.

Sears (1939) discussed several possibilities for the use of chromosome aberrants in a broad genetic analysis of T. vulgare. An effort was initiated to determine homologies among the monosomic individuals by using T. durum (n=14) as a common parent. Another area of study is the immediate effects on the plant resulting from duplications and deficiencies of chromosomes and parts of chromosomes. Monosomics and trisomics may also be used to locate genes on specific chromosomes of common wheat.

By 1944 Sears (1944) had obtained seventeen of the 21 possible nullisomics and monosomics. From observations of the nullisomic individuals, the factors governing seed color, pubescent nodes, squareheadedness and suppression of speltoidy, and awn development, were assigned to specific chromosomes. Sears' observation of the frequency of transmission of the nullisomic and monosomic conditions

from selfing monosomic plants indicates the nullisomics are produced in frequencies ranging from 3 to 10 percent while monosomic frequency ranged from 75 to 85 percent. Burnham (1962) points out that in the production of female gametes there is approximately 50 percent chance of the univalent being lost in the first meiotic division and a 50 percent chance of it being lost in the second meiotic division. The final percentage of cells that receive the univalent may be about 25 percent. Deficient female gametes, against which there is no apparent selection, occur on an average of 75 percent of the time resulting from this transmission - not as a result of there being a 50-50 chance of the univalent passing to one pole or the other at both divisions.

Male transmission of (n-1) is reported by Sears (1944) as ranging from 1 to 15 percent depending on the chromosome. This low number of functioning deficient male gametes is presumably due to the elimination of deficient pollen through competition with normal pollen.

From these studies of transmission frequency, Sears (1958) was able to predict breeding behavior of a typical monosomic plant for the common wheat variety Chinese Spring (n=21).

Table I. Breeding behavior of a typical monosomic plant.

Female	Male	
	21 Chromosome Pollen 96%	20 Chromosome Pollen 4%
21 chromosome eggs 25%	21_{II} plants 24%	$20_{II} + 1_{I}$ plants 1%
20 chromosome eggs 75%	$20_{II} + 1_{I}$ plants 72%	21_{II} plants 3%

By 1958 Sears reported the final complete grouping of all 21 chromosomes into 7 homologous groups and also designated the genome to which most chromosomes belonged.

MATERIALS AND METHODS

I. Cytogenetics

In the present study, twenty-one monosomic lines, each monosomic for a different chromosome, of the hard red winter wheat Triticum aestivum L. variety Kharkoff MC22 (subsequently referred to as KMC22), were obtained as seed from Dr. B. C. Jenkins, Department of Plant Science, University of Manitoba, Winnipeg, Canada. These lines had been produced by a backcrossing program with KMC22 as the recurrent parent on original crosses with Chinese Spring monosomics. The backcrosses had been completed for 8 or 9 generations to insure relatively pure genetic recovery of the Kharkoff complement. One exception to this was the line monosomic for chromosome 6D which had a history of only 3 backcrosses.

The chromosome numbering system used in this study is explained as follows: The first numeral and capital letter refer to the specific chromosome involved, the subscript letter refers to one of two families developed for each substitution line and the final numeral designates the original plant in this line from which the progenies for this study were propagated. As an example, 1Da-2 would designate substitution line 1D, family a, and the second plant.

The twenty-one monosomic lines, perpetuated by selection, were grown on an individual plant basis during the 1965-66 season at the Agricultural Research Station, Bozeman, Montana. Head samples for pollen mother cell cytological analysis were taken on plants of

monosomic lines about 3 days prior to emergence from the boot. Head samples were preserved and refrigerated in Newcomer's fixative (Newcomer 1953). Slide preparations were made according to Belling's (1926) method of staining. Chromosome counts were made when possible, otherwise the occurrence of a univalent in the cell was taken as an indication of a monosomic condition.

Absolute identification of monosomic lines of chromosome 4Aa-3 and chromosome 1Db-2 was made uncertain due to the presence of unusual chromosome configurations at meiosis. These two lines were not included in the study. Line 7Ba-1 was not included because nonsegregating progeny indicated the original parent was a disomic.

The correct meiotic stage for cytological identification was unavoidably missed in certain plants. These plants were allowed to self pollinate and mature. Five plants from each of the unidentified parents were grown in the greenhouse and examined cytologically. If the parent was a monosomic the progeny would segregate for the aneuploid condition in an approximate ratio of 3 monosomic to 1 disomic. (Witnessed deviation from this ratio may be explained by small sample size and irregularities of univalent transmission inherent in the monosomic line.) If an examined plant was monosomic, indications are that the parent was monosomic, therefore, no other samples were observed from the other 4 plants. On the other hand, if a plant was disomic the other 4 plants were examined also. If they were all disomic the parent was considered a disomic; if all

were not 2n the parent was considered a monosomic. The possibility of misclassifying a parent as a nullisomic or a monosomic was disregarded for this study because morphological identification of the nullisomic plant is possible, and cross pollination in a nullisomic is an infrequent occurrence.

II. Extraction, Antigen Treatment, and Protein Determination

Concurrent with cytological sampling, the leaves were clipped from field plants in about the 6 leaf stage. The plant tissue, after being washed and blotted dry, was confined within a stainless steel cylinder, semipulverized and subjected to 3,000 pounds per square inch of pressure. The crude plant sap was drawn off and frozen within 20 minutes. All plant samples of expressed sap were identified and kept separate throughout the study.

The antibody sources were the Single plant selection and the selection 1Da-2. Selection 1Da-2 is a derived disomic from segregating monosomic and disomic lines of KMC22. The selection referred to as a "Single" plant line is a propagation of a single plant selection of the variety KMC22 from the Montana State Experiment Station breeding program. These two selections were grown under greenhouse conditions, vernalized and allowed to reach the 4-6 leaf stage before procedures were repeated for sap expression. Five grams of green tissue was found to yield approximately 2 ml. of plant sap.

The expressed sap from each selection was divided into 2 lots. One lot of each selection was retained in the native crude state and the other lot was treated by ammonium sulfate protein saturation

methods (Colowick and Kaplan, 1955). The frozen sap was thawed and centrifuged at 4,000 xg. for 5 minutes. The supernatant liquid was diluted to twice its volume with 0.05 Molar phosphate buffer pH 7.0 and saturated to 20 percent with ammonium sulfate. After twenty minutes of constant agitation under refrigeration, the mixture was centrifuged at 15,000 xg. for 10 minutes. The supernatant portion was then made to 60 percent saturated with respect to ammonium sulfate and, after being agitated as before, was centrifuged at 12,000 xg. for 10 minutes. The supernatant liquid from this fraction was discarded, the precipitate was taken up in 10 mls. of the phosphate buffer, and dialyzed overnight at 2° centigrade in 1,500 mls. of the buffer. After 2 hours of dialysis, the buffer was replaced by fresh phosphate buffer. The prepared material was then placed in closed vials and frozen.

The biuret method of protein determination outlined by Cambell et al. (1964) was used for quantitation of the treated preparations. The treated extracts were diluted to measure 1 milligram of protein per ml. for injection.

III. Serology

To produce the antiserum, four rabbits, all litter mates, were maintained. The animals were bled previous to any injection and the resulting non-immune serum was frozen. Included is the injection schedule (Table II).

Table II. Injection schedule.

Rabbit Number				No. Days From Initial Injection (0)				
Treated		Crude						
1Da-2	Single	1Da-2	Single	0	14	16	18	20
01				1 ml	2 ml	2 ml	2 ml	2 ml
	02			1 ml	2 ml	2 ml	2 ml	2 ml
		03		1 ml	2 ml	2 ml	2 ml	2 ml
			04	1 ml	2 ml	2 ml	2 ml	2 ml

The thawed crude sap was centrifuged to remove chloroplasts before injection. Subcutaneous injections were administered in 4 places at each injection, over the shoulders and in the lumbar region.

A suitable antibody level was generally obtained the seventh day after the fifth injection at which time the animals were bled following the method of Creel, et al. (1965). Immediately following each bleeding a 2 ml. booster injection was given to maintain antibody titer.

A modification of the gel-diffusion method described by Ouchterlony (1948) was used. The gel-diffusion plates were 90 mm. in diameter with agar poured to a depth of approximately 2 mm. The Ionagar No. 2 was prepared as a 0.6 percent (w/v) solution. The solvent for the agar was prepared according to Creel, et al. (1965). A preservative of sodium azide, 1 part in 2000, was added. After dissolving the agar in the solvent, 11 ml. were pipetted into the sterile petri dishes. The plates were allowed to stand several hours before the well and trenches were made.

Gel-diffusion plates were constructed reacting each antiserum with its homologous antigen. Comparisons could then be made involving serum specificity differences between animals as well as differences, if any, between crude or treated and 1Da-2 or Single antigen preparations.

Experiment I. Unabsorbed antisera with selected monosomic and disomic extract preparations.

Wells and trenches were made in the agar plates with a template made according to Fig. 1. Wells were cut in the cooled agar with a number 3 cork borer (8 mm. in diameter) 0.8 mm. apart and 7.5 mm. from the trench edge. The trench measured 5 x 76 mm.

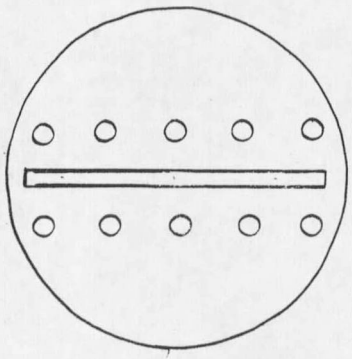

Fig. 1 Template Design for Experiment I.

A total of ten extract samples from monosomic and disomic plants within each of the eighteen monosomic lines were chosen for study (Table III). The crude sap was allowed to thaw, then each well was filled with appropriate crude extract. One plate accommodated plant samples from 1 monosomic line. The trench was filled with antiserum