I. GENETIC STUDIES OF MOUSE SERUM PROTEIN TYPES

II. MOLECULAR HYBRIDIZATION OF SHEEP HEMOGLOBINS

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ABSTRACT

Investigations were conducted to detect serum protein variant systems in mice and to define their genetic control. Three systems were detected. A pair of codominant alleles at a locus in the second linkage group controls electrophoretically different transferrin types. An electrophoretic difference among inbred lines in the presence or absence of a prealbumin is controlled by a single autosomal locus without dominance. A serologically detected system is controlled by a single locus closely linked or identical to the H-2 locus in the ninth linkage group. The variant component seems to be an α-globulin of high molecular weight. The principal difference between serum types is quantitative; qualitative differences are not excluded. The serum component is not detectably related serologically to H-2 erythrocyte antigens. The effects of development, pregnancy and stress upon these serum components were studied. Using these three serum variants as markers, the sera of homologous radiation chimeras were examined for donor type proteins. These occasionally appear transitorily, but long-term survivors having entirely donor erythrocytes have only host type serum proteins.

Molecular hybridizations were performed with the two known sheep hemoglobin variants. Radioactive and ferriheme labels showed some exchange of subunits between variant types, but less than expected. Incomplete exchange is apparently due to inadequate asymmetric dissociation or to partial incompatibilities between both subunits of the two hemoglobins. The electrophoretic difference between the two types resides mainly, if not entirely, in one of the subunits.

CONTENTS

I. GENETIC STUDIES OF MOUSE SERUM PROTEIN TYPES

A. INTRODUCTION

Elucidation of the basic mechanisms of gene action, the mechanisms by which genetic information coded in nuclear DNA is translated into the complex of reactions and interactions in the whole organism, is an important area of research in genetics. One approach seeks detailed structural and functional definition of the heterocatalytic products of the genes and the principal mediators of reactions, the enzymes and other proteins. Studies in this area encompass a wide range of species and techniques. The most efficient and elegant studies will as a rule probably be done with microorganisms. For example, in the E. coli tryptophan synthetase system (1), the tyrosinase (2) and glutamic dehydrogenase (3) systems of Neurospora, and the phage lysozyme system (4), structures of the proteins are being studied. Complementing these studies are the genetic fine structure studies in bacteriophage (5, 6), Salmonella (7) and E. coli (1), which promise to be useful in the investigation of genetic coding, one important aspect of the mechanism of gene action.

In order to verify the universality of basic principles established in studies of lower forms, and to apply them usefully in medicine and agriculture, it is important that similar studies be carried out in mammals and other higher forms. This will require detailed definition of the genetic control and of the function and structure of biochemically important specific proteins in particular species. Significant progress is being made in mammalian biochemical and physiological genetics, and in some areas, for example in the studies of the human hemoglobins, despite the advantages of

microorganisms for genetic study the pioneering basic work has been done in mammals. Many biochemical markers are becoming known in numerous mammalian species. These promise to be useful in physiological and biochemical as well as genetic studies, in transplantation and immunogenetics, somatic cell genetics, in investigations of protein synthesis, perhaps eventually in chromosomal fine structure studies and developmental genetics.

Mammalian biochemical genetics had its origin nearly 60 years ago, not long after the rediscovery of Mendel's work, in the work of Garrod (8, 9). Garrod's studies and the later classic experiments of Beadle and Tatum (10, 11) were based upon essentially the same principle, the recognition of mutation by the detection of a physiological or biochemical abnormality, traceable to a loss of or structural aberration in an enzyme concerned with a specific biochemical reaction. This relationship of gene and enzyme was extended in 1949 by the genetic studies of Neel (12) and the chemical studies of Pauling et al. (13) on sickle cell anemia, to a relationship of the gene to other biochemically important proteins.

The discovery by Pauling and his coworkers that the basis for sickle cell anemia is an aberration in the molecular structure of the hemoglobin led to the concept that genes control the specificity of proteins in general, and that the effect of a mutation at a specific locus is to alter the structure of the protein controlled by that locus. It also introduced a general tool for biochemical genetic research, electrophoresis, which has made it possible to recognize and study a new class of biochemical mutants. As distinguished from the class showing some physiological

abnormality traceable to a variant protein, this class includes macromolecules having altered physical or antigenic properties, even though they may produce no detectable physiological handicaps. These are, in other words, isoallelic systems.

This class of mutants is important for a number of reasons. It makes possible the demonstration of the validity of the one-to-one principle earlier established with material criticized as being highly selected by the detection methods (14), now with systems which have no obvious selective value at all. It provides material for a new appraisal of evolutionary mechanisms, based upon subtle, gradual changes, rather than drastic one-step alterations, and raises new questions as to the mechanisms maintaining polymorphisms under very low apparent selective pressure. Subtle changes in protein structure are probably importantly involved in "normal" genetic variability or "biochemical individuality" (15).

Extensive biochemical genetic studies have been carried out in a number of mammalian species, aimed at the detection of protein variant systems or protein "polymorphisms". These have centered upon systems of the isoallelic type recognizable by changes in physical or antigenic properties of the protein, and principally upon proteins of the blood. It will be of value to consider these studies with regard to the specific proteins involved, the techniques employed, the principles established, and the new questions raised, as a basis for evaluating the results of the studies subsequently presented in this thesis.

The contributions made to the understanding of the role of the gene in control of protein structure by the studies of the human

abnormal hemoglobins are well-known and will be but briefly described. The demonstration by Pauling and coworkers (13) that sickle cell anemia was due to a molecular change in the hemoglobin, detectable by a change in electrophoretic properties, stimulated a search for similar cases of abnormal hemoglobins, and many were found. Nearly 30 different types have been described, differing in electrophoretic mobility, spectra, alkaline denaturation, or other properties. Several of these have been rather thoroughly studied genetically, and the inheritance of any given abnormal hemoglobin type seems always to be under single gene control.

X-ray diffraction studies by Perutz et al. (16) and chemical studies by Schroeder and coworkers (17, 18) established that the hemoglobin molecule consisted of two kinds of paired polypeptide chains, each with a different N-terminal amino acid. These are called the α and β chains, and the hemoglobin molecule may be represented by the formula, $\alpha_2\beta_2$. The chemical studies of Ingram and other workers (19-30) have established rather convincingly that each difference between an abnormal hemoglobin and normal hemoglobin A involves the substitution of a single amino acid at a point in either the α or the β chain. The technique of "fingerprinting" introduced by Ingram (19) has been of great value to workers in this field, as well as to workers with other proteins. Genetic studies of individuals having two abnormal hemoglobin types have shown that each chain is under the control of a separate genetic locus, and that these loci are effectively unlinked (30-32). Two important basic genetic principles seem to have emerged from this work. The first is that a mutation need involve no more than a single amino

acid in a chain of 150, and that such a seemingly minor change can have a major effect upon the organism. Such a change is envisioned as resulting from a change in a single base pair in the DNA controlling the amino acid sequence of a polypeptide chain (20). The second point is that each polypeptide chain in a protein molecule consisting of two or more such chains is apparently determined by a discrete piece of genetic information, presumably analogous to the "cistron" demonstrated by Benzer (5), and that these cistrons need not be adjacent.

Chemical studies of the hemoglobin of the human fetus have established that the molecular formula is $\alpha_2 \gamma_2$ (33-35), and it seems reasonably clear that the gamma chain is controlled by a third locus, which functions instead of the beta locus until birth, while the alpha locus probably functions continuously. These findings are of interest in developmental genetics. Studies of some of the minor components usually present in red cell lysates have suggested that at least one additional locus, the δ, is probably concerned with the structure of still a third partner for the alpha chain resulting in the formation of hemoglobin A_2 (36-40). The technique of molecular hybridization (reviewed in detail in Section II. A.) has also made important contributions in these areas of study.

Interest has also centered upon the kinds of evolutionary mechanisms which must act to maintain polymorphism for abnormal hemoglobins which are deleterious in the homozygous individual. High mutation rates have been suggested, but seem improbable (41). The more reasonable explanation is that the individual heterozygous for an abnormal hemoglobin has some selective advantage relative

to the homozygote. There is evidence that in the case of sickle cell hemoglobin the heterozygote may be more resistant to malaria than normal homozygotes(41-44). Such systems have been assigned the term "balanced polymorphism".

Hemoglobin variants in numerous other species (45) also pose a problem with regard to the maintenance of polymorphism. Cattle (46-50), sheep (51-58) and mice (59-65) each have two alternative types recognized electrophoretically. These variants have been rather extensively studied, and there is no obvious indication that any phenotype confers either significant advantage or disadvantage under ordinary conditions. Thus, although there is no apparent selection pressure against either allele, a mechanism would still seem to be required which prevents fixation of one or the other type. An indication of the kind of subtle selective mechanism which may operate in such cases is seen with the sheep hemoglobins. Here one of the types has a somewhat higher affinity for oxygen, and it appears that this type is more frequent at higher altitudes (57). A similar type of mechanism might perhaps be postulated in cattle, where one type is more frequent among breeds usually found in hotter climates (49-50). The possibility of correlation with resistance to trypanosomiasis has been suggested (49), but there is as yet no firm evidence bearing upon either possibility. In mice, the distribution of types among wild populations is not well known except that the two common types occurring in laboratory strains are both found (64), so nothing can be concluded about possible selective mechanisms. Chemically and genetically, the mouse hemoglobins offer some very interesting problems. The structural change does not lead to a simple change in electrophoretic

mobility as in all other species studied (59), and there is some evidence of complexity of loci involved in the genetic determination (64). The mouse hemoglobins have found a useful application in radiation research as markers for following erythrocyte repopulation in radiation chimeras (66).

An interesting case of hemoglobin polymorphism occurs in the horse. No genetic differences have been detected in the horse, but all horses seem to have two major hemoglobin components, differing in electrophoretic mobility (67). Fingerprint studies of these two hemoglobins suggest that they probably differ in only a single peptide (68). The case may be similar to that of hemoglobin A_2 in humans, except that here there are two major components rather than a major and a minor component. It may be relevant that the horse has no fetal hemoglobin detectable by fingerprint studies (69).

Following the lead of the hemoglobin workers, many investigators are now studying electrophoretic and also antigenic variants in other proteins. Studies of a variety of proteins in numerous species increase the probability of uncovering new facets of gene action.

In general, the most useful non-hemoglobin variant systems found thus far have been in the proteins of the blood plasma. The most important exception, the beta-lactoglobulin system in the milk of cattle, was first reported by Aschaffenberg and Drewry in 1955 (70). This involves two electrophoretically different proteins, controlled by a pair of codominant alleles (71). The physical-chemical properties of the two proteins have been rather extensively studied (72-76), and both appear to consist principally of dimers, probably of a single subunit. The two types appear, on the basis

of titration studies, to differ in only a single carboxyl group in the subunit (74). Molecular hybridization attempts have been unsuccessful, suggesting that the difference between the two subunits results in complete incompatibility between them, and supporting the concept of a dimer of a single subunit (76). Another difference in cow's milk involves the alpha-lactalbumin (77), but detailed studies of this difference have not been reported.

The first serum protein variant system to be described in detail was the haptoglobin system in man, detected by Smithies with his starch gel electrophoretic technique (78, 79). The genetic control of the trait has been shown to involve principally a pair of alleles (80-82), although several modified phenotypes remain to be defined (83-86). Both principal alleles occur frequently in most human populations, and no apparent abnormality is associated with either (80-83, 85, 87-91).

The patterns of the haptoglobins on starch gel electrophoresis and the chemical implications of the patterns are of particular interest. One type, Hp 1·1, involves a single hemoglobin-binding component on starch gel, while the homozygote of the other type, Hp 2·2, has a series of bands of decreasing intensity regularly spaced from the major component to the origin. The heterozygote, Hp 2·1, has a weak band matching the single component of Hp 1·1 and a series of bands, none of which matches in mobility any of the Hp 2·2 bands. Because the bands of a series are not separated genetically, there would seem to be involved a molecular pleiotropism, and a series of hybrid proteins in the heterozygote. It has been demonstrated that this is not an artifact due to the starch gel technique, even though no such series of bands is seen

by other electrophoretic methods (92, 93). The components represented in the series of bands are apparently polymeric forms of the same subunit, which are separable on starch gel electrophoresis because of a molecular sieve effect of the gel. Allison has proposed an association mechanism which could account for this phenomenon (94), and chemical studies support the association theory (95, 96).

Among other species, variant haptoglobin phenotypes have been observed in monkeys (97, 98) and in the Alaskan fur seal and marmot (99). In neither instance is any information available concerning the mechanism of inheritance. In the cynomolgus monkeys the phenotype found in 7 of 30 individuals tested resembles the Hp 2-1 (mod.) phenotype of humans (97).

The single protein of mammalian serum which has thus far shown the most extensive polymorphism in terms both of species in which it occurs and numbers of phenotypes is the iron-binding beta-globulin, transferrin. Beta-globulin variation was first demonstrated in cattle by Ashton (100), and shortly thereafter in humans by Smithies (101). These variants were later shown to bind iron, and were consequently assumed to be transferrins (102, 103). Transferrin polymorphisms, or beta-globulin polymorphisms presumed to involve transferrin, have also been demonstrated in sheep (104-106), goats (105), horses (107), swine (108, 109), rhesus and cynomolgus monkeys (98, 110-113), and chimpanzees (114). The genetic control of these polymorphisms has been studied in humans (86, 103), cattle (115-117), and swine (108).

In the human system, eight alleles are believed to be involved (118), each controlling an electrophoretically unique component. The C component is most frequent in all populations

studied, and unlike the haptoglobins, the other types occur only relatively rarely in certain populations (88, 91, 93, 119). The wide occurrence of polymorphisms for this particular component in so many species raises the question whether some important general selective mechanism or principle may be involved in their maintenance, but there is as yet no evidence to support such an hypothesis. The in vivo and in vitro iron-binding capacities of several of the human types have been studied (118), and there seems to be no difference between them in this regard, suggesting that any selective advantage of homozygosity or heterozygosity for the rare components may lie in subtle adaptive capacity under specific circumstances. Ashton has reported data in cattle which might suggest maternal-fetal incompatibility due to transferrin difference, but the evidence is not compelling (120). Ashton has also reported somewhat more convincing evidence for an effect of one of the transferrin alleles on milk production in dairy cattle (121).

A number of other electrophoretic differences in serum proteins have been noted, with more or less evidence for their genetic control. Ashton has reported variation in minor components in the serum of cattle which he calls "thread proteins", with preliminary evidence for their genetic determination (122). He has also reported that in some cattle the S-alpha$_2$-globulin component is absent (123), with evidence for genetic control based upon three animals of this type.

Thompson et al. (124) reported that among sera from seven lines of mice tested by moving boundary electrophoresis, one showed a unique shoulder of beta$_1$-globulin, presumably due to a differing

component. Hybrids between this line and another showed the same electrophoretic pattern as the differing line. The trait was therefore assumed to be genetically controlled by one or more dominant genes. Reciprocal crosses resulted in no differences between F_1's regardless of sex, so it was assumed that sex linkage was not involved. Because it was necessary to pool serum from ten or more mice for each moving-boundary experiment, no data were collected on segregants.

In man, cases have been reported involving variation in several other serum protein components. At least one form of the serious human condition called agammaglobulinemia is apparently inherited (125). Individuals have been observed who have no detectable albumin in their serum, but this seems not to be a clinically important condition (126). Two reports in the literature describe families in which several members showed a condition called "bisalbuminemia" (127, 128); the serum of each affected individual contained two electrophoretically distinguishable albumin species. The family data suggest that the trait is inherited. Another general condition for which there is evidence of genetic control, perhaps involving several loci, is afibrinogenemia (129). Smithies has reported variation in the postalbumins of human serum, detected by the vertical starch gel technique, but no reports have yet been made on the inheritance of this variation (130).

Three enzymatic differences in mammalian serum have been reported, with evidence for genetic control. One is in human serum, in which two alleles control different pseudocholinesterases having different enzymatic activities (131). The two enzymes apparently have very slightly different electrophoretic mobilities (132).

The assay procedure has been well-developed (131), and the genetic evidence seems convincing (133, 134). An esterase difference has also been reported in the serum of swine with evidence for genetic control by a multiple allelic system (135), but full details of the genetic data are not yet available. A third system involving presence or absence of an atropine esterase in the blood of rabbits has been rather well studied and apparently is under the control of a pair of codominant alleles at a single locus (136).

The application of electrophoretic methods has made possible significant advances in mammalian biochemical genetics. More recently the application of a new criterion for detection of protein variants has become possible. Immunogenetic studies of cellular blood types have been in progress since the early discovery of human blood groups by Landsteiner (137). However, the techniques of blood grouping were, with one important exception, not adaptable to soluble antigens, and the precipitin methods for study of soluble antigens were not well adapted for genetic studies. The breakthrough came when Oudin (138) and Ouchterlony (139) developed their techniques for immunodiffusion in agar. The basic Ouchterlony double diffusion method has been modified in many ways to make it useful for many kinds of studies. Probably one of the most useful adaptations has been the combination of this technique with electrophoresis, the method now called immunoelectrophoresis (140, 141). Through the application of these tools several interesting variant systems have been detected and defined.

Oudin has shown genetic variation in the antigenic specificities of rabbit gamma-globulins using the single diffusion technique in agar (142). He finds six different "allotypes" controlled

by two loci. Dray and Young (143, 144) using immunoelectrophoresis and Dubiska et al. (145) using precipitin methods and the Ouchterlony technique have shown similar variations.

Dray has reported differences in human gamma-globulins detectable with monkey antisera and immunoelectrophoresis, but these studies are preliminary (146). One system in human serum involves an electrophoretic difference detected by serological methods. This is the Gc system found by Hirschfeld (147), recognized by immunoelectrophoresis as a difference in position along the path of electrophoretic migration of the precipitin band formed by reaction of a specific serum component with antibodies against it in rabbit or horse anti-human globulin antisera. Genetic studies of the difference indicate control by a single locus having no relation to the haptoglobin, cholinesterase, transferrin, or Gm systems, or to a number of the blood groups (148, 149).

Several earlier studies were carried out on antigenic differences among sera of related species, using the classical precipitin methods, but without really clearcut results. Cumley and Irwin (150-152) reported specificity differences among species of doves. Parental specificities all seemed to be present in hybrids and seemed to segregate in simple Mendelian fashion in backcrosses. Antigenic variations were also observed in human sera, but no genetic studies were reported (153). Scheinberg (154), using precipitin methods as well as starch gel electrophoresis, has reported similar results with pigeon-dove and cattle-bison species crosses.

An antigenically variant system has been found in human serum which it is possible to study by the methods of red cell typing. This is the Gm system, discovered by Grubb (155). It was

found that the serum of some rheumatoid arthritis patients had the capacity to agglutinate human erythrocytes first coated with an incomplete anti-Rh_o serum. The serum of some individuals inhibits this reaction if added to the agglutinating sera. The occurrence of this inhibitor, now called the Gm factor, is under genetic control (156-158), and the inhibitor has been shown to be a gamma-globulin (156, 159). At present four alleles are known, distinguished by reactions with different agglutinating sera (160-162), and two new, apparently independent systems of the same general type have been reported (163, 164). Gm heterogeneity has also been reported in chimpanzees (165).

Many potentially useful protein variant systems are therefore available for study in a number of mammalian species. One of the most useful mammalian species for studies of basic genetic significance has, however, been rather neglected in this regard. The mouse offers advantages in formal genetic definition, genetic constancy of experimental material, and facility in genetic studies presently unmatched by other mammalian species. Its usefulness is limited only in instances in which large amounts of material are required, and even this disadvantage is minimized by the availability of highly inbred lines.

This thesis describes the detection and definition of biochemical markers in the serum of the mouse, employing the techniques demonstrated above to be most powerful for study of genetically determined protein variants: starch gel electrophoresis, immunodiffusion and immunoelectrophoresis.

B. Electrophoretic Variant Systems

1. Materials and Methods

a. Animals

The inbred lines employed in these studies are listed below. The line designations are followed by the abbreviations which will be used for them throughout this dissertation, the generations during which they have been maintained by continuous brother-sister matings, (e.g. F112 indicates 112 consecutive generations of brother-sister mating), the sources of the stocks and the history of maintenance in this colony.

1. A/HeJax, (A/He), (F112). From Pedigreed Expansion Stocks originally obtained from R. B. Jackson Memorial Laboratory (hereafter abbreviated as Jax) at F109, maintained for three generations by brother-sister matings.

2. A/Jax, (A/J), (F?,+4). From Jax Production Stocks, maintained for several generations by random mating within the line, then for four generations by brother-sister matings.

3. AKR/Jax, (AKR), (F57). From Pedigreed Expansion Stocks at Jax at F55, maintained for two generations by brother-sister mating.

4. BALB/cJax, (C), (F approx. 98). From Jax Production Stocks, not maintained in this colony.

5. CBA/Jax, (CBA), (F106-109). From Jax Pedigreed Expansion Stocks, at F103-106, maintained for three generations by brother-sister mating.

6. C3H/?, (C3H), (F?). Obtained from local dealer, subline and history unknown, not maintained in colony.

7. C57Bl/6Jax, (B/6), (F approx. 65,+4). From Jax Production Stocks, maintained by four generations of brother-sister matings.

8. C57Bl/10Gn-lu, (B/10), (N27). Obtained from Dr. Margaret C. Green at 27 generations of backcrossing to C57Bl/10Gn, not bred in colony for these studies.

9. C57Br/cdJax, (BR), (F approx. 101). Obtained from Jax Production Stocks, not maintained in colony.

10. DBA/1Jax, (D/1), (F approx. 36). From Jax Production Stocks, not maintained in colony.

11. DBA/2, (D/2), (F67). From Jax Pedigreed Expansion Stocks, maintained for three generations by brother-sister mating.

12. Flexed, (Flx), (F11). Not a standard inbred line. Obtained from Dr. Elizabeth S. Russell at F5, maintained for six generations by brother-sister mating.

13. RF/Jax, (RF), (F?+18). From Jax Production Stocks, not maintained in colony.

14. WB/Re, (WB), (F34,+4). From Dr. Elizabeth S. Russell at F34, after one generation of random mating within the line, maintained for four generations of brother-sister mating.

15. WC/Re, (WC), (F29,+4). From Dr. Elizabeth S. Russell at F29, after one generation random mating within the line, maintained for four generations by brother-sister mating.

Breeding animals were housed in stainless steel pens. One male and one to three females made up each breeding unit. Litters were usually reared in the breeding pens until additional litters were born in the same pen. The mice were weaned at four to six weeks. At weaning they were individually identified by ear markings, and their sex and phenotype with respect to any visible markers

recorded. They were then either placed in new breeding units, or held in groups of 25 to 35 for later use in experiments or for testing.

In the three genetic studies to be described, animals were never classified for serum type until they had reached at least six weeks, and in most cases eight weeks of age. Where possible, segregants from $F_1 \times F_1$ and reciprocal backcross matings were pooled in the holding pens, and were not identified as to cross until after testing. This eliminated possible bias in assigning phenotypes because of knowledge of the type of cross. When females at a known stage of pregnancy or animals at a known stage of fetal life were required, these were obtained by timed matings, in which males were allowed with the females only during the night, and the females were checked the following morning for vaginal plugs.

b. Blood samples

For routine collection of samples of serum or red blood cells, the mice were first warmed 15 to 20 minutes under a lamp, then bled from the ventral tail vessel. For serum samples, the blood was collected in 1 ml. centrifuge tubes coated with silicone. The silicone coating enhanced retraction and shrinkage of the clot, and significantly decreased hemolysis. When cells were desired, the blood was collected in isotonic citrate-saline solution (2.0% sodium citrate, 0.5% sodium chloride), and subsequently washed three or more times in 10 to 20 volumes of isotonic saline. In general it was possible to obtain as much as 1.2 ml. of whole blood per mouse, and an average of about 0.6 ml. with less than 1% mortality. For routine testing of hemoglobin types, whole blood was absorbed

on small pieces of No. 1 filter paper, which were then introduced into starch gels for electrophoretic separation as described later.

For studies of very young animals, blood samples were obtained by decapitation. The blood was collected immediately in a capillary pipette and transferred rapidly to a silicone-treated tube. By this method it was possible to obtain 0.1 to 0.2 ml. of whole blood from animals as small as 18-day fetuses. As a rule it was possible to collect samples of 0.1 to 0.2 ml. from the tail of animals past the age of about 20 days without mortality.

Serum samples were stored in ¼-dram shell vials at 4°C., if they were to be tested within two days, otherwise at -10°C. indefinitely. Careful observations have verified that no changes occur in the samples with respect to the components of interest during storage at -10°C. for at least two years.

c. Starch gel electrophoresis

Electrophoresis was carried out by the horizontal method described by Smithies (79). Separations were in borate buffer, pH 8.6 (0.020 to 0.024 M H_3BO_3, 0.008 to 0.0096 M NaOH). Starch was obtained from Connaught Medical Research Laboratories, hydrolyzed for use in electrophoresis, and used at a concentration of 12 to 14 g. per 100 ml. of buffer. Exact concentrations of starch and buffer varied with different preparations of hydrolyzed starch, and were determined by the procedure outlined by Smithies (79).

The starch was solubilized by suspending it in the buffer in a filter flask and heating to boiling for about one minute. A vacuum was applied to remove air bubbles and the hot milky solution poured into plastic trays of 40 x 300 x 6.5 mm. inside dimensions.

These were covered at once with a polyethylene film (Saran Wrap) to prevent evaporation, and allowed to cool at least 45 minutes at 4°C. Upon cooling, the solution polymerizes into a firm opalescent gel.

Serum samples were introduced into slots cut out of the gel with metal cutters. Slot size varied with the test and the kind of sample. In general five samples were placed on each gel in slots 1 x 7 x 6.5 mm. The origin was at a point one-third the length of the tray from the cathode. Samples of 0.03 to 0.08 ml. whole serum were placed in the slots with a capillary pipette. The slots were then sealed with a mixture of two parts petroleum jelly and one part mineral oil melted by heating to about 50°C. The gel was covered with Saran Wrap, leaving an area at either end of the tray 2 cm. in width exposed for contact with a wick of cloth toweling, which was immersed in buffer in the electrode cells. The cells contained borate buffer (0.3 M H_3BO_3, 0.12 M NaOH). The compartments into which the wicks dipped were separated from the electrode compartments, containing platinum electrodes, by perforated baffles.

Duration of electrophoresis varied with the preparation of starch and the systems being tested, but in general totaled 800 to 900 volt hours/cm., usually 8 or 9 hours at 9 or 10 V/cm., supplied by a variable voltage power supply. Electrophoresis was always carried out at 4°C. At the end of each run, the gels were removed from the trays, marked for identification, then sliced horizontally into two halves. The bottom half was stained with Amido Black 10B, the top half either discarded or utilized for other specific stains. Details of the staining procedures are described later.

For studies of prealbumin types, the procedure was the same, except that the buffer was adjusted to pH 9.0, and the samples were

placed in slots 6.5 x 6 x 2 mm., each containing 0.08 ml., with six samples per tray. Separations were for 8 hours at 9.5 volts per cm. The result was an almost complete loss of resolution of all components except the pre-albumin components, whose resolution was markedly improved.

For determination of hemoglobin types, samples of whole blood were absorbed on filter paper as previously described. The filter paper squares were then inserted into a slit made transversely across the width of the gel. Five rows of four samples each were usually tested on one gel. The electrophoretic conditions were identical to those employed for serum, except that the duration of each run was only three hours. No stain was necessary.

Since separations by starch gel electrophoresis depend upon molecular weight as well as electrophoretic mobility (93), the protein bands on the starch gels were identified with respect to their mobilities in the classical serum electrophoretic patterns by two-dimensional electrophoresis (166). Separations were made first on Whatman 3MM paper using borate buffer of the same composition as the gel buffer, for six hours at 4°C., 10 V/cm. in a horizontal apparatus. At the end of the run each paper strip was cut into longitudinal halves, and one half stained. The remaining half was then cut into 10 x 6 mm. sections which were placed in a gel in the same manner as for hemoglobin testing, adjacent to strips of the same size soaked in whole serum for comparison. In this way a given component on the starch gel could be classified as albumin, alpha-, beta- or gamma-globulin. Alternatively the first electrophoresis was done in agar, details of which are described in Section C. 1. d., p. 67. Subsequent steps were the same as for paper electrophoresis.

Several techniques were employed for the identification of specific components by some known activity. The gels were stained for total protein by immersing in a saturated solution of dye, either brom phenol blue or Amido Black 10B in a solution of 50 parts methanol, 50 parts water and 10 parts glacial acetic acid. The strips were left in the dye for two to five minutes, then destained in a solution of the same composition, but containing no dye, and stored indefinitely in this solution.

The transferrins were identified by the autoradiographic technique described by Giblett et al. (102). To each sample to be tested 6 μc. of $^{59}FeCl_3$ were added per ml. whole serum. The samples were allowed to stand for about 24 hours at 4°C., then placed in gels and separated in the usual way. After the run, the gel was sliced, one half stained, the other wrapped in Saran Wrap and placed in contact with a piece of Kodak No-Screen X-ray Film. After 10 to 12 hours, the film was removed and developed in D-8 Developer for 3 minutes.

Peroxidase activity characteristic of hemoglobin and hemoglobin-haptoglobin complexes was detected by the method described by Owen et al. (167). 0.2 g. benzidene base was dissolved in 100 ml. warmed distilled water to which 0.5 ml. of glacial acetic acid had been added. Just before use, 0.2 ml. of 30% H_2O_2 was added. The gel was immersed in this solution for about 15 minutes, during which time the active bands become deep blue and quite sharp. The gels were then rinsed several times in distilled water, wrapped in Saran Wrap and stored in the refrigerator.

For detection of serum oxidase activity attributable to the copper-binding protein ceruloplasmin (168), the technique of Uriel

(169), described for agar gels, was used with slight modifications. 40 mg. of p-phenylenediamine was dissolved in 100 ml. of 0.1 M acetate buffer, pH 4.7. The starch gel strip was placed in this solution and left for 2 hours at room temperature, then rinsed in acetate buffer. N, N-dimethyl-p-phenylenediamine may be substituted for p-phenylenediamine. The former gives a pink band which develops more rapidly, but is less intense. The latter develops a brown, rather diffuse band. The gels can be stored only very short times. The oxidation product diffuses rapidly.

The stain for esterase activity was carried out with only a single substrate, although many may be used, giving somewhat different results (170). In this procedure, described by Paul and Fottrell (171), the gels were first rinsed for 15 to 20 minutes in phosphate buffer, 0.25 M, pH 6.25. They were then transferred to a solution of 1% alpha-naphthyl acetate and 25 mg. Red B Salt in 50% acetone, and allowed to develop for 30 min. to 1 hr., following which they were rinsed in the destaining solution employed with the standard protein stain. The advantage of the final step is that it shrinks these gels to the same size as those stained with Amido Black 10B, permitting close comparison of results.

Cytochrome oxidase activity, according to Lawrence et al. (172), was detected by the following procedure. The gels were incubated in a solution of 50 ml. phosphate buffer, 0.1 M, pH 7.5, 2 ml. 1% alpha-naphthol in 40% ethanol, and 2 ml. 1% aqueous N, N-dimethyl-p-phenylenediamine for 2 hrs. at room temperature. This procedure gives several weak bands, but appears to be rather non-specific.

Although the gels, after the total protein and certain of

the other staining procedures, can be stored almost indefinitely, for permanent records and for convenience the results were photographed. This was done on Plus-X film, with photoflood illumination and a +3 accessory lens on a 35 mm. camera.

2. Results and Discussion

a. The starch gel electrophoretic pattern of mouse serum proteins

To facilitate description and discussion of the mouse serum protein components as detected by starch gel electrophoresis, the following information is presented on the identification and nomenclature of components. Figure 1, Appendix I, p. 179, shows the typical pattern of an adult serum, and a diagrammatic representation showing all of the components separable when optimum resolution is obtained for each by varying electrophoretic conditions. Those which can be identified by either physical properties or chemical or biological activity as homologous with previously named components in the sera of mice, humans or other mammals are given the same names. Components which have not been identified relative to homologues in other species are given an arbitrary letter designation for identification.

The gamma-globulins are identified on the basis of their characteristic range of electrophoretic mobilities and their precipitation behavior in ammonium sulfate. The limit of the gamma-globulin zone on the anodic side of the origin is indefinite, but probably is at least as distant as the S-alpha$_2$-macroglobulin band. Component F, which is usually resolved but has not been identified, lies within the gamma-globulin region. A number of components, which have not been indicated, are shown by starch gel immunoelectrophoresis to remain at the origin. These have not been identified, but it is clear from comparative immunoelectrophoresis studies of the type described in Section C. 2. d., p. 82, that at least some of these components represent proteins also found at other points on the

starch gels. Perhaps this is partially denatured material which retains antigenic specificities but does not migrate.

The S-alpha$_2$-macroglobulin component has been identified in two-dimensional electrophoresis by its characteristic mobility differences on paper and starch gel, and by its lipoprotein properties (93). The positions of the two haptoglobin bands have been reported by Blumberg (98), using the methods of Smithies (79). The transferrins were identified as described on p. 22. A component having strong esterase activity seems to underlie the major transferrin component, but this is not due to the transferrin itself because minor transferrin components do not show it, and it does not change in position with a genetically determined change in position of the transferrin.

The positions of the three hemoglobin-haptoglobin complexes have been determined by the peroxidase activity method described on p. 22. Similarly the approximate location of the single serum oxidase component, presumed to be homologous with the ceruloplasmin of human serum, has been identified by the technique described on p. 22. This component lies in a region which will be termed the alpha-beta-globulin complex (Nos. 11-19 in Figure 1), which includes three relatively strong resolvable components, designated C, D, and E, and a number of others too weak or too diffuse to be clearly seen. A number of enzymatic activities have been demonstrated in this region, but it has not been possible to relate particular bands to particular activities with any degree of certainty. Lawrence et al. (172) have shown in this region the occurrence of cholinesterase, aminopeptidase, alkaline phosphatase and oxidase (ceruloplasmin) activities. The esterase activity has also been shown in this region by several other workers (172, 173), and in the present study

by the method described on p. 23.

The F-alpha$_2$-globulin component, which, like the S-alpha$_2$ component, seems common to sera from almost all mammalian species studied (93), moves just behind the albumin zone. The albumin is obvious on the bases of both its mobility and its quantity. A rather prominent pre-albumin zone moves ahead of the albumin. By two-dimensional electrophoresis, this component has been shown to be the pre-albumin component in separations by classical electrophoretic methods as well, and is presumed to be homologous with the tryptophan-rich pre-albumin component of human serum (174). At least two other very minor components move ahead of the prealbumin. The fastest of these is usually resolved under normal conditions, the second can be well-resolved only under special conditions as discussed in Section 1. b., p. 20. Thus far nothing is known about the true electrophoretic mobilities of these components, because they are present in quantities too small to be detected with two-dimensional methods, or about their homologies with known components in other species, or their biological functions. The technique of Lawrence et al. (172) for detection of cytochrome oxidase activity, shows activity in pre-albumin bands, apparently the major pre-albumin component and component A. The A component and another just behind it, which is not clearly detectable with protein stains, show rather strong esterase activity with the method described. It is rather clear that component B (discussed in more detail in Section B. 2. d., pp. 51-63), does not have significant esterase activity.

b. Non-genetic variation in serum electrophoretic patterns

To define unambiguously inherited variations in a given

trait, one must also define carefully the kinds of non-genetic variations which can occur due to changes in environment, physiological state, or state of maturity. Without such information, errors in interpretation of observed differences, or in assignment of phenotypes may result. One should have some reservations about assertions concerning the genetic determination of individual differences, whether morphological or biochemical, unless these are supported by genetic evidence from studies in which age, environment and physiological state are controlled, or at least noted and considered in interpretation of the results. Since the early work of Smithies (78), many reports have appeared on differences both within and between species, based on studies of a few individuals of unspecified age and sex, despite evidence that large quantitative and occasionally apparent qualitative non-genetic changes occur in serum proteins. The appearance of new components during pregnancy, or during the ovarian cycle in oviparous animals, has been reported in species representing three different classes of vertebrates (175-178). Studies of various body proteins during development have been reported and always indicate changes (176, 179-181). During postnatal development, changes from a fetal to an adult type are known to occur in the hemoglobins of most mammalian species studied (45). The occurrence of fetuin in the serum of calves, which disappears in the adult, has long been known, as well as significant quantitative variations of other serum components in many species (182). A prealbumin is present in the serum of the chick embryo, which disappears at about 7 days of age, as new α-globulins appear (176).

Several investigators have reported changes in quantity of mouse serum protein components under various conditions of stress

or disease (173, 183-187). The work previously cited by Thompson et al. (124) was facilitated by the fact that after infection with Salmonella typhimurium the electrophoretically differing β_1-globulin component was increased in quantity and more easily detected. Popp (173) noted that in radiation chimeras the serum usually contained an increased quantity of a component identified as F α_2-globulin by starch gel electrophoresis. A similar change was noted by Goranson et al. (186). Clausen and Heremans (184, 185) describe a number of changes occurring in the serum of mice, detected by immunoelectrophoresis, and resulting from either disease conditions or the presence of certain types of tumors. Spectacular increases in quantities of certain specific serum fractions are noted by Potter and Fahey (187) in mice bearing specific plasma cell neoplasms.

In the present study, an attempt was made first to define the variation in serum protein components during postnatal development. Samples were systematically collected from birth until about 30 days of age, when the pattern usually becomes that of the adult. The principal study has been done with B/6 and CBA lines with samples collected on successive days; the D/2 and A/He lines have been spot-checked at intervals of 3 to 4 days during this period and found to have no significant differences in the pattern of developmental changes. There is, of course, much individual variation in these developmental patterns, generally associated with environmental conditions. For example, the patterns become those of the mature adult less rapidly in mice from unusually large litters or reared under crowded conditions. Thus a range of days is usually indicated for the times of appearance and disappearance of components

or the initiation and termination of quantitative changes.

Figure 2, Appendix I, p. 180, shows typical serum electrophoretic patterns after separation at pH 8.5, for B/6 and CBA mice at the ages indicated. A number of striking differences from the adult pattern are seen in the serum from 18-day fetuses. The quantity of gamma- and of S α_2-globulin is less, the "haptoglobins" show more clearly in their usual positions even when significant amounts of hemoglobin are present, as though they do not yet have a hemoglobin-binding capacity. These may actually be other components migrating at the same rate. Peroxidase stains at this age do not show the adult pattern of hemoglobin-haptoglobin complexes. The quantity of the major transferrin component is at least doubled, while only very small amounts of minor transferrin components are detectable. Several very sharp bands can be seen in the usually unresolved region, but nothing is known of their functions at present. There is essentially no oxidase activity in these sera; this activity does not appear until several days of age. A new component is seen between the F-alpha$_2$-globulin and the albumin. One might speculate that this may be analogous to the fetuin of bovine serum, a component present in the newborn which is qualitatively different from anything in the adult, and which gradually disappears as the animal matures (182), but there is no evidence for this. In the mouse, the quantity of the component progressively decreases with age, until it is no longer detectable at 15 to 25 days. Another striking difference in the serum of the 18-day fetus is the small quantity of albumin.

The electrophoretic patterns of the sera of B/6 and CBA

18-day fetuses are essentially identical, except for the two components in which these lines have inherited differences. The quantity of transferrin and the relative amounts of minors and major are the same in the two lines, but the mobility is different, as will be discussed in the next section. Also, very obvious at this stage (although not clearly visible in the photograph) is the difference in the pattern of the prealbumins. B/6, which lacks prealbumin-B during adult life, as will be shown in Section B. 2. d., pp. 51-63, also lacks it during fetal life, whereas the fetus of the CBA has even larger quantities of this component than the adult, such that it is clearly distinguishable after separation even at pH 8.5.

The pattern of the serum from newborns is not essentially different from that of the 18-day fetus, except that somewhat more albumin is present and the transferrin minors seem to have increased in quantity slightly at the expense of the quantity of major. This trend with respect to the transferrins continues until about 20 to 30 days of age, when the pattern may show significantly more of the first minor component than of the major. At this point the trend is reversed, and the relative quantity of major transferrin increases again until the adult pattern is reached at from four to six weeks. The genetically determined transferrin phenotypes and pre-albumin phenotypes are very clearly distinguished at birth and tests of segregating litters at birth have verified that the individual's genotype, rather than its mother's, is being expressed at this age.

The quantities of gamma-globulin, S α_2, some of the unresolved components, the albumin, and the major prealbumin all increase gradually from birth until adult concentrations are reached

at about 25 to 30 days. In some instances, however, when the mice are crowded or diseased and grow slowly, these changes may require as long as two months.

Another effect to be noted is a rather nebulous sex effect. It is seen clearly in the 8 and 9-day old females in Figure 3, Appendix I, p. 180, which shows gels into which the samples have been inserted by absorbing the serum on a piece of filter paper, as described for the hemoglobin typing procedure. The animals in this case were seven to thirteen days of age. It is seen in other females as late as 16 days of age. The interpretation of this time difference is not yet clear. It may be that two different effects are being observed. The occurrence and significance of such apparently sex-limited components during development have not yet been adequately defined, and can not be discussed further at present.

Another source of variation in serum electrophoretic patterns is pregnancy. To define these effects in the mouse, females of the B/6, A/He, D/2 and Flx lines were tested at various stages of pregnancy. The CBA line was checked at intervals, but at less precisely defined stages. No line differences in the effects of pregnancy were detected.

Figure 4, Appendix I, p. 181, shows typical electrophoretic pattern changes of sera from B/6 females at indicated days of pregnancy or postpartum. The most significant effect is the presence in the serum of a new component between the F α_2-globulin and the albumin, present early in pregnancy and decreasing to very little by parturition. This is the position in which a new component appears in the serum of late fetal and early postnatal stages. This could perhaps be a material produced in the mother and passed to the

offspring, but no further studies have been conducted to test this possibility. An increase in material in the unresolved region is also rather obvious, and a significantly greater oxidase activity is present during pregnancy. Another component is seen in this region in about the same position as that appearing in the sera of developing females, particularly apparent in Figure 4 in the sample taken at parturition. There is some indication of a small increase in transferrin during pregnancy. By a different technique Clausen and Heremans noted no significant change (185).

Finally, some observations on changes in serum electrophoretic patterns under various conditions of stress or disease should be recorded. These do not represent systematic studies, as did the previous observations, but are rather occasional observations on interesting material which happened to be available. The observation of Popp (173) and others (186) on the increase of an alpha-globulin component during stress conditions has been confirmed, but it appears that this increase is in a component in the alpha-beta complex region, rather than the F-alpha$_2$. This has been observed in animals receiving injections of bacteria, in radiation chimeras, in X-ray controls, in homologous animals in parabiosis, in animals suffering induced anemias, and in slow-growing runted or diseased animals. Much of the change in such conditions is in the alpha-beta complex region, so nothing can presently be stated about particular components affected.

A more striking effect in most of these conditions is in the transferrins. The usual observation is that under stress the quantity of the minor components increases relative to the major component. It is not possible to state conclusively that there is also

an absolute decrease in total transferrin, but there usually appears to be. This condition has been seen to reach an extreme in several animals from experiments in which an anemia was induced by a presumed autoimmune mechanism. In these instances the relative quantities of the three components were almost completely reversed. There seems also, in many of these cases, to be an increase in the quantity of Sα_2-globulin. No physiological basis for this change can be offered at this time.

c. Transferrin variants

1) Genetic studies.--The mouse serum protein variant system first detected and most thoroughly studied at this time involves the iron-binding beta-globulin serum component called transferrin or siderophilin. The following reprint of a published paper describes the variant system and detailed studies of its inheritance. Similar studies of this system with essentially identical results have been independently reported by Cohen (188), in a paper appearing shortly after this report.

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*GENETIC CONTROL OF SERUM TRANSFERRIN TYPE IN MICE**

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Relationships between genes and proteins are being defined in detail through genetic studies of the fine structure of the chromosome and chemical analyses of the molecular structures of genetically variant proteins. The most extensive chromosomal fine structure investigation has been done with bacteriophage.[1] The best evidence concerning the effects of mutation on protein structure has come from chemical and genetic studies of human hemoglobins.[2] Eventually genetic fine structure studies and protein structure analyses will be combined for given genetic loci and the proteins they control. The most elegant studies of this type will probably be done with microorganisms, because of their advantages for genetic study. It is important, however, also to extend our understanding of gene-protein relationships in higher animals, particularly mammals. The hemoglobin work has demonstrated that such investigations are possible in mammals. It should be worthwhile to analyze other protein variant systems in the same way, in order better to understand the detailed mechanisms and relationships involved in the genetic control of a variety of different proteins. Such studies require variant systems which can be readily isolated and purified, which have been shown to be genetically determined, and which, preferably, occur in species adapted to intensive genetic study.

A genetic difference has been observed in the β-lactoglobulins of cow's milk,[3] and a number of physicochemical studies of these differing proteins have been carried out.[4–6] Genetically determined antigenic differences in the γ-globulins of rabbits have been studied by gel diffusion and immunoelectrophoresis.[7–9] Chemical investigations of rabbit γ-globulins have been in progress for many years. Investigations of fractions obtained by papain digestion by Porter[10] and by peptic digestion by Nisonoff *et al.*[11] offer hope for identification of the antigenic differences with specific portions of the molecule. Observations with starch gel electrophoresis of a number of genetically determined serum protein variant systems in humans (reviewed by Smithies[12]), cattle,[13, 14] sheep,[14] goats,[14] horses,[15] and swine[16] have provided materials which may prove useful for structural studies. Adequate techniques are available so that many of these serum proteins could be isolated and purified without serious difficulty. However, with all of the species in which serum protein variants have been identified, as well as with the human hemoglobin studies, there is difficulty in conducting intensive genetic studies of the sort which would be desirable for the most precise definitions of gene-protein relationships in mammals, in terms of allelism, linkage, intragenic recombination, etc.

The most favorable mammalian species available for such investigations is the mouse. Well-defined linkage groups, adequate markers, the availability of highly inbred lines, and a short generation interval make it very useful for genetic study. Hemoglobin variants have been observed in the mouse and have been well studied genetically.[17] However, no other well-defined protein variants have been reported in the mouse. Thompson *et al.*[18] have described an apparent genetic difference

between certain lines of mice in quantity of β_1-globulin, determined by moving boundary electrophoresis, but this might involve only an increase in some lines of a component common to all, rather than a molecular difference. The genetic evidence is based only on F_1's; segregating progeny could not be tested because it was necessary to pool sera from a large number of mice for each moving boundary run.

To detect useful protein variant systems in mice, sera from a number of inbred lines have been screened, utilizing two of the most powerful techniques presently available for detection of molecular differences in proteins—starch gel electrophoresis and the Ouchterlony gel diffusion technique. Presented here are the results of a genetic study of one of several serum protein differences which have been detected, an electrophoretically distinguishable serum transferrin variant.

Materials and Methods.—Thirteen inbred lines of mice were surveyed: A/Jax, A/He, AKR, BALB/c, CBA, C3H, C57B1/6, C57Br, DBA/2, Flexed, RFM, WB, and WC. For genetic study of the transferrin difference, reciprocal crosses were made between CBA and C57B1/6 (B/6) to produce F_1 progeny. $F_1 \times F_1$ crosses and reciprocal backcrosses to each parental line were made with both groups of F_1's. Progeny were weaned and individually identified at four to five weeks, but no mouse was tested for serum protein type until it had reached at least eight weeks of age. Mouse sera exhibit a number of qualitative and quantitative changes from birth to three to four weeks of age;[19] therefore testing was delayed until it was certain that only adult characteristics would be observed.

Blood samples were collected from the ventral tail vein in silicone-treated tubes. Sera were tested fresh or after storage at $-10°C$. Only slight changes in electrophoretic patterns of the sera were noted after freezing.

The serum components were separated by horizontal starch gel electrophoresis.[20] Gels were prepared with approximately 13 gm. hydrolyzed starch (Connaught Medical Research Laboratories, Toronto, Canada) per 100 ml borate buffer, pH 8.5, approximately 0.02 molar in borate. Exact starch and buffer concentrations varied with different lots of starch. Fifty μl of each serum sample were pipetted, without supporting material, into a slot, cut out of the gel after cooling. Separations were carried out at 4°C for nine hours at 10V/cm. Under these conditions, despite the lack of supporting material in the slots, the electrodecantation effect observed by Smithies[21] in horizontal gels was negligible. Upon termination of a run, the gels were sliced in half and stained with Amido Black 10B.

Hemoglobin samples for linkage studies were collected by absorbing a drop of tail blood on a 5 × 9 mm piece of Whatman No. 1 filter paper. The pieces of filter paper were inserted into starch gels and separations carried out under conditions identical to those employed for serum, except that the duration of each run was only three hours.

The variant components were identified as β-globulins by the combined paper and starch gel electrophoresis technique described by Poulik and Smithies.[22] They were identified as transferrins by autoradiography with ^{59}Fe, as described by Giblett *et al.*[23]

Results and Discussion.—Several serum protein differences recognizable either electrophoretically or immunologically occur between the lines tested. One of these involves the iron-binding, β-globulin component, usually called transferrin

or siderophilin. Upon separation of the serum components by starch gel electrophoresis, the CBA line differs in this major β-globulin component from all of the other lines thus far tested, in that a protein more negatively charged at pH 8.5 replaces the component found in the other lines (Fig. 1, *A* and *D*). This more rapidly migrating protein found in the CBA line has been designated Trf-1, its slower homologue, Trf-2. These designations will be used also for the phenotypes of individuals which show these components.

Sera from F_1 progeny of crosses between CBA and B/6 contain both Trf-1 and Trf-2 (Fig. 1, *B*). This phenotype will be referred to as Trf-1·2. The two proteins are present in approximately equal amounts, with the Trf-1 band somewhat

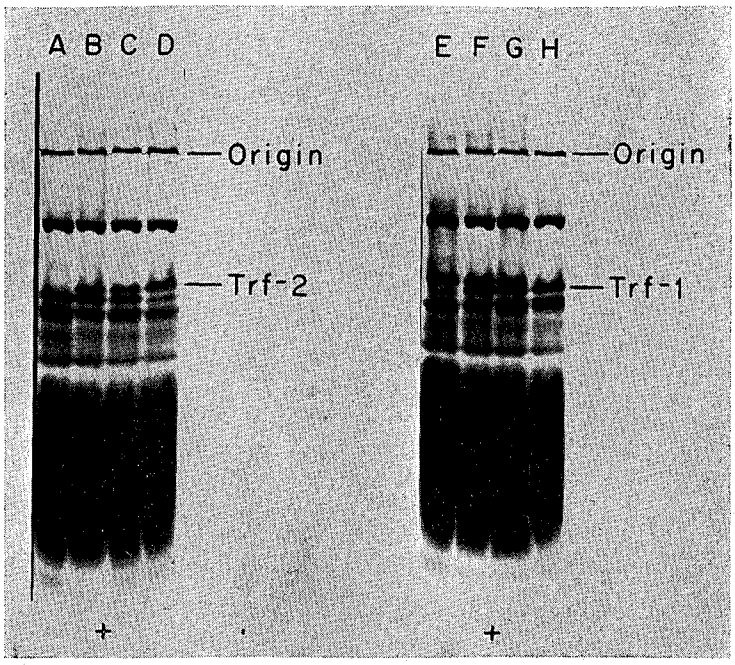

FIG. 1.—Amido Black-stained gels after separation of sera from: (*A*) CBA, (*B*) CBA × B/6 F_1, (*C*) mixture of CBA plus B/6, (*D*) B/6, (*E*) CBA × B/6 F_2 (Trf-2), (*F*) CBA × B/6 F_2 (Trf-1·2), (*G*) CBA × B/6 F_2 (Trf-1·2), (*H*) CBA × B/6 F_2 (Trf-1).

more intense. Each protein seems to be reduced in quantity to about one-half its level in the parental lines, as indicated by comparison of the F_1 sera with a mixture of equal quantities of CBA and B/6 sera (Fig. 1, *C*). Results of tests of F_1 progeny are recorded in Table 1. The results are the same regardless of sex or the direction in which the cross is made, so it may be assumed that the trait is not sex-linked.

Figure 1, *E*–*H*, shows the electrophoretic patterns of sera from four F_2 progeny. Only the three transferrin phenotypes shown are observed among the F_2 and the backcross progeny; these phenotypes are indistinguishable from those of the parental and F_1 individuals shown in Figure 1, *A*, *B*, and *D*. The simplest hy-

pothesis of genetic control is that the two proteins are determined by a pair of codominant alleles.

The results of the tests of F_2 and backcross progeny are shown in Table 1. Data from males and females have been pooled, tests for homogeneity revealing no significant deviations. The sex ratio of progeny from all crosses is very close to unity. The Chi-square and probability values obtained when the data are tested for fit with the 1:1 and 1:2:1 ratios expected with the codominance hypothesis are also shown in Table 1. The fit is good, although there are excesses of *Trf*-1·2 types in the backcrosses. The data support the hypothesis of genetic control by a pair of codominant alleles. This is further supported by preliminary results from a CBA × DBA/2 cross, in which five F_1's and 42 F_1 × DBA/2 backcrosses show the same phenotypes as the analogous CBA × B/6 crosses.

TABLE 1

DISTRIBUTION OF PHENOTYPES AMONG PROGENY FROM FIRST AND SECOND GENERATION CROSSES BETWEEN CBA AND C57B1/6

Cross		Phenotype					
Dam	Sire	Trf-1	Trf-1·2	Trf-2	Total	χ^2	P
C57B1/6 × CBA		0	26	0	26		
CBA × C57B1/6		0	22	0	22		
Total		0	48	0	48		
F_1 × C57B1/6		0	33	32	65		
C57B1/6 × F_1		0	44	38	82		
Total		0	77	70	147	0.33	0.5
F_1 × F_1		55	115	66	236	1.18	0.5
F_1 × CBA		18	23	0	41		
CBA × F_1		34	43	0	77		
Total		52	66	0	118	1.68	0.2

It is proposed that this locus be designated the transferrin locus (*Trf*), and that the two alleles identified be called Trf^1 and Trf^2, corresponding to the protein types which they control. This symbolism is consistent with that adopted for the hemoglobin locus in mice.[24]

Progeny from backcrosses to B/6 have been analyzed to determine whether the transferrin locus is linked to either of two other loci at which the CBA and B/6 lines differ. The CBA line is agouti, *A*, and has diffuse hemoglobin, Hb^2, the B/6 line is non-agouti, *a*, and has single hemoglobin, Hb^1. The results of these linkage tests are shown in Table 2. The recombination frequencies between the transferrin locus and the hemoglobin and agouti loci are 0.51 ± 0.042 and 0.56 ± 0.041 respectively, neither of which is significantly different from 0.5. In several of the classes in Table 2, rather large deviations from the expected occur. These could indicate viability differences due to interactions between loci or incompatibilities between dam and offspring, or may reflect aberrant segregation of the sort which seems to occur occasionally in certain crosses in mice.[25] Additional backcross progeny are being produced to ascertain whether these deviations have any real basis. It seems probable, however, that the transferrin locus is not in linkage groups I or V, unless it is near or outside *Hk* in I or *Sd* in V.

Figure 2 shows an autoradiogram obtained after electrophoresis of serum samples, containing added ^{59}Fe, from CBA, F_1, F_2 (Trf-1·2), and B/6 individuals, and the same gel stained with Amido Black 10B. Autoradiograms of samples from other F_2 progeny give identical results. There can be little doubt that these proteins

TABLE 2

BACKCROSS LINKAGE RESULTS

Cross			Phenotype Hemoglobin Locus			
			Diffuse		Single	
Dam	Sire	Sex	Trf-1·2	Trf-2	Trf-1·2	Trf-2
F_1 × C57B1/6		Male	7	8	11	4
		Female	8	7	7	13
C57B1/6 × F_1		Male	12	16	7	7
		Female	9	3	14	11
Total			36	34	39	35
			Agouti	Agouti Locus	Non-agouti	
			Trf-1·2	Trf-2	Trf-1·2	Trf-2
F_1 × C57B1/6		Male	5	6	13	6
		Female	7	14	8	6
C57B1/6 × F_1		Male	12	11	7	12
		Female	10	9	15	6
Total			34	40	43	30

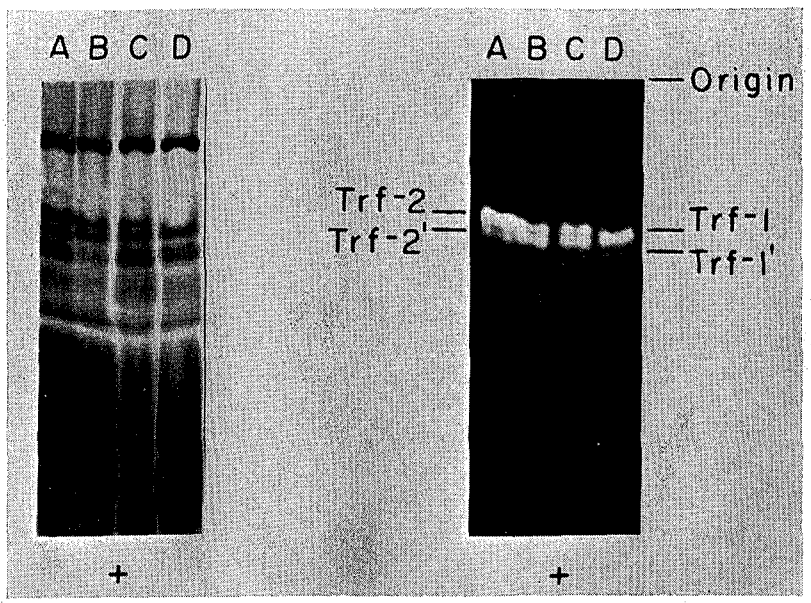

FIG. 2.—Amido Black-stained gel and autoradiogram after separation of samples containing ^{59}Fe. (*A*) B/6, (*B*) CBA × B/6 F_2 (Trf-1·2), (*C*) CBA × B/6 F_1, (*D*) CBA.

are the iron-binding components of the serum, and that only three phenotypes occur in the segregating progeny. It is apparent, however, that in addition to the major components designated Trf-1 and Trf-2 there is associated with each a second, more rapidly migrating, minor component, which also binds ferric ions, and which varies in its mobility as the major component varies. These minor components can be seen in the Amido Black-stained gel shown in Figure 2, as well as in the autoradiogram. Blumberg[26] has also shown a major and a minor transferrin component, but observed no genetic variation in either component. The minor components have been tentatively designated Trf-1′ and Trf-2′. The consistently greater intensity of the Trf-1 band in heterozygotes suggests that this band overlaps a Trf-2′ component. A reduced intensity of Trf-1′ in heterozygotes suggests that less of this component is present in the serum of these animals than in the

Trf-1 phenotype. It appears that the minor components exhibit phenotypic patterns analogous to those of the majors, and that they are controlled by the same kind of genetic mechanism.

The electrophoretic results from the F_2's and backcrosses have been carefully examined for recombination between the major and minor components, e.g., a recombinant type containing Trf-2 and Trf-1′. This might be somewhat difficult to determine conclusively in the Trf-1·2 and Trf-1 phenotypes, but should be readily apparent in the Trf-2 phenotype. No such recombination has been observed in 107 Trf-1, 258 Trf-1·2, and 136 Trf-2 backcross and F_2 progeny. Several explanations are apparent for this phenomenon. The major and minor components could be structurally different and determined by different, but closely linked loci. They could be structurally different, but both controlled by the same locus. They could represent the same structural unit, controlled by the same locus, with one of them conjugated with another unit common to both genotypes, or with major and minor components representing polymeric forms of the same unit. The latter seems the most reasonable explanation, but further physical and chemical analyses, and possibly further genetic studies, will be required to resolve this problem.

The major transferrin components probably correspond to the $\beta_{2\text{-}1}$ component of Clausen and Heremans,[27, 28] identified by immunoelectrophoresis of CBA × DBA/2 F_1's as a transferrin. These workers have reported the identification of only one component having iron-binding properties. Presumably, therefore, the mutation changing the electrophoretic mobility does not affect the antigenic combining site. If this had been altered, two separate transferrin bands should have been seen after immunoelectrophoresis of serum from these mice, which are of the Trf-1·2 phenotype. If the combining site is the same on both Trf-1 and Trf-2, although the components should separate electrophoretically on the agar gels used in immunoelectrophoresis, they would probably not separate far enough to produce any marked change in the precipitin band. The observation of only a single transferrin band further suggests that the minor components might be artifacts due to particular properties of the starch gel or the borate buffer, or again that they have a polymeric relationship to the major components. In immunoelectrophoresis, different polymeric forms of the same unit might precipitate in the same band.

The serum transferrin variants in mice could be useful in several ways. The transferrin locus may be useful as a genetic marker, particularly when its linkage group has been determined. The potential value of detailed chemical analyses of such variants has been discussed. The method of Boettcher *et al.*[29] for isolation of the human transferrins may be applicable for isolation of the mouse transferrins as well, since they have a similar mobility in starch gel and have been shown by Clausen and Heremans[28] to cross-react with antisera against human transferrin. The transferrin variants might be of value in transplantation studies, as markers for detecting repopulation in radiation chimeras, although Popp and Smith[30] were unable to demonstrate repopulation by serum protein-synthesizing cells with rat-into-mouse heterologous chimeras.

Summary.—Sera from mice of 13 inbred lines have been compared by starch gel electrophoresis. The CBA line differs from the other lines tested in the substitution of a more rapidly migrating β-globulin component, a transferrin, for the slower-migrating transferrin component found in the other lines. Sera from 48

F_1 progeny from reciprocal crosses between CBA and C57B1/6 contain both components in approximately equal amounts, but each is reduced in quantity relative to the parental lines. Tests of 501 F_2 and backcross progeny show segregation of the parental and the F_1 transferrin phenotypes with the frequencies expected from genetic control of the trait by a pair of codominant alleles. The locus controlling the trait has been called the transferrin locus (*Trf*), and the alleles, Trf^1, in the CBA line, Trf^2 in the other lines. Tests for linkage to the agouti and hemoglobin loci are negative. Autoradiography with ^{59}Fe has shown that each major transferrin component has associated with it a more rapidly migrating minor transferrin component, which appears to be under control of the same locus.

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Because the nomenclature in the preceding report and in that by Cohen (188) differed slightly, it seemed desirable to revise the nomenclature to a system mutually acceptable. The results of the revision are included in Appendix II in a manuscript submitted for publication as a short note in Genetical Research. The revised symbols will be used in the remainder of this report.

Rather large excesses or deficiencies were noted in certain classes in the (CBA x B/6)F_1 x B/6 backcross linkage results in Table 2 of the publication, e. g., compare the numbers of males and females in the diffuse hemoglobin, Trf-2(Trf-b) class of the B/6 x F_1 cross. Although the results taken as a whole show no significant dependence between loci, it seemed possible that interaction between specific Hb and Trf alleles or maternal-fetal incompatibility might be involved, so additional backcross progeny were produced and tested. The results are presented in Table 1. These data reveal no significant dependence and the excesses or deficiencies noted in the previous sample are not apparent. The two samples are homogeneous, and when pooled likewise show no significant dependence and no large deviations in specific classes. It may thus be assumed that the deviations in the first sample were not indicative of significant interaction or incompatibility.

To establish more firmly the hypothesis of genetic control of the transferrins by a single locus, a second generation was produced from the CBA x B/6 segregants. The results of tests of small numbers of progeny from these crosses are presented in Table 2. These indicate that only the expected phenotypes occur, in about the frequencies expected. No new phenotypes were detected. These data also represent progeny tests, verifying the validity of the

Table 1

Additional backcross segregation data for transferrin with hemoglobin and agouti loci

Cross				Phenotype			
				Hemoglobin Locus			
				Diffuse		Single	
Dam	Sire		Sex	Trf-ab	Trf-b	Trf-ab	Trf-b
F_1 x	B/6		Male	4	6	3	5
			Female	6	4	5	4
B/6 x	F_1		Male	5	6	8	6
			Female	4	8	6	5
Total				19	24	22	20
				Agouti Locus			
				Agouti		Non-agouti	
				Trf-ab	Trf-b	Trf-ab	Trf-b
F_1 x	B/6		Male	2	6	5	5
			Female	6	4	5	3
B/6 x	F_1		Male	8	10	5	2
			Female	5	6	5	7
Total				21	26	20	17

Table 2

Transferrin phenotypes of F_3 CBA x B/6 progeny

	No. of progeny			
Mating*	Trf-a	Trf-ab	Trf-b	Total
Trf-a x Trf-a	9	0	0	9
Trf-a x Trf-ab	6	6	0	12
Trf-a x Trf-b	0	12	0	12
Trf-ab x Trf-ab	5	5	4	14
Trf-ab x Trf-b	0	8	12	20
Trf-b x Trf-b	0	0	7	7
				74

*Reciprocal crosses pooled.

phenotypic classification of the 19 parents of these animals.

Two more inbred lines, D/1 and B/10, have been tested since the preceding publication and found to be of the Trf-b phenotype. The number of segregants among which no recombination has been observed between major and minor components has now reached 1118.

The clear-cut, easily determined, codominant nature of the transferrin difference, and the fact that the variant type occurs in a standard inbred line, suggest potential usefulness as a genetic marker. Consequently tests were carried out to determine the linkage relationships of the Trf locus. As demonstrated in the published study, the locus is not detectably linked to the agouti or hemoglobin loci, in linkage groups V and I, respectively. Crosses were made to a number of available stocks carrying markers in known linkage groups: dilution, d, (II); dominant spotting, W, (III); hairloss, hl, and belted, bt, (VI); brown, b, (VIII); and flexed, f, (XIV). Reciprocal crosses were made between CBA and the marked lines, and the F_1's backcrossed to the recessive parent. In all cases the marked lines were demonstrated to have Trf-b.

Only small numbers were produced and tested in these linkage testcrosses, except in that between CBA and D/2, involving the d and b loci. This was the first cross to produce backcross progeny, and early results indicated relatively close linkage of Trf with d. Extensive testing has confirmed this. Table 3 shows the results of three backcross linkage tests carried out to determine the recomination frequency between Trf and d. The first cross is a simple backcross utilizing the two inbred lines, CBA and D/2. This is designated as Coupling No. 1. The recombination frequency in F_1 females is 0.145 ± 0.045, in F_1 males 0.053 ± 0.036.

Table 3

Backcross segregation data for the Trf and d loci

Cross	Sex	Phenotypes: Intense Trf-ab	Intense Trf-b	Dilute Trf-ab	Dilute Trf-b	Total	r	S.E.
Coupling No. 1								
F_1 x D/2	Male	17	2	4	12	35		
	Female	15	1	2	9	27		
	Total	32	3	6	21	62	0.145	0.045
D/2 x F_1	Male	8	0	2	10	20		
	Female	8	0	0	10	18		
	Total	16	0	2	20	38	0.053	0.036
Coupling No. 2								
$\frac{D\ Trf^a}{d\ Trf^b}$ x $\frac{d\ Trf^b}{d\ Trf^b}$	Male	18	4	2	12	36		
	Female	17	5	2	18	42		
	Total	35	9	4	30	78	0.166	0.042
$\frac{d\ Trf^b}{d\ Trf^b}$ x $\frac{D\ Trf^a}{d\ Trf^b}$	Male	9	2	1	8	20		
	Female	12	1	1	11	25		
	Total	21	3	2	19	45	0.111	0.050
Repulsion								
$\frac{D\ Trf^b}{d\ Trf^a}$ x $\frac{d\ Trf^b}{d\ Trf^b}$	Male	4	16	13	1	34		
	Female	4	13	9	4	30		
	Total	8	29	22	5	64	0.203	0.050
$\frac{d\ Trf^b}{d\ Trf^b}$ x $\frac{D\ Trf^b}{d\ Trf^a}$	Male	2	6	7	0	15		
	Female	2	8	4	0	14		
	Total	4	14	11	0	29	0.138	0.064

A number of instances of spurious linkage involving the dilution locus have been reported, summarized by Michie (189). These are attributed by Michie (190) and Wallace (191) to a phenomenon of dependent segregation of chromosomes derived from the same or sometimes from both parental gametes, based upon non-random orientation of centromeres on the spindle at meiosis, which these authors have called "affinity". In the cases reported, the effect disappears after one generation. Although none of the reported cases of spurious linkage with dilution involved recombination frequencies as low as 15%, it seemed desirable in designing further linkage crosses to avoid this possibility.

The first of these crosses was that designated in Table 3 as Coupling No. 2. This was a cross of CBA by dilute-segregating progeny ($\underline{\text{d Trf}^b}/\underline{\text{d Trf}^b}$) from the third generation of a cross involving B/6, A/J, and D/2. Such a stock might reasonably be expected to exhibit no affinity. The possibility that affinity operates in the gamete contributed by the CBA parent is not eliminated here. F_1's of genotypes $\underline{\text{D Trf}^a}/\underline{\text{d Trf}^b}$ produced by this cross were testcrossed to the D/2 line. Except for a somewhat, though not significantly, higher recombination frequency in heterozygous males, the result in this cross is essentially the same as that previously obtained.

To discount any possible affinity due to the CBA parent and to eliminate any bias in the data due to a small differential viability evident in the Coupling No. 1 cross, a third test was designed. In this instance, recombinants from the Coupling No. 1 cross of $\underline{\text{d Trf}^a}/\underline{\text{d Trf}^b}$ type were crossed to B/6 ($\underline{\text{D Trf}^b}/\underline{\text{D Trf}^b}$). The progeny were tested for Trf type, and the $\underline{\text{d Trf}^a}/\underline{\text{D Trf}^b}$ individuals selected for the linkage test. These were testcrossed to D/2, and

the results of this Repulsion test are presented in Table 3. Again, although higher, the recombination frequencies are not significantly different from those obtained in the previous two crosses. Pooling of the data from these three crosses yields an estimated map distance of 14.6 ± 2.0 between d and Trf.

2) Other observations.--Additional autoradiography experiments have shown that a third minor component, Trf-b", also binds iron as do Trf-b and Trf-b', and Trf-a and Trf-a'. However, while Trf-b, Trf-b', Trf-a and Trf-a' seem to bind iron in direct proportion to concentration, as manifested by uptake of Amido Black, this minor seems to bind less iron relative to its concentration. This has been observed by autoradiography of serum from animals suffering a severe induced anemia. In such animals, as well as in animals under other kinds of stress, the relative proportions of the major and minor components change drastically. Individuals can be found in which Trf-b" is present in quantity as great as either Trf-b or Trf-b'. It is not at all uncommon to find individuals in which the first minor is present in greater quantity than the major.

3) Discussion.--Most of the points discussed in the publication are still pertinent. In the light of additional data on segregating progeny, on a third generation of progeny, on linkage, and the independent corroboration of the study by Cohen (188), there can be no doubt about the mode of inheritance. The additional data on backcross progeny from B/6 indicate that the deviations previously noted in the data on segregation with the Hb and A loci were not significant, so the previous suggestion of possible effects of interaction, incompatibility or aberrant segregation is no longer applicable.

The possible uses of the transferrin system discussed in the publication bear further discussion at this time. The locus can most certainly now be useful as a genetic marker, since its linkage relationship with dilution has been rather thoroughly defined, and will be more useful when linkage tests with another marker in linkage group II are complete. It would seem to be particularly useful because the markers previously available in linkage group II on either side of dse were complicated by such factors as infertility and incomplete penetrance.

A chemical analysis of the variant proteins would still seem likely to yield important information. Probably of particular interest is the chemical and physiological relationship of major and minor transferrin components, in view of the observations on changes of these components under stress and during development. The failure to detect recombination between major and minor components in over 1000 segregants makes it probable that these are controlled by a single locus, rather than separate loci or pseudoalleles. The polymeric relationship previously proposed may be correct, but the recent findings of Parker and Bearn (192), concerning the effects of the binding of sialic acid residues on mobility of the human transferrins may be more applicable here. Binding of some other component under certain conditions could also conceivably produce this effect. Chemical studies of these components might suggest the physiological significance of these changes.

Also of physiological as well as evolutionary interest is the very general occurrence of transferrin polymorphisms in almost every species studied. There must be some physiological advantage either for the heterozygote or for each homozygote in a different

environmental situation, resulting in balanced selection pressures which tend to retain two or more alleles in a population. Giblett et al. (118) have studied relative binding capacities in vivo and in vitro for several of the human transferrin variants and find no significant differences, under normal physiological and environmental conditions. Subtle selective mechanisms might, however, become obvious only under very specific conditions.

For more intensive study of such physiologically and evolutionarily important mechanisms, the mouse would be an ideal species. Before the difference in mice can have significance in this connection, however, it will be necessary to show that the polymorphism exists under natural conditions. Feral populations have not yet been studied. Trf-a has been found only in the CBA line. This could suggest that the variant in this line is the result of a mutation since its separation from related lines. If this were the case, then the polymorphism in this species might be of no physiological significance at all. The CBA line exhibits no deleterious effects due to the variant allele, so there would seem to be no reason why it could not have been fixed after arising by mutation.

The previous suggestion that the variants might be useful as markers in radiation chimeras, as the hemoglobin variants have been used, has been tested and on the basis of the results presented in Section D. 3., pp. 114-119, must now be withdrawn. If repopulation by cells synthesizing transferrin can be obtained at all in significant amounts, it is clear that a system must be designed for this specific purpose. The marker is not of use in routine studies.

To the extent that the inbred lines thus far tested represent in any reliable way the gene pool of mouse populations in

general, the finding of additional transferrin variants of an electrophoretic nature in large numbers does not seem probable. However, the serological and other techniques by which other classes of variants might be detected are virtually unexplored for the transferrins. It is impossible to predict the extent of polymorphism which might be detected by such methods. As discussed in the introduction, and as will be seen in a subsequent section, the application of serological techniques to other serum proteins has been fruitful in several species, including the mouse. Other techniques which might be suggested are solubility methods, applied with success to the mouse hemoglobin variants (66), chromatography, tests for iron-binding capacity, and ultimately, perhaps chemical analysis of amino acid composition and sequence. All of these approaches would be facilitated by availability of the isolated transferrins in relatively pure form. This consideration makes the application to mouse transferrins of the Rivanol precipitation technique, which has been useful with the human and cattle transferrins (193, 194), of some importance. This has been undertaken, but is only in the preliminary stages.

4) Summary.--1. An electrophoretic difference has been detected in a major globulin component of mouse serum having an increased mobility in one of fourteen inbred lines tested.

2. Two-dimensional electrophoresis and autoradiography with ^{59}Fe have demonstrated that the component showing electrophoretic variation is a beta-globulin which binds ferric ions. This is taken as evidence that the component is the serum transferrin or siderophilin.

3. Genetic analyses have established that the trait is

controlled by a pair of codominant alleles at a single autosomal locus. This locus has been designated the transferrin (Trf) locus, with the two alleles designated Trf^a in the single variant line, CBA, and Trf^b in the other lines.

4. The Trf locus has been shown to be in the second linkage group of the mouse, 14.6 ± 2.0 map units from the d locus.

5. Striking changes have been observed in the relative proportions of major and minor transferrin components during postnatal development and under conditions of stress. These changes may have physiological significance.

d. Pre-albumin variants

1) Results.--A second electrophoretically distinguished serum protein variant system, involving the component designated prealbumin-B (Section B. 2. a., p. 27), was detected during the course of genetic studies of the transferrin system. This component at pH 8.5 usually appears as a sharpened leading boundary on the principal pre-albumin zone in the starch gel electrophoretic pattern of CBA serum, but does not appear to be present in B/6 serum. This is a very minor component, in concentration perhaps less than 100 μg. per ml.; consequently observation of it depends upon specific conditions. At pH 8.5 it is not always possible to detect the difference. However, at pH 9.0 it is possible to increase the mobility of this component, relative to the principal prealbumin, sufficiently to separate it from that component and to classify parental, F_1, and segregant phenotypes with good repeatability. When artificial mixtures are prepared with sera from CBA and B/6, the presence of the component can be detected in concentrations of CBA in B/6 as low

as 12.5%. The mixture of equal quantities is comparable to that obtained in CBA x B/6 F_1 hybrids.

Three phenotypes have been tentatively assigned, designated Pre-a, Pre-ao and Pre-o. Pre-o is the phenotype of the B/6 line, Pre-a the phenotype of the CBA line, and Pre-ao that of the F_1 between these two lines. The distribution of the two presumed homozygous phenotypes among other inbred lines is as follows: Pre-a: AKR, C, D/1, D/2, RF, WB; and Pre-o: A/He, A/J, Flx, WC. The phenotypes are shown in Figure 5, Appendix I, p. 181. They appear relatively discrete, but distinction between Pre-a and Pre-ao is sometimes difficult, and weak Pre-ao types may occasionally be misclassified as Pre-o. In any instance of doubt, a new sample must be collected and tested. After sufficient experience in evaluating results, it is possible to classify most individuals repeatably.

A portion of the CBA x B/6 segregants employed in the transferrin studies was used in the study of the genetic determination of the pre-albumin trait. The results of tests of F_1's, and of F_2's and backcross progeny are shown in Table 4. Six F_1's of each sex in each of the two reciprocal crosses exhibited no detectable differences in phenotype, so the trait is probably not sex-linked. The data from these segregants, with one exception, do not deviate significantly from expectations, assuming single gene control, but are not as conclusive as might be desired. In no case is the probability that the observed deviations could be due simply to chance greater than 0.3. The data from the backcross to B/6 are reasonably good, but in the F_2 an obvious difference appears in the distribution of phenotypes between sexes. A Chi-square test for homogeneity between sexes yields a probability between 0.1 and 0.05. In the data from

Table 4

Distribution of prealbumin phenotypes among first and second generation CBA x B/6 progeny

Cross	Sex	Phenotype Pre-a	Pre-ao	Pre-o	Total	χ^2	P
CBA x B/6	Male	0	6	0	6		
	Female	0	6	0	6		
	Total	0	12	0	12	-	-
B/6 x CBA	Male	0	6	0	6		
	Female	0	6	0	6		
	Total	0	12	0	12	-	-
F_1 x B/6	Male	-	2	3	5		
	Female	-	3	6	9		
	Total	-	5	9	14	0.64	0.3
B/6 x F_1	Male	-	13	16	29		
	Female	-	12	15	27		
	Total	-	25	31	56	0.64	0.3
Total		-	30	40	70	1.43	0.2
F_1 x F_1	Male	20	28	20	68		
	Female	9	40	21	70		
	Total	29	68	41	138	2.11	0.3
F_1 x CBA	Male	4	2	-	6		
	Female	1	11	-	12		
	Total	5	13	-	18	2.72	0.1
CBA x F_1	Male	11	15	-	26		
	Female	11	17	-	28		
	Total	22	32	-	54	1.85	0.1
Total		27	45	-	72	4.50	0.036

the two kinds of backcrosses to CBA a significant deviation from expected occurs. The very large deviation among females in the F_1 x CBA backcross, which is principally responsible for the failure to fit expectation, cannot be ascribed to simple sex-linkage. One possible explanation that could be suggested is a maternally transmitted effect. This seems unlikely, as does any sort of maternal-fetal incompatibility in this case. This is the only class in which such a striking deviation is seen.

Another possible basis for the deviations in these data, among females in particular, might be that somewhat less of this material is produced in females than in males. Although this is not obvious in the F_1's or the parental inbred lines, in such a case it is conceivable that some Pre-a individuals could have been classified as Pre-ao, and some Pre-ao as Pre-o. There is a rather consistent trend in the data in this direction. It is important to note here, however, that only the phenotypes expected were observed in the backcrosses. This is true despite the fact that F_2's and backcross progeny were randomly mixed at weaning, and testing was done without knowledge of the origin of any individual until completion of classification. There was therefore no possibility of bias in assigning phenotypes. Consequently the fact that no individuals in the backcrosses to CBA were misclassified as Pre-o when they should have been Pre-ao argues against any consistent misclassification in this direction. Alternatively, there may be viability differences between phenotypes in this combination.

Since the CBA and D/2 lines are both Pre-a, it is apparent that the segregants from the repulsion test for linkage of Trf to d, described in Section c., p. 46, should also segregate for Pre as in

a simple backcross. These animals were classified for Pre type to test whether an independent cross might give more reliable results. These results are presented in Table 5, and indeed are much more convincing.

The genetic background of D/2 may make possible the expression of somewhat more of the Pre component, or perhaps the intercrosses between CBA and D/2 prior to the testcross studied increased the vigor of the animals and with it the degree of expression of the Pre trait. At any rate, distinction between Pre-a and Pre-ao in this cross could be made much more reliably and repeatably than in the previous crosses, and it was possible to classify these animals after separation of the samples at pH 8.5. These results would seem to make it possible to accept an hypothesis of single gene control much more confidently, and would suggest that the deviations in the CBA x B/6 data may have been due to viability differences or simply to chance.

To test further the mechanism of inheritance and the validity of the phenotypic classifications, the F_3 progeny produced for the transferrin studies were also tested for pre-albumin type. The parents used in these crosses, taken from the various classes of second generation progeny, were carefully tested for pre-albumin type. The assigned phenotypes are as well-established as is possible under present test conditions. The results of tests of these third generation progeny are shown in Table 6. It is clear that, as in the second generation data, only those phenotypes expected were observed, again with blind testing of samples whose origin was indicated only by a code number. The numbers in the various groups are rather small for ratios to have significance, but these are

Table 5

Distribution of prealbumin phenotypes among segregants in the repulsion linkage tests

Cross	Sex	Phenotype Pre-a	Pre-ao	Pre-o	Total	χ^2	P
F_1 x DBA	Male	19	15	-	34		
	Female	12	18	-	30		
	Total	31	33	-	64	0.03	0.8
DBA x F_1	Male	8	7	-	15		
	Female	6	8	-	14		
	Total	14	15	-	29	0.02	0.8

Table 6

Prealbumin phenotypes of F_3 CBA x B/6 progeny

Mating*	No. of progeny Pre-a	Pre-ao	Pre-o	Total
Pre-o x Pre-a	0	6	0	6
Pre-o x Pre-ao	0	12	9	21
Pre-ao x Pre-ao	7	18	3	28
Pre-ao x Pre-a	7	10	0	17
				72

*Reciprocal crosses pooled.

approximately what would be expected. There is, however, a consistent excess of heterozygotes.

The F_3 data also support the validity of the classification procedure. All of the expected and no unexpected phenotypes were found in the three critical tests where exceptions should have been detected had any of the 17 parents involved been misclassified. These data on the third generation progeny therefore also seem to strengthen appreciably the hypothesis of single gene control.

The data from the CBA x B/6 crosses have been examined for dependence in segregation with other loci. Markers available in this cross were A, Hb, Trf, and Ss (discussed in Section C). The distribution of Pre types among the classes of segregants for the other loci, and the probability values from tests for dependence, are shown in Table 7. Although many of the probability values are low, none is as low as the 5% level. The deviations from expected may be real, indicating linkage or interacting effects on viability, or may reflect merely random deviations. In the F_1 x CBA cross, it will be noted that in the data on segregation with Ss the Pre-a, Ss-hl class is quite small compared with the other three classes. It is conceivable that the deviations in this backcross previously discussed could be in part attributable to low viability due to interaction of these genotypes.

Although the possibility of misclassification of Pre phenotypes previously pointed out would tend to decrease apparent linkage between loci, it would not be likely to obliterate close linkage. There is no evidence for linkage in these data. When the data from the F_1 x B/6 and F_1 x CBA backcrosses are pooled, the recombination frequency between Pre and Ss, 0.446 ± 0.047, is not significantly

Table 7

Segregation data for *Pre* with other loci

Cross*	Sex	Phenotype: Hemoglobin locus: Diffuse Pre-ao	Diffuse Pre-o	Single Pre-ao	Single Pre-o	Total	χ^2	P	r	S.E.
F_1 x B/6	Male	10	15	5	4	34				
	Female	6	6	9	15	36				
	Total	16	21	14	19	70	0.05	0.8	0.500	0.060
		Agouti locus: Agouti Pre-ao	Agouti Pre-o	Non-agouti Pre-ao	Non-agouti Pre-o	Total				
F_1 x B/6	Male	7	9	8	10	34				
	Female	6	8	9	13	36				
	Total	13	17	17	23	70	0.05	0.8	0.486	0.060
		Trf locus: Trf-ab Pre-ao	Trf-ab Pre-o	Trf-b Pre-ao	Trf-b Pre-o	Total				
F_1 x B/6	Male	9	10	6	9	34				
	Female	10	12	5	9	36				
	Total	19	22	11	18	70	0.49	0.5	0.471	0.060

*Reciprocal crosses pooled.

Table 7 (Continued)

Cross*	Sex	Phenotype Ss locus: Ss-hl Pre-ao	Ss-hl Pre-o	Ss-h Pre-ao	Ss-h Pre-o	Total	χ^2	P	r	S.E.
F_1 x B/6	Male	8	9	4	8	29				
	Female	8	7	5	9	29				
	Total	16	16	9	17	58	1.37	0.2	0.431	0.065

		Trf locus: Trf-ab Pre-ao	Trf-ab Pre-a	Trf-a Pre-ao	Trf-a Pre-a	Total				
F_1 x CBA	Male	9	5	8	10	32				
	Female	16	6	12	6	40				
	Total	25	11	20	16	72	1.48	0.2	0.431	0.058

		Ss locus: Ss-hl Pre-ao	Ss-hl Pre-a	Ss-l Pre-ao	Ss-l Pre-a	Total				
F_1 x CBA	Male	7	5	9	10	31				
	Female	10	1	13	6	30				
	Total	17	6	22	16	61	1.58	0.2	0.459	0.064

*Reciprocal crosses pooled.

Table 7 (Continued)

Cross*	Sex	Phenotype: Brown locus: Black Pre-ao	Black Pre-a	Brown Pre-ao	Brown Pre-a	Total	χ^2	P	r	S.E.
Repulsion	Male	9	16	13	11	49				
	Female	13	7	13	11	44				
	Total	22	23	26	22	93	0.26	0.6	0.580	0.051

		Dilution locus: Intense Pre-ao	Intense Pre-a	Dilute Pre-ao	Dilute Pre-a	Total				
Repulsion	Male	12	15	10	12	49				
	Female	17	10	9	8	44				
	Total	29	25	19	20	93	0.02	0.8	0.473	0.052

		Trf locus: Trf-ab Pre-ao	Trf-ab Pre-a	Trf-b Pre-ao	Trf-b Pre-a	Total				
Repulsion	Male	13	13	9	14	49				
	Female	11	8	13	12	44				
	Total	24	21	22	26	93	0.52	0.4	0.462	0.052

*Reciprocal crosses pooled.

different from 0.5.

Also contained in Table 7 are the segregation data from the repulsion linkage test (Section c., p. 47) which give information on relationships with the brown (b), d and Trf loci. Brown may be confidently used as a marker here because all of the recombinant individuals mated to B/6 to produce the heterozygous parents for this cross were also homozygous for brown (b/b). No significant linkage between Pre and the other loci is evidenced in these data. It therefore appears that rather large segments of linkage groups I, II, V, VIII and IX may be eliminated as possible locations of the Pre locus.

2) Discussion.--The evidence for genetic control of the pre-albumin trait by a pair of alleles at a single autosomal locus is reasonably convincing when all of the data are considered. The two presumed alleles may be tentatively designated Pre^a and Pre^o. The Pre^o allele has been so designated because it produces no detectable protein homologous with that produced by Pre^a. It is possible, of course, that Pre^o controls an altered protein, the electrophoretic mobility of which is slower than Pre-a and similar to that of some other component, such as the albumin, and is therefore buried beneath this component. Further studies of the sort discussed subsequently will be necessary to distinguish these possibilities. The designations proposed are only tentative, due to the fact that nothing is known about the nature of the component. When this is known, the name and symbols should be changed to a more specific designation.

At present the Pre variant does not promise to be particularly useful. Detection and classification with the electrophoretic method and a general protein stain are difficult and perhaps not

entirely reliable even after a great deal of experience with the system. If the component has some specific biological or chemical activity which would make possible a specific stain on the starch gel or more reliable detection by some other method, then the variant system might become more useful as a tool in biochemical and physiological studies, or as a genetic marker when its linkage relationships have been determined. Attempts to find such a specific or even semi-specific staining property or other activity have thus far been unsuccessful. The methods applied include iron-binding, cytochrome oxidase activity, ceruloplasmin activity, non-specific esterase activity, peroxidase activity and lipid staining. Attempts to adapt glycoprotein staining procedures for general use with starch gels have been unsuccessful. Immunoelectrophoresis has been attempted as a means for detection and distinction, but no precipitin band attributable to this component can be detected. Too little of the material is present in the serum to make attempts at isolation and characterization a practical possibility.

The rapid migration of the component on starch gel, relative to other components, suggests that it either has a very low isoelectric point or is of low molecular weight. The relative increase in mobility with increase in pH would tend to support the latter. It has not been possible to determine the true mobility of the component. Because it is present in such small amounts, it cannot be detected by the relatively insensitive two-dimensional procedure.

3) Summary.--1. A difference has been detected among 12 inbred lines of mice in the presence or absence of a minor component at the leading edge of the principal pre-albumin zone.

2. The difference is probably under genetic control by a

pair of alleles at a single autosomal locus with no dominance. The alleles are tentatively designated Pre^{a} and Pre^{o}, with each occurring in several inbred lines.

3. Analyses for linkage relationships indicate no close linkage with the A, Hb, Trf, Ss , d or b loci.

4. The identity, function and properties of the component are not known.

C. A Serologically Detected Variant System

1. Materials and Methods

a. Animals

The mice used in these experiments were as described and designated in Section B. 1. a., p. 16 of this thesis.

b. Antisera

Seven different antisera were used in this study, six of them provided by Dr. R. D. Owen. The first reagent was prepared in the following way. A rabbit was injected intramuscularly with 10 ml. of an alum precipitated mouse serum globulin preparation. This was prepared by precipitation of the globulins from whole serum from Swiss mice at 50% saturation with ammonium sulfate. Three subsequent 1 ml. injections of the soluble preparation, without alum precipitation, were given intraperitoneally at daily intervals beginning 21 days after the intramuscular injection. The following day 1 ml. was given intravenously and the rabbit was bled after ten days. This antiserum, called T12, was the principal reagent in the early stages of the study. The rabbit was reimmunized about nine months later with a newly prepared but similar globulin preparation. An initial injection of 1 ml. was given intraperitoneally, followed by three injections of 0.5 ml. each intravenously at two-day intervals. The rabbit was bled at eight and ten days after the last injection. This antiserum will be called AMG, and has constituted the standard reagent for most of these studies.

Four additional antisera used in certain tests were prepared against whole plasma from four inbred lines of mice. The injection

schedule was three intravenous injections per week for three weeks, each injection consisting of 0.5 ml. of a preparation approximately one-half plasma in isotonic citrate-saline. The four rabbits were reimmunized three months later with a single intraperitoneal injection of 0.5 ml. plasma at ½ dilution, followed on two alternate days by intravenous injections of 0.5 ml. of the same material. The rabbits were bled ten days after the last injection. The antisera are designated as follows: R21, anti-A/J; R22, anti-C57Bl/10; R23, anti-CBA; R24, anti-C57Bl/10-H-2^d. The seventh antiserum was prepared by subcutaneous injections of whole AKR serum in Freund's adjuvant. The antiserum used in these studies, obtained after a third immunization, is designated IT.

c. Diffusion plates

General principles for diffusion plates were first described by Ouchterlony (139). The details of the technique used were taken from Ridgway (175). Agar base was poured in two layers of 8 ml. each into molded 10 x 100 mm. petri plates. The composition of the agar base was as follows:

15 g. Difco Bacto-Agar
7.2 g. Sodium chloride
6.0 g. Sodium citrate
10 ml. Merthiolate solution (1% aqueous)
10 ml. Trypan Blue solution (1% aqueous)
980 ml. distilled water.

The agar, sodium chloride and sodium citrate were added to the water, which was boiled to melt the agar. The pH of the hot solution was adjusted to 6.7 with 0.1 N HCl, using indicator paper. After pH

adjustment the merthiolate and trypan blue solutions were added, and the hot solution was pipetted into 18 x 150 mm. culture tubes in quantities of 8 ml. each. These tubes were either used immediately in pouring plates, or were stored at 4°C. for later use. The agar was melted after storage by heating 20 min. in an autoclave.

The first layer of agar was poured into the plate rapidly from the tube without swirling. One-half hour was allowed for the agar to solidify, then stainless steel or porcelain penicylinders were positioned on top of this layer in a standard hexagonal pattern to serve as the antigen or antiserum reservoirs. The distance between center and outer reservoirs in this pattern is 2 cm. The positioning of the penicylinders was facilitated by the use of Lucite guides with holes bored at the proper positions. The penicylinders were simply dropped through into proper position. After the reservoirs were in place, the second layer of agar was poured from a tube at nearly boiling temperature. When properly poured, the second layer covered the plate uniformly and flowed evenly around the penicylinders.

Fifteen to twenty minutes after pouring of the second layer, the reservoirs could be charged. In routine testing for serum type, 0.1 ml. of undiluted AMG antiserum was placed in the center well and 0.1 ml. of each mouse serum diluted to ¼ in isotonic saline was placed in the outer reservoirs. On occasion other arrangements of antigen and antiserum were employed. These will be described with the specific experiments in which they were used.

For inhibition methods, the procedure was exactly the same except that in preparing the agar base, the quantity of agar, merthiolate and trypan blue to be added was calculated on the basis of

8 ml. quantities for each layer, whereas water, sodium citrate, and sodium chloride were added for only 7 ml., the remainder being made up by the addition of 1 ml. of the inhibiting material, diluted, if necessary, in isotonic saline. Both layers of agar contained the added inhibiting material. Tubes containing 7 ml. of the special melted agar base were held in a water bath at 56°C. Just before pouring, 1 ml. of the inhibiting preparation was pipetted into the tubes, which were mixed rapidly and poured as quickly as possible. It was usually necessary to tilt the plates somewhat to obtain a uniform covering of the surface of the plates. All other steps of the technique were as described for the standard plates.

The plates were sealed with rubber tape and developed in an incubator at 37°C. Complete development of the typical mouse serum pattern required 14 days. After 18 to 20 days, the plates were removed from the incubator and photographed at once, or were stored at 4°C. for up to two weeks until a convenient number had accumulated for photography.

The plates were viewed and photographed on an indirect lighting apparatus described by Klontz et al. (195). Photographs were taken on Plus-X film with a 35 mm. camera, equipped with a +3 portrait accessory lens, mounted above the viewer.

d. Immunoelectrophoresis

Immunoelectrophoresis was performed in two ways. The first procedure will be termed comparative immunoelectrophoresis. After separation of serum samples by starch gel electrophoresis, and slicing and staining of the gels, as described in Section B. 1. c., p. 19, the unstained half of the gel was cut into 1 cm. squares, each

identified as to its original position on the gel. These squares were placed in a freezer for at least an hour and not more than 24 hours, then positioned on a standard diffusion plate, in the place of a penicylinder, after pouring of the first layer. The second layer was poured in the usual way. Appropriate arrangements of starch gel squares and whole serum samples in adjacent penicylinders made it possible to relate any precipitin band on the diffusion plates to a particular 1 cm. section of the starch gel electrophoretic pattern, and by reference to the stained gel it was usually possible to determine which component produced a particular precipitin band. The freezing of the gel segments before introduction into the plates greatly enhances the diffusion of proteins out of the gel and into the agar. The pouring of the second layer of hot agar around the starch gel sections makes close contact, and has no apparent denaturing effect on the protein.

For some experiments the same comparative procedure was followed with preliminary separation of the sera by agar electrophoresis. Agar electrophoresis was carried out in the apparatus used for starch gel electrophoresis (see Section B. 1. c., p. 19). The gels were prepared with 1.5% agar in veronal buffer at pH 8.6 and ionic strength 0.0125. Separations were carried out for four hours at 8 V/cm. in an ambient temperature of 4°C. All procedural details after electrophoretic separation were identical to those described for starch gels, except that the agar gel sections were not first frozen.

The second general method was in principle the same, but gave results more comparable to orthodox immunoelectrophoretic techniques (140, 141). It was possible however to obtain superior electrophoretic

separations by employing starch gel instead of agar. The serum samples to be tested were separated in the usual way on starch gel. The gels were then sliced and one half stained as usual. The other half was cut into sections 5 cm. long by 1 cm. wide. It was possible to divide the entire electrophoretic path of the sera into three such sections. Each section was then frozen and placed on a petri plate into which a very thin layer of the standard agar base had been poured. Two such strips from the same gel, usually containing two different serum samples were placed at 1 cm. on either side of a 3 mm. x 5 cm. piece of glass rod positioned to form a center trough. A second layer of agar base was then poured between the gel strips and around the glass rod. After cooling the rod was removed, leaving a trough into which about 0.2 ml. antiserum could be pipetted. Such plates when incubated at 37°C. usually developed final patterns after about five days.

e. Hemagglutination tests

H-2 antigenic types of mouse red cells were determined by the Gorer-Mikulska technique (196), employing dextran and human serum as the suspension medium. The hemagglutinating isoantisera were prepared from mice of one line sensitized by a skin graft from another line against which antiserum was desired, followed by an intraperitoneal injection of spleen cells after sloughing of the graft. Serum was collected by sacrifice about 10 days after the injection. Antisera used were BALB/c-anti-BAF_1, DBA-anti-AKR, AKR-anti-DBA, and RF-anti-A. (I am indebted to Dr. R. D. Owen for these antisera.) The antisera were diluted in 2% dextran (prepared from Intradex, 6% dextran, salt-free, in 5% glucose) in isotonic saline;

the cells for test were prepared as 2% suspensions of washed red cells in a medium of human serum diluted to 1/2 in saline. One drop of the cell suspension was added to one drop of the diluted antiserum in a 13 x 100 mm. tube, and the contents mixed and allowed to stand for 1½ to 2 hours. The tests were read for agglutination by streaking the pellet on a microscope slide with a capillary pipette, gently rocking the slide to disperse the pellet, and inspecting under a dissecting microscope.

For saline agglutination tests, the rabbit antisera were diluted in isotonic saline, and the washed test cells were prepared as 2% suspensions in saline. One drop of cell suspension was added to two drops of antiserum in a 13 x 100 mm. tube. The contents were mixed and the tubes allowed to stand 15 min. at room temperature. The tubes were then centrifuged at low speed for 30 sec. and read macroscopically for agglutination.

2. Results

a. Detection and description of phenotypes

This system was detected during screening by the double diffusion technique for variants among inbred lines. Figure 6, Appendix 1, p. 182, shows the results of tests of CBA, B/6 and (CBA x B/6)F_1's, and of CBA, D/2 and (CBA x D/2)F_1's with the AMG antiserum. The B/6 serum has a component which forms a precipitin band midway between the antigen and antiserum wells. This band is continuous on one side with a very faint one forming quite near the antigen well containing CBA serum, and on the other side with a strong band opposite the well containing F_1 serum. The weak band formed by the CBA serum is consistently detectable only when adjacent to a sample giving a strong reaction, when it usually appears as a banding of the adjacent band. The band formed by reaction with the homologous component in the F_1 serum is a strong one, but is displaced toward the antigen well relative to the B/6 band. The position of the band formed by an F_1 serum is indistinguishable from that of a mixture of equal quantities of B/6 and CBA sera. Tests of CBA, D/2 and (CBA x D/2)F_1's give similar results. Apparently the inbred lines, which are presumably homozygous for different alleles at at least one locus, represent two extreme phenotypes, while the F_1, which is heterozygous for all autosomal differences between the two lines, is intermediate between them. The observed differences in band position in the different phenotypes are most simply interpreted as the result of a concentration difference in a specific serum component. This is tested and discussed more fully in later sections.

b. Non-genetic individual variation

The earlier discussion concerning the importance of defining non-genetic electrophoretic variations in studies of protein variant systems applies equally to the serological technique, and particularly to the variant system just described, because this appears to involve a quantitative difference. Changes in other components have not been extensively studied because of difficulties in identifying specific components by this technique not encountered in electrophoretic studies. The serum samples studied electrophoretically (Section B. 2. b., pp. 27-34) were also used to define the effects of age and pregnancy upon the serological variant system.

Only CBA and B/6 were included in the developmental studies. Samples at age intervals of two to four days from 18 days of fetal development to 30 days after birth were studied under standard test conditions, with adult sera on each plate for comparison. It was found that in the B/6 line the variant component is present at 18 days of fetal development in a concentration about 50% less than that in the adult. This concentration increases until at 10 to 12 days after birth it is significantly greater than in the adult. This condition persists until about one month of age, when the quantity begins to decrease again to the adult level. In the CBA's, samples placed adjacent to B/6 adult serum showed the typical bending of the precipitin band near the antigen well at all ages, but at no stage could any significant quantitative difference be detected. Certainly there is no increase in concentration greater than two to four times that of the very low adult level; anything greater than this could have been easily and consistently detected.

Two components unrelated to the serological variant are

apparently entirely absent in the 18-day fetus and up to about 10 days after birth in one case, about 18 days in the other. Nothing is known at present about the identity of these components. This absence of components was noted in both the CBA and B/6 lines.

The effects of pregnancy on CBA, Flx, B/6 and D/2 have been studied in a similar fashion. In this case it has not been possible to show any consistent change in the serological variant in CBA, A/He or B/6 at any stage of pregnancy. Similarly, no serologically detectable quantitative or qualitative changes occur in other components.

Stress apparently affects the variant component, resulting in an increased concentration detectable in both CBA and B/6 lines as well as several other lines tested. The result is a change in precipitin band position indicative of as much as a two-fold increase in concentration. This has been observed in radiation chimeras and controls, in parabionts, and in diseased animals. Possible changes in other components have not been carefully checked, but there are some quantitative differences.

c. Genetic studies

1) Inheritance.--Since the difference was first found to occur between CBA and B/6, among other lines, it was again possible to utilize the CBA x B/6 segregants available from the transferrin studies. These animals were tested in an attempt to determine the mode of inheritance of the trait. F_1 males and females from reciprocal crosses show no differences, so it may be assumed that an autosomal difference is involved.

Preliminary tests of F_2 and backcross segregants with

antiserum T12 suggested that the three phenotypes seen in the parental and F_1 individuals appeared as discrete phenotypes in the subsequent generation. Tests of parental and F_1 sera in serial dilutions against various dilutions of the superior AMG antiserum showed that with undiluted AMG and mouse sera diluted to ¼, the distinction among the three phenotypes was clear and reliably reproducible. Further tests under these conditions confirmed the presence of only three discrete phenotypes among the segregating progeny, although instances of apparent individual non-genetic variation resulted in occasional overlaps of the homozygous B/6 phenotype and the F_1 type. The three phenotypes have been designated Ss-h for the B/6 phenotype, Ss-l for the CBA type, indicating high and low levels of the serum factor, and Ss-hl for the intermediate type. In routine tests of segregants the plates were inspected on three alternate days after complete development at about 14 days. Each sample was independently classified each time into one of the three phenotypes. If all three classifications were not identical, a new sample was collected and the questionable animal retested. About 15% of the animals required one retest to be certain of proper classification, but many of these were due to the location of several Ss-l samples adjacent to each other and the consequent failure of formation of a detectable Ss band. In almost all cases, retesting with new samples and different arrangements of samples made possible a correct assignment of phenotype. As in the previous genetic studies, the samples were never identified as to the specific type of cross until after testing was completed.

The results of tests of 284 F_2 and backcross progeny are shown in Table 8. These data fit rather well the ratios expected

assuming the trait to be controlled by a pair of alleles at a single locus with no dominance. The only serious exception occurs in the cross of CBA x F_1 in which two females were classified as Ss-h, an impossible phenotype in this cross under the proposed mechanism of genetic control. It is assumed in this instance that these two individuals were overlaps, of the sort previously discussed. They had died by the time of testing and were not available for retest. Three other inconsistent individuals of the same kind upon retesting were classified as Ss-hl, so it may be reasonably assumed that these also were misclassifications.

The two presumed alleles controlling the trait have been tentatively designated $\underline{Ss}^l$ and $\underline{Ss}^h$, corresponding to the homozygous phenotypic designations. The available segregation data on these animals have been analyzed for possible linkage of the Ss locus to A, Hb and Trf. The analysis is presented in Table 9, and it is clear that there is no significant association among these loci, although the data for segregation with Hb approach significant dependence. The data for segregation with Pre have been presented in Table 7 and discussed in Section B. 2. d., p. 57, and likewise indicate no significant association.

2) Association with H-2 locus.--All inbred lines available at the time of detection of this variant system were tested for Ss type. The lines originally tested and the types determined are as follows: Ss-l; CBA, AKR, C3H and Flx; Ss-h: B/6, D/2, WB, A/J, A/He and C. It was noted that the three Ss-l lines of known H-2 type, CBA, AKR and C3H, have in common the $\underline{H\text{-}2}^k$ allele at the Histocompatibility-2 locus, while none of the Ss-h lines of known H-2 type have this allele. To test a possible relationship to the

Table 8

Distribution of serum antigen types among first and second generation CBA x B/6 progeny

Cross	Sex	Phenotype Ss-h	Ss-hl	Ss-l	Total	χ^2	P
CBA x B/6	Male	-	5	-	5		
	Female	-	5	-	5		
	Total	-	10	-	10		
B/6 x CBA	Male	-	5	-	5		
	Female	-	7	-	7		
	Total	-	12	-	12		
F_1 x B/6	Male	2	1	-	3		
	Female	4	4	-	8		
	Total	6	5	-	11	0.1	0.7
B/6 x F_1	Male	10	17	-	27		
	Female	12	16	-	28		
	Total	22	33	-	55	2.20	0.1
Total		28	38	-	66	1.52	0.2
F_1 x F_1	Male	13	38	19	70		
	Female	23	30	16	69		
	Total	36	68	35	139	0.1	0.9
F_1 x CBA	Male	-	4	6	10		
	Female	-	4	6	10		
	Total	-	8	12	20	0.8	0.4
CBA x F_1	Male		15	21	36		
	Female	(2)	9	14	23		
	Total	(2)	24	35	59*	1.86*	0.2*
Total		(2)	32	47	79*	2.85*	0.1*

*Does not include the two inconsistent Ss-h individuals (see text).

Table 9

Segregation data for serum antigen type with other loci

Cross*	Sex	Agouti locus: Agouti Ss-hl	Agouti locus: Agouti Ss-h	Agouti locus: Non-agouti Ss-hl	Agouti locus: Non-agouti Ss-h	Total	χ^2	P	r	S.E.
F_1 x B/6	Male	10	5	8	7	30				
	Female	9	6	11	10	36				
	Total	19	11	19	17	66	0.75	0.3	0.424	0.061

		Hemoglobin locus: Diffuse Ss-hl	Diffuse Ss-h	Single Ss-hl	Single Ss-h	Total				
F_1 x B/6	Male	14	8	4	4	30				
	Female	7	3	11	12	33				
	Total	21	11	15	16	63	1.92	0.1	0.413	0.062

		Trf locus: Trf-ab Ss-hl	Trf-ab Ss-h	Trf-b Ss-hl	Trf-b Ss-h	Total				
F_1 x B/6	Male	10	7	8	5	30				
	Female	13	11	7	5	36				
	Total	23	18	15	10	66	0.10	0.7	0.500	0.061

		Trf locus: Trf-ab Ss-hl	Trf-ab Ss-l	Trf-a Ss-hl	Trf-a Ss-l	Total				
F_1 x CBA	Male	10	15	8	12	45				
	Female	10	10	3	10	33				
	Total	20	25	11	22	78	0.98	0.3	0.462	0.056

*Reciprocal crosses pooled.

H-2 locus, segregating progeny from several crosses between H-2k lines and lines of other H-2 types were classified for both Ss type of the serum and H-2 type of the red cells, determined by hemagglutination. The results of these tests are summarized in Table 10.

In all but two of 91 animals, the Ss trait and H-2 type segregated together. These two animals both appeared to be heterozygous for the serum trait, but homozygous for one of the H-2 alleles. These exceptions could possibly be due to non-genetic variation in the serum trait as seen in the CBA x F_1 segregation data (although in the case of the (RF x A/J)F_1 x RF segregant this is most unlikely), or to errors in classification by the red cell tests, or these mice are recombinants. A third individual, noted in Table 10, was classified as Ss-h, but came from a cross in which the only expected phenotypes were Ss-h1 and Ss-1. This is probably another case similar to the exceptional individuals noted in the CBA x F_1 cross, however retesting of this individual three times failed to show any phenotypic change. The animal was nearly two years old and may have represented some quantitative variability due to advanced age. Because this animal and the two apparent recombinants were past breeding age, the genotypes could not be verified by progeny tests. Whatever the significance of these exceptions, however, the data indicate at least a very close linkage between the Ss trait and the H-2 locus. It is possible that the immunodiffusion test of the serum is merely detecting another expression of the complex H-2 region. Recombination has been reported to occur within the H-2 complex with a frequency of over 1% (197,198).

Two further lines of evidence also suggest a close relationship or identity of the Ss locus with the H-2. One of these, already

Table 10

Segregation data for Ss and H-2 loci

Cross	Phenotypes				Total	χ^2	P
	H-2ak		H-2k				
	Ss-hl	Ss-l	Ss-hl	Ss-l			
(RF x A/J)F_1 x RF	6*	0	1	5	12	8.6	0.0005
	H-2bk		H-2k				
	Ss-hl	Ss-l	Ss-hl	Ss-l			
(CBA x B/6)F_1 x CBA	13	0	0	21	34	34.0	10^{-4}
	H-2bk		H-2b				
	Ss-hl	Ss-h	Ss-hl	Ss-h			
(CBA x B/6)F_1 x B/6	13	0	1	7	21	17.1	10^{-4}
	H-2dk		H-2d				
	Ss-hl	Ss-h	Ss-hl	Ss-h			
(CBA x D/2)F_1 x D/2	9	0	0	15	24	24.0	10^{-4}

*Includes one individual classified as Ss-h (see text).

pointed out, is the occurrence of the Ss-1 phenotype exclusively in those inbred lines thus far tested which have the H-2^k allele, and the occurrence of Ss-h only in those lines having an H-2 allele other than H-2^k. The initial relationship between the two traits on the basis of common types in CBA, C3H and AKR has now been extended to other lines. The F1x line, which had not previously been typed for H-2, was tested for red cell type by hemagglutination with the available reagents. Although such tests cannot definitely prove that the line is H-2k, the reactions of F1x cells are identical to those of CBA, and the occurrence of or failure of agglutination with various reagents definitely eliminates H-2a, H-2b and H-2d. The supposition is therefore strong that this line is also H-2k. A fifth line has been shown indirectly to have the Ss-1 phenotype. This is the RF line, known to be H-2k. Animals of the RF line were not available for test, but the (RF x A/J)F_1 x RF progeny (Table 10) segregate Ss-1 and Ss-h1. The A/J line has been shown to be Ss-h, so the RF must be Ss-1. Thus five Ss-1 lines are rather conclusively also H-2k.

The association applies to non-H-2k lines as well. All of the Ss-h lines have either been shown to be non-H-2k or may be reasonably presumed to be. The D/1 line, which is H-2q, has been added to those originally tested and shown to be Ss-h. The H-2 types of the WB and WC lines have not been reported nor tested in these studies, but the closely related WH line is H-2d and the history of these lines suggests that if these lines are not also H-2d they are probably H-2b.

After the relation to H-2 became apparent, the non-H-2k lines were studied more carefully for possible quantitative serum

factor differences which might be related to differences among other H-2 alleles. It was found that a definite and repeatable difference in band position could be detected between B/6 and the A/J, A/He, D/1, D/2 and C lines. The B/6 line is H-2b, while the other lines are H-2a, H-2d or H-2q. On a quantitative basis the other lines have approximately twice the concentration of Ss component as the B/6. While the difference in only one H-2b line is not conclusive, it does indicate another possible line of evidence for control of the serum characteristic by H-2. Segregants from crosses between B/6 and these other lines have not been tested, but the pattern of serum from (B/6 x A/J)F_1 hybrids is clearly intermediate.

In addition to the quantitative differences among Ss-h lines, differences between sexes within lines also occur. In all but B/6, males appear to have about 50% more of the Ss component than females. The same effect is usually seen in B/6, but in the opposite direction and of somewhat smaller magnitude. The females apparently exceed the males in this line by about 25%. It has not been possible to detect sex differences in the Ss-l lines, but a difference as much as two-fold could probably occur without being detected.

d. Physical and antigenic characterization

It is, of course, of importance to define the nature of the component involved in any variant system. However, with reference to possible control by the H-2 locus, two specific questions are of particular interest. Is there any qualitative difference between the Ss component in Ss-l lines and that in Ss-h lines? Does the serum component have any antigenic specificity in common with cellular H-2 antigens, or is it involved in the structure of these antigens

in any way? The studies to be reported are preliminary. They do, however, bear upon the questions raised above, and serve to indicate the directions in which further studies should move.

1) Immunoelectrophoretic studies.--These experiments have provided some information on the physical properties of the component. An attempt was made to identify the Ss component with some component resolvable by separation of the serum by starch gel electrophoresis. This was done by the comparative immunoelectrophoresis method, with preliminary starch gel electrophoresis in borate buffer at pH 8.5. The object was to detect reactions of identity between some component in the starch gel and the Ss precipitin band.

The successful result of such an experiment is shown in Figure 7(a), Appendix I, p. 182. This shows very clearly a reaction of identity between several components in those portions of starch gel which came from the anode side of the origin, and components in the whole serum. The Ss precipitin band can be identified in this figure by the characteristic reaction with CBA serum placed in the bottom well. It is clear that the Ss component is present in this portion of the starch gel in both CBA and B/6 sera. Careful studies of the entire length of the starch gel electrophoretic separation showed that this section and the corresponding section from the opposite side of the origin are the only portions of the gel containing detectable amounts of Ss component. It also appears that approximately the same quantitative relationships exist between B/6 and CBA sera after electrophoresis as in the whole unseparated serum.

Figure 7(a) also shows that most of this component has moved from the origin very little. The upper surfaces of the gel, those facing the antigen well in the 12 o'clock position, were the surfaces

of the slots into which the samples were introduced for electrophoresis. The definite slope of the precipitin band from the origin side outward toward the gel on the opposite side indicates that the main concentration of the component is in a very narrow portion of the gel just at the origin.

This result suggests either that at pH 8.5 the component is near its isoelectric point, or that its molecular weight is too high to permit it to migrate through starch gel, in which a decided molecular filtration effect operates (93). To test this possibility, a similar experiment was carried out, but with electrophoretic separation at pH 4.7. If the component did not previously migrate because it was at its isoelectric point, it should migrate at this pH. The result was identical to that previously described. All of the Ss material was still at the origin.

This suggested more strongly that the component did not move on the starch gel because of a high molecular weight. The alternatives are that the component is not a protein at all, but remained at the origin because it was electrically inert, or if a protein, that it was entirely lacking in histidines and its terminal α-amino group was blocked, so that nothing was titrated between pH 4.7 and pH 8.5. To test this, the same experiment was carried out at pH 8.6, but this time with electrophoresis in agar. Since the material can diffuse through agar in an Ouchterlony plate, if it has an electrical charge it should move during electrophoresis on agar. In this experiment the component was found concentrated in the α_1-globulin region, just behind the albumin (see Figure 7(b), Appendix I, p. 182). In this case, there is no question that the component migrated under the influence of an electrical field. The

most reasonable interpretation of these experiments at present is that the component, whether a protein or not, has a surface charge which would classify it electrophoretically as an alpha-globulin, but is of such high molecular weight that it cannot enter the relatively small pore spaces in a starch gel. Ultracentrifugal analysis might throw some light on this, but has not yet been carried out.

It is noteworthy that the agar immunoelectrophoresis experiment also showed no electrophoretic difference between CBA and B/6 with respect to this component. This fact bears upon the question whether the observed difference between these lines is due to a qualitative or a quantitative difference in the Ss component, which will be discussed more fully in a later section.

2) Relative concentrations in different phenotypes.--The simplest interpretation of the difference observed on the diffusion plates between sera of Ss-h and Ss-l lines is that both phenotypes have the same component, but in different concentrations. On this assumption, the quantitative relationships between lines can be estimated by dilution studies. This has been done, comparing B/6 serum with CBA serum. The relative positions of the precipitin bands formed by serial dilutions of the two sera in saline were compared, and it was found that when B/6 serum is diluted in saline to 1/8 or 1/16, the Ss band matches in position the Ss band of undiluted CBA serum. If B/6 serum is diluted to ½ in saline the Ss band matches in position the F_1 serum, as does an equal mixture of CBA and B/6 sera.

However, when B/6 serum is diluted in CBA serum, at dilutions

above ¼ a small but significant difference can be seen between the band position of a given mixture of the two sera and the band position of B/6 serum at the same dilution in saline. This suggests that the CBA serum is contributing material, in quantities which become significant at lower dilutions of the B/6 material.

Similar comparisons of B/6 with A/He and D/2 sera indicate that the concentration of Ss in these sera is about twice that in B/6 and 16 to 32 times that in CBA. This is based upon a comparison of males. As previously observed the B/6, A/He and D/2 females are not very different. B/6 x A/J hybrids give bands in positions intermediate between the parental lines, while D/2 x CBA and A/J x RF hybrids have bands in the same position as B/6. On the basis of simple quantitative differences, these results indicate that the approximate concentrations of Ss component in the various lines and their hybrids might be represented in arbitrary units as follows: CBA = 1, B/6 = 12, A/He and D/2 = 24, B/6 x A/J = 18, D/2 x CBA and A/J x RF = 12 = B/6.

3) Antiserum comparisons.--The AMG antiserum has been the most useful reagent for these studies principally because it contains the highest titer of anti-Ss. However, a number of other reagents of various types were also tested. While any interpretation concerning the nature of an antigen based upon the character of an antiserum produced against it is subject to serious reservation, the results obtained with these different antisera appear to be of some significance and should be recorded and considered. These antisera and their designations have all been previously described (Section C. 1. b., p. 64). In tests against various inbred lines,

with AMG included in an outer reservoir for comparison, it was found that R21 and R24, prepared against lines which are H-2a and H-2d, have higher anti-Ss titers than R22, prepared against C57Bl/10, which is H-2b. None of these sera had a titer as high as AMG, which was prepared against globulins from a stock of Swiss mice. Tests of this stock of Swiss mice for H-2 type by hemagglutination and for Ss type showed them to be probably principally H-2a and entirely Ss-h. It is also noteworthy that R24 was prepared against serum from C57Bl/10-H-2^d, which is coisogenic with C57Bl/10 (H-2^b) used for R22.

Two antisera prepared against Ss-1 lines, R23 and IT, show a much more striking difference. In these two antisera, if anti-Ss is present at all, its concentration is so low, when tested against any mouse serum, that the precipitin band cannot be detected. Thus with seven antisera there seems to be a definite correlation between concentration of Ss in the immunizing mouse serum and concentration of anti-Ss in the antiserum. It would not be expected that antibody titer should be so dose dependent, particularly when several different immunization procedures were employed. It might be suggested that this constitutes evidence for a qualitative difference between these lines or even complete absence of the component in Ss-1 sera. However, on qualitative grounds it would be expected that different antisera should react most strongly with the immunizing mouse serum. In fact, in the five antisera which give detectable reactions with Ss, the relative positions of the Ss bands formed by sera from different lines of mice are the same as previously described for AMG.

4) Absorption analyses.--A more direct approach to the study of possible qualitative differences is absorption analysis,

using various antigen preparations from different inbred lines. If the serum component has a relationship to the H-2 antigens of red cells and tissues, the anti-Ss in the antiglobulin sera might be specific for certain H-2 types. To test this possibility, AMG, R22, R24 and a normal rabbit serum were tested for saline agglutinins against cells from A/He, B/6, CBA and D/2. The saline agglutination titers of the three antisera were between 160 and 640, the normal serum 40, with no evidence of specificity. Aliquots of each antiserum were absorbed with cells from each of these inbred lines, two absorptions each with one-half volume of cells, and again tested against these cells. A few of the absorbed sera gave weak reactions at a dilution of ½, again without specificity, but titrations showed no agglutination at higher dilutions by any antiserum regardless of the source of absorbing cells. The failure to find differences is not unexpected. The agglutination by unabsorbed antisera may be entirely due to cross-reactivity, or to reaction with adsorbed mouse globulins. If the serum component does have a common antigenic specificity with red cells, it need involve no qualitative difference between lines. Furthermore, there are many specificities on the red cell, and these heteroimmune sera might be expected to react principally with species antigens.

On the other hand, if there are antigenic sites on the red cells similar to those on the Ss component, then one would expect that by absorption with cells antibody against common combining sites would be removed from the antiserum, and a reduction in titer of anti-Ss could be detected on diffusion plates when the red cell-absorbed antisera are tested against mouse serum. Further, if specificity differences exist between lines, reactivity with certain

lines might be expected to be changed by absorption, while with other lines it might be unchanged, depending upon the original antiserum and the kind of cells with which it was absorbed. Obviously, not all of the anti-Ss would necessarily be directed against such common sites, since the Ss component probably has several different combining sites. However, the sensitivity of the diffusion plate method is such that a decrease in antibody of the order of 30% could reliably be detected. Tests of the rbc-absorbed sera, with unabsorbed antiserum on each plate for comparison, showed no detectable change, either absolute or relative, in the positions of the Ss precipitin bands formed by B/6, (A/J x B/6)F_1, A/He, CBA and D/2 sera. It may therefore be concluded that absorption with red cells changes neither the titer nor the specificity of the anti-Ss in these sera in any significant way. The possibility of some cross-reactivity at a level below the detectable limit cannot be excluded.

Another absorption approach tests more directly the possibility of qualitative differences in the Ss component, without reference to the relationship of its specificities to those of cell surfaces. This is the obvious procedure, absorption of the rabbit antisera with mouse serum from the various inbred lines. The absorptions can be done in either of two ways, on the plate itself or before introduction of the antiserum into the plate. Each procedure has certain advantages. The inhibition technique of Bjorklund (199), makes possible the absorption of an antiserum by any quantity of antigen, without the difficulties which may arise due to dilution of the antiserum if it is absorbed before introduction into the plates. The inhibition technique presumably immobilizes the antibody by precipitation. Absorption before addition of the antiserum

leaves soluble complexes and an excess of certain antigens in the mixture, which can complicate the results. On the other hand, preabsorption of the antiserum avoids the diffuse halo of precipitation around the antiserum well characteristic of inhibition plates, which may sometimes obscure a precipitin band forming by reaction with antigen from the antigen wells. More importantly, preabsorption makes possible the identification of a particular component by comparison of the precipitin bands formed by absorbed and unabsorbed antisera on the same plate. Both procedures have been employed, with consistent and complementing results.

Figure 8, Appendix I, p. 183, shows the result obtained when AMG is tested against sera from inbred lines of both serum types on an inhibition plate in which 1/32 volume of CBA whole serum has been added to the agar. It appears that the CBA serum in the agar has absorbed out all antibody except that reacting with one component, which gives a pattern similar to the Ss pattern obtained with unabsorbed antiglobulin. Such a result superficially suggests a true qualitative difference between the component in Ss-l and Ss-h lines, such that after absorption with CBA serum, all antibody is removed except anti Ss-h, which reacts with the Ss-h sera, but not with the Ss-l serum. Closer inspection, however, shows that the typical banding of the band at the CBA and Flx antigen wells still occurs, whereas, if all antibody capable of reacting with the component in the CBA serum had been removed such a reaction could not occur. Tests of AMG on agar containing 1/32 concentration of A/He, B/6 or D/2 sera yield no detectable reactions.

The most reasonable interpretation of this result is that there is not enough Ss component in CBA serum at the concentration

used in the agar to inhibit any significant amount of anti-Ss. It could be that there is no true Ss component in the CBA serum at all, and the bending of the band at the CBA well is a non-specific reaction with some other component. If the absolute position of the precipitin band formed by the Ss component of a given line is compared with that on a standard plate, it is found nearer the center well in the inhibition plate, suggesting removal of some antibody. If twice as much CBA serum is added to the agar, the same kind of pattern is obtained, and the precipitin bands are still nearer the center well, suggesting that more antibody has been inhibited by this quantity of serum. If four times as much CBA serum is added, the precipitin band can no longer be seen, and if present is obscured by the background of other precipitation around the center well. These results suggest that the difference between these sera, at least as recognized by the rabbit antiserum, is principally a quantitative one, with the concentration of the component in Ss-l sera so low that extremely large amounts of serum are required to inhibit all of the anti-Ss. The possibility of a weakly cross-reacting non-homologous component is not entirely excluded by these results. It could conceivably have the same effect as a low level of the Ss component. When sera from the Ss-h lines are added to the agar, the results are consistent with the quantitative estimates of Ss concentration. In these tests, enough Ss component is apparently present to inhibit all anti-Ss.

Similar tests of R22 show no detectable reactions in any combination. Presumably the antibody titer in this antiserum is so low that this amount of CBA serum, as well as serum of other lines, is sufficient to inhibit the anti-Ss. The antisera against Ss-l

sera give no detectable reaction when inhibited by any mouse serum under identical conditions, similarly arguing against any qualitative difference. If such a difference existed, it would be expected that inhibition by Ss-h serum would leave specific anti-Ss-1 antibodies capable of reacting with Ss-1 serum. It is possible, however, that if such a reaction occurs it is too weak to be detected, since the initial anti-Ss titer in these antisera is at best very low.

Another interpretation of the results with inhibition plates is also possible. The precipitin band observed may not be the Ss component at all, but some other serum component which gives the same kind of pattern. It is desirable to exclude this unlikely possibility. This can be done directly by preabsorption of the antiserum, then testing on a standard plate with unabsorbed antiserum in an adjacent well as a reference by which the Ss precipitin band can be identified. This test is shown in Figure 9, Appendix I, p. 183. The AMG antiserum was preabsorbed with 1/3 volume of whole CBA serum, and incubated 1 hour at room temperature, then 24 hours at 4°C. The precipitate was sedimented by centrifugation, and the supernatant poured off. This absorbed antiserum was introduced into the center well and tested against Ss-h and Ss-1 sera as before, except that unabsorbed AMG was put into one well for comparison. The reaction of identity between the single precipitin band produced by reaction with the absorbed AMG and the Ss band identifiable in the pattern of bands formed with unabsorbed AMG proves that the absorption results here and on the inhibition plates involve the Ss component.

A result very similar to that shown in Figure 9 was obtained when R24 was absorbed with CBA serum (1/3 volume) and tested against the same pattern of mouse sera with unabsorbed AMG for comparison.

In this case, however, a detectable precipitin band was formed only against the A/He serum. Thus it would again appear that some antibody remained after absorption of this antiserum by CBA serum.

The reactions between unabsorbed AMG and the mouse serum components remaining in the absorbed antiserum after absorption are also of interest. In general the pattern indicates a very large antigen excess, as may be seen in the series of bands between the top and center wells in Figure 9. This pattern is not much different from that on either side of the top well where unabsorbed AMG reacts with whole mouse sera. When AMG is absorbed with 1/3 volume of Ss-h (B/6) serum and tested as in Figure 9, no precipitin bands appear between the absorbed serum and the test mouse sera. The pattern of reaction between the remaining Ss material in the center well and the unabsorbed antiserum at the top is not the typical reaction seen in a standard plate, but is more like the reaction of an Ss-l serum. This suggests that at these levels of B/6 serum and AMG antiserum, the antigen and antibody are near equivalence, so that no detectable antibody remains. The effect is not seen when absorptions are carried out with sera from A/He or D/2 nor when R22 or R24 are absorbed with B/6. This supports the evidence for a quantitative difference between B/6 and the other Ss-h lines noted earlier.

It remains to define more thoroughly the parameters of these observations, by inhibition and preabsorption with lower levels of Ss-h sera and higher levels of Ss-l sera, to determine upper and lower limits at which these results hold.

5) Erythrocytes as a source of Ss.--If the Ss trait is controlled by the H-2 locus, the Ss component might have some direct structural relationship to red cells or other cells of the body.

The component in the serum might, for example represent a degradation product of cells contaminating the serum samples. This can be reasonably eliminated because the variability is not great enough. Variations of 25% or less can be detected on the plates, while variations of perhaps 50- or 100-fold or more must occasionally occur in the amount of contaminating cell debris. The possibility does exist, however, that the component represents some in vivo degradation product of cells. It has been observed rather consistently that the apparent quantity of Ss component in X-irradiated animals increases by 50 to 100% within one to two weeks after irradiation. However, other factors, such as hemoconcentration, could explain this change.

To test the possibility that erythrocyte breakdown might be the source of Ss material, seven CBA mice were injected with a single large dose of A/He cells. Each animal received 0.4 ml. washed packed red cells in 0.7 ml. total volume, injected over a period of several minutes. All of the animals survived, as did two control animals identically treated with CBA cells. Serum samples from these mice were collected at 3, 5, 11 and 20 days, and tested on diffusion plates. No change was detected in the Ss band at any time, even though this volume of cells, constituting at least 1/3 of the total red cell population of these animals, over this period of time should have contributed significantly to the quantity of Ss component, if erythrocytes were the source. This, of course, excludes only erythrocytes, but these might be expected to be a major source of Ss component if it were derived from cellular material.

6) Studies with isoantisera.--Another approach to the study

of possible qualitative differences between the Ss-1 and Ss-h components is isoimmunization. This is important particularly because with rabbit antisera subtle differences between components may not be recognized as differences, and therefore distinguishing antibodies may not be formed. Rabbit antisera against red cells of mice are rather poor reagents for classification of red cell differences; the most reliable tests employ isoantisera. On the other hand, the mouse seems in general to be a rather poor precipitin and agglutinin producer, so the probability of obtaining a precipitating antibody if differences do exist cannot be predicted.

The first tests of isoantisera utilized reagents prepared for hemagglutination tests. If the Ss component shares specificities with the red cells, and a qualitative difference does occur in these Ss components, then it might be possible to detect a reaction with isoantiserum definitely known to contain antibody, even though it is an incomplete hemagglutinin. Several of these isoimmune sera were first tested in a standard diffusion plate with the isoimmune reagents in the center well, and AMG in an outer well for comparison. The reagents tested were RF-anti-A/J, D/2-anti-AKR, AKR-anti-D/2, and AKR-anti-(AKR x D/2)F_1. None of these reagents gave detectable precipitin bands, when tested against normal sera from a number of inbred lines.

These reagents were also tested for incomplete, blocking antibody. This was done by mixing normal serum from a given line with an isoantiserum prepared against cells from that line in various proportions, 1:7, 1:3, 1:1, 3:1. These mixtures were placed in the antigen reservoirs adjacent to an identical mixture of normal sera from the two lines involved, and tested against AMG. The presence

of incomplete antibody reactive with the Ss component should result in either a blocking of combining sites, some of which should be recognized by rabbit antibodies, or in a reduction in rate of diffusion due to formation of antigen-antibody complexes, or both, resulting in a displacement of the precipitin band toward the outer well relative to the identical mixture of normal sera. Although difficulties arise due to the low probability that a change would be detectable in some combinations, in most instances there is no such ambiguity. No changes were observed in any case. There thus seems to be no gross similarity between the Ss specificities and those recognized on the cells by these isoantisera.

Attempts were also made to isoimmunize directly with serum. This work was done jointly with Dr. S. Tokuda. Six CBA mice were injected with 0.02 ml. B/6 serum in 0.03 ml. Freund's adjuvant in each footpad. The injection was repeated after two weeks. The same procedure was carried out with 6 B/6 mice given CBA serum. After one and two months following the second injection the mice were bled, and the sera tested for the presence of precipitins or blocking antibody as previously described. No detectable antibody was found in any individual of either line, at either bleeding, of either precipitating or blocking type. Extensive ring tests also gave negative results. If antibody of any sort is present, a much more sensitive test, perhaps complement fixation, will be necessary to detect it.

3. Discussion

The segregation data leave little doubt that the Ss trait is controlled at a single locus by two alleles exhibiting no apparent dominance. Non-genetic quantitative variation may lead to occasional overlap of the Ss-h and Ss-hl phenotypes, but does not seriously affect this interpretation of the genetic control. Control by a single locus has been demonstrated only for the difference between Ss-h and Ss-l lines as originally classified. The difference between B/6 and the other Ss-h lines has not been studied genetically, so it is not known that the same locus is involved or that this difference is not due to a dissimilar genetic background in B/6 rather than to diversity at a specific locus.

The evidence for some association with the H-2 locus is indisputable. The precise nature of this association is not known. In 88 of 91 cases the traits definitely segregate together. One of the exceptional individuals is clearly no ordinary recombinant. The other two exceptions could be either recombinants or cases of misclassification for one or the other trait. The question is thus still open, as indeed it would have been had there been no exceptions, whether the serum trait is controlled by the H-2 locus or by a separable but closely linked locus. In a sense, any attempt to distinguish between these possibilities at present may be fruitless in view of the evidence that the H-2 locus is a complex region, within which recombination occurs with a frequency of 1% or more (197, 198, 200, 201).

There is clearly a paradox here. On the one hand, the association of Ss^{l} with $H\text{-}2^{k}$ and of Ss^{h} with other H-2 alleles in inbred lines suggests that the serum trait may be controlled directly

by the H-2 locus. On the other hand, there is the evidence that intragenic recombination occurs, resulting in new combinations of substituent H-2 specificities on the cells, and in this study there is the indication that recombination may occur between H-2 and Ss. If intragenic or intergenic recombinations do occur, why should an association of Ss^{l} and $H\text{-}2^{k}$ always be found among inbred lines? One answer would be that all of the inbred lines studied received this region of their ninth chromosomes from common original sources, with the alleles at the two loci in their present relationships, and rapidly fixed one or the other type before serum and cellular traits recombined. Such a situation could have occurred if all of these inbred lines arose from rather restricted sources, but their breeding histories do not indicate this (202). Perhaps this association is related in some way to the unexpectedly frequent occurrence of alleles $H\text{-}2^{b}$, $H\text{-}2^{d}$ and $H\text{-}2^{k}$ in presumably unrelated lines. It has been suggested that these alleles may be favored by selection (203). An alternative would be to consider the apparent recombinations observed in this study either as errors in classification, or as recombinations within the H-2 region, some portion of which controls the Ss trait. An attractive hypothesis would be that the serum trait is controlled directly by some portion of the region in which alternative sets of information determine either cellular H-2k type and serum Ss-l type, or non-H-2k and Ss-h. There is evidence for two principal divisions of the H-2 region, one determining essentially H-2d type, the other H-2k (203). One might then assume that the serum trait is controlled by the k portion. The association of the traits in inbred lines supports such an hypothesis; the apparent recombinations between $H\text{-}2^{k}$ and Ss^{l} would seem to contradict it.

If the exceptional individuals in this study are true recombinants, the precise nature of the recombination is poorly defined in any event, and need not necessarily exclude control of Ss by a k portion of the H-2 region. The reagents employed in these studies would be expected to have antibodies against at least four of the well-defined specificities, different combinations of which make up the different H-2 red cell types (197), but it is not known whether agglutination was due to reaction principally of one type of antibody or an equal reaction of all. It might be assumed that the serum trait is controlled by a portion of the H-2 region which determines, for example, specific H-2 factor F, and that the antiserum used to classify H-2 type was principally anti-D, where D is a specificity also found in some H-2 types other than H-2k. Then an intragenic recombination separating regions determining the D and F specificities would appear to be a recombination between $H\text{-}2^{k}$ and Ss^{1}, when in fact the exchange only separated factors recognized by the test reagents, one factor unique to the k portion and determining Ss type but giving no significant hemagglutination reaction, the other characteristic of several H-2 alleles, but representing in this case the only criterion of H-2k type recognized by the hemagglutination reagent. One approach to this problem would be to test segregants of known serum type with the battery of hemagglutination reagents which distinguish specifically the different antigenic factors constituting an H-2 type. It might be possible to show, for example, that the serum trait is always associated with presence or absence of the F factor on the cells, even if recombination occurs within the H-2 region. Considering the uncertain nature and difficulties of interpretation of such recombinations, as well as their infrequent

occurrence, this does not at present seem a practical undertaking.

Whatever its precise relationship to the H-2 locus, the serum trait should be useful in further studies of this region. Routine use could be made of serum tests in classifying for H-2 type, particularly in classifying individuals as $H\text{-}2^k/H\text{-}2^k$ (Ss^1/Ss^1) versus $H\text{-}2^k/H\text{-}2^{"x"}$ (Ss^1/Ss^h). This distinction is quite easy and reliable, and recombination, if it occurs, is infrequent. This test has the advantage that serum samples can be stored for later testing, whereas red cells are difficult to store. If actually separable from H-2, the Ss locus could perhaps prove to be the long-needed marker on the opposite side of the H-2 locus from T, Fu and tf. This must, of course be tested in crosses involving these markers, and may be a difficult undertaking because of the low recombination frequencies between these markers. However, if borne out, this could prove a most useful aspect of the serum trait, making possible studies of the complexities of the H-2 region with the proper chromosomal definition which has heretofore been lacking.

An aspect of these results not entirely distinct from the question of genetic control concerns the structural nature of the Ss component itself, and the nature of the difference between serum types. The immunoelectrophoretic studies have yielded useful preliminary information about the component, but a thorough characterization is needed. Although it seems probable, it cannot now be stated with assurance even that the component is a protein.

Present evidence seems rather convincing that the difference between serum types recognized by the rabbit antisera is a quantitative one. The expression of the trait on diffusion plates is inconsistent with any interpretation invoking a qualitative difference

in antigenic sites as a simple alternative to a quantitative difference. It might be argued, for example, that the Ss-1 component is lacking several combining sites, so that the rabbit antiserum recognizes fewer sites on the Ss-1 component and finds an optimum proportion of antigen for precipitation only very near the antigen well. According to theory, the Ss-1 precipitin band in such a case should still form in the same position as the Ss-h band, but should be weaker in intensity due to less precipitation of antibody, and spur formation should result due to reaction of unprecipitated antibody against sites not on the Ss-1 material with Ss-h material in an adjacent well. This principle has been convincingly demonstrated in several systems utilizing haptens or cross-reacting proteins (137, 204-208). The Ss-1 component might, on the other hand, be assumed to be of much higher molecular weight, e. g. a dimer, or altered in tertiary configuration such that it would diffuse more slowly, but without any alteration in antigenic sites which could lead to spur formation. Such a model would probably fit the observed diffusion plate pattern, but would not fit the inhibition and absorption results, unless a secondary quantitative difference were also assumed.

Perhaps there is no Ss material in the Ss-1 lines at all, and the weak reaction in Ss-1 sera is due to the presence of a cross-reacting protein present in low concentration or diffusing slowly. In such a case, the points raised above concerning qualitative differences in an homologous component would also seem to apply. Further, the fact that the material detectable by immunoelectrophoresis has the same mobility in both serum types argues against such a situation. It would be a coincidence to find that the cross-reacting material also has an indistinguishable electrophoretic mobility.

The same observation also suggests that no qualitative difference in mobility exists between the Ss-h and Ss-l substances; however, it is probable that a change of one or two charges could not be detected by the method employed.

The evidence from absorptions is consistent only with a quantitative difference as detected by the rabbit antisera. The results of isoimmunization attempts and of precipitin and blocking tests with hemagglutinating sera fail to support any qualitative difference, at least involving antigenic sites. The apparent correlation of antibody titer with Ss type of immunizing serum in different rabbit antisera might be construed as evidence for qualitative differences, but this would seem best ascribed to chance or to a real antigen dosage effect, until more compelling evidence indicates otherwise.

Obviously this question cannot be fully and unequivocally resolved until the detailed structure of the Ss component from both serum types is known. The fact that qualitative differences have not been detected in no sense proves that they do not occur. The conclusions from tests to detect them must be tempered with the considerations that mice are rather poor precipitin formers, that the blocking tests may not be sufficiently sensitive, that rabbit antisera may not recognize subtle differences, and that subtle qualitative differences may not be serologically detectable under any conditions.

Something, however, definitely controls the observed quantitative difference. Is this simply a regulatory function by the Ss (or H-2) locus, without any specification of structure at all? If the difference is purely quantitative, then it would be expected

that the structural information would be at another locus. If so, then what is the mechanism for control? Perhaps the Ss material is produced at a constant rate in all mice, and is utilized by H-2k animals, but not by non-H-2k individuals. This would suggest that it could be involved in H-2 antigenic specificities. The evidence in this regard will be reviewed shortly. Perhaps a product controlled by the $\underline{H\text{-}2^k}$ allele has an inhibitory effect upon synthesis of Ss material. It might be proposed that the Ss material is an enzyme involved in the attachment of H-2 specificity-determining mucoid or lipoid groups to a macromolecular backbone, or that it is the backbone molecule itself, and that its high concentration in the serum of non-H-2k mice reflects a greater requirement for this material. Many mechanisms of this sort might be proposed for regulation of a simple quantitative difference. On the other hand, perhaps a subtle qualitative difference in structure controlled by a difference in genetic information at the Ss locus would be sufficient to alter the rate of synthesis of the Ss-1 component, as occurs with many of the abnormal human hemoglobins (13, 30, 31).

The search for some common antigenic specificity between the serum factor and H-2 antigens has been unsuccessful. Although probably not very sensitive, the tests of rabbit antiglobulins absorbed with erythrocytes and of hemagglutinating isoantisera show that no gross similarity exists. The possibility that the component represents cellular degradation products seems rather conclusively disproven, at least for erythrocytes. The failure to detect any change when erythrocytes of Ss-h mice were injected into Ss-1 mice, and the lack of any significant change in Ss levels in H-2k (Ss-1) mice several weeks after the establishment of essentially complete

transplants of erythropoietic tissue from H-2b (Ss-h) mice (see Section D. 3., pp. 114-119), argue strongly against this possibility. The result in chimeras would seem also to exclude other possible direct relationships of the type discussed in the preceding paragraph, such as enzymatic activity of the Ss component. However, it must be noted that the only criterion for repopulation in the chimeras was hemoglobin type. It is not known with certainty from these studies that the H-2 specificities of the cells were characteristic and complete H-2b specificities. The studies of radiation chimeras do not exclude possible relationships, involving either antigenic specificities or other mechanisms, with tissues other than the erythropoietic. It has been suggested that some H-2-determined antigens concerned with transplantation reactions occur in certain tissues, but are not detectable on the erythrocytes (209).

Probably the most direct approach to many of these difficulties is to isolate and purify the Ss component from both genetic types. Promising methods suggested by these studies include ultracentrifugation and specific precipitation by preabsorbed antisera of the type employed in the inhibition and absorption studies.

4. Summary

1. A variant system in the serum of mice has been detected by the Ouchterlony immunodiffusion technique, involving an apparent quantitative difference between certain inbred lines.

2. The non-genetic influences upon this component have been studied. The component is present as early as 18 days of fetal development, and shows some fluctuations in quantity until about six weeks when the adult pattern is established. Some variation occurs in adults as well.

3. Genetic studies have verified that the trait is controlled by a pair of alleles without dominance.

4. The locus controlling this trait has been shown to be closely linked to, or identical with, the H-2 region in the ninth linkage group. A consistent association of specific phenotypes for the two traits among all inbred lines tested suggests that the serum trait might be directly controlled by the H-2 region, but apparent recombination between these phenotypes tends to contradict this.

5. Immunoelectrophoretic analyses suggest that the component is an alpha-globulin, probably of high molecular weight.

6. Studies designed to characterize the serological difference between serum types indicates that the difference recognized by rabbit antisera in immunodiffusion is a quantitative one. No evidence supporting qualitative differences has been obtained, but the possibility is not excluded.

7. Attempts to show an antigenic relationship between the serum factor and the H-2 antigenic specificities of the red blood cells have been unsuccessful. Erythrocyte degradation has been excluded as a possible source of the serum factor.

8. Regardless of its precise relationship to the H-2 locus, the serum trait offers a promising tool for further studies of genetic diversity in this region.

D. Studies of Serum Protein Types in Homologous Radiation Chimeras

1. Introduction

It has been demonstrated that mice subjected to a lethal dose of X-irradiation can be treated with injections of heterologous, homologous or isologous bone marrow or other hematopoietic tissues (210-219), and that the injected cells form effective and frequently permanent transplants of erythropoietic tissue (220-231). Consideration of the immunological implications of transplants of foreign tissue in an incompatible host led to much debate of the question whether the secondary disease occurring in incompatible combinations is due to reaction of the host against the graft, the graft against the host, or both (232). In connection with this point, several studies were carried out to determine whether antibody or other serum proteins of donor type could be found in the serum of heterologous rat-into-mouse chimeras.

Weyzen and Vos (233) used ring tests with chicken anti-rat serum, absorbed by mouse serum, to study the sera of 15 heterologous chimeric mice at about 100 days after treatment with rat bone marrow. All of these chimeras' sera gave rather weak positive reactions, and immunoelectrophoresis showed precipitation in the gamma-globulin region. Grabar et al. (234) used absorbed rabbit antisera against both rat and mouse serum, in immunoelectrophoresis of sera from 16 heterologous chimeras and five isologous controls between 25 and 100 days after treatment, and found that in the heterologously treated animals reactions occurred between the anti-mouse serum and a component in the albumin region, and between the anti-rat serum and two components in the gamma-globulin region. They concluded that the chimeras had rat gamma-globulin. Makinodan (235)

and Gengozian (236) made reciprocal immunizations between rats and mice to obtain specific antisera without the necessity of absorptions and tested these by Ouchterlony immunodiffusion and ring tests against the sera of heterologous chimeras up to several months after treatment. They found no evidence for rat proteins, but Gengozian (236) observed that a supra-lethal dose of X-irradiation to an established chimera resulted in the release of detectable quantities of rat protein into the serum. Popp and Smith (173) studied by starch gel electrophoresis the occurrence of rat-type proteins in the serum and in extracts of other tissues in rat-into-mouse chimeras. They found high proportions of rat globulins in some tissues for about six weeks after treatment, at which time mouse proteins began to appear again in larger amounts. They found no rat proteins in the serum at any time, however. These results by different workers are obviously in conflict, and the conflict is apparently still unresolved, although Popp and Smith (173) have suggested that the rat serum protein detected in earlier studies (233, 234) might have been a rat erythrocyte protein resulting from hemolysis, rather than rat gamma-globulin.

Aside from the immunological implications, it is of interest from the standpoint of physiological definition of the effects of radiation to know whether plasma protein-synthesizing cells are as severely damaged as erythropoietic tissue by lethal X-ray doses, and if so whether losses of these cell types are restored by transplants. It would be of particular interest to study this in homologously treated animals. No studies of the type described above have been reported for homologously treated chimeras because no markers have been available for serum protein differences.

The three serum protein variant systems described in Sections B and C are useful for this purpose. Studies have been carried out to determine whether donor type variants of these three serum components occur in homologous chimeras established by standard procedures.

The animals in the studies reviewed were treated with bone marrow. It has been shown that homologous fetal liver also gives good protection and in some strain combinations seems to circumvent the severe secondary disease (215-219). In the studies to be reported here, both bone marrow and fetal liver are used. It is reasoned that the proper cell types may be included in fetal liver, if not in bone marrow, because most of the serum albumin and globulins, except gamma-globulin, are thought to be synthesized principally in the liver (237-238). It is known from the studies reported in Sections B. 2. b., pp. 27-34, and C. 2. b., pp. 72-73, that at 18 days the fetus definitely has the three proteins to be used as markers, and it has been firmly established that at least the Trf and Pre types of the serum in the fetus are determined by the individual's own genotype. There can therefore be no question that the fetus at this stage has the necessary differentiated cells.

For studies of Trf and Ss components, the strain combination best suited is B/6 tissue injected into irradiated CBA recipients. The Trf-a of CBA is faster electrophoretically than the Trf-b of B/6, so Trf-b can be easily and sensitively detected in a Trf-a individual without interference from minor components. Similarly the CBA line is Ss-l, meaning a low concentration of the Ss component. Donor type Ss-h material can be more sensitively detected as an increase in a low existing level of Ss component, and low levels of donor contribution can be more precisely estimated. This

combination is not useful for Pre, however, since the CBA line is Pre-a, with a detectable component, while B/6 is Pre-o with no detectable component. Therefore a different strain combination, C tissue injected into (B/6 x A/J)F_1 recipients, hereafter called BAF_1, was employed for this marker, making it possible to look for the appearance of the Pre-a protein of line C at a position in the starch gel electrophoretic pattern where there is none in the Pre-o BAF_1. Both strain combinations involve hemoglobin differences, so the course of erythrocyte repopulation can be followed by the method of Popp and Cosgrove (66).

2. Materials and Methods

a. Animals

The genetic constitutions of the four lines used in these studies for the markers to be investigated are as follows: Recipient - CBA ($\underline{Trf}^a$, $\underline{Ss}^1$, $\underline{Pre}^a$, $\underline{Hb}^2$), Donor - B/6 ($\underline{Trf}^b$, $\underline{Ss}^h$, $\underline{Pre}^o$, $\underline{Hb}^1$); Recipient - BAF_1 ($\underline{Trf}^h$, $\underline{Ss}^h$, $\underline{Pre}^o$, $\underline{Hb}^1/\underline{Hb}^2$), Donor - C ($\underline{Trf}^b$, $\underline{Ss}^h$, $\underline{Pre}^a$, $\underline{Hb}^2$). (I am indebted to Mr. A. P. Roberts who provided samples from chimeras of the second combination, which had been prepared for other investigations.)

The serum types of the following groups were studied: CBA - 31 radiation controls, 13 treated with B/6 fetal liver (HFL)*, 32 treated with B/6 bone marrow (HBM)*, 8 treated with CBA fetal liver (IFL)*, and 10 treated with CBA bone marrow (IBM)*; $\underline{BAF_1}$ - 10 radiation controls, 21 treated with C bone marrow, 16 treated with BAF_1 bone marrow; B/6 - 5 radiation controls. None of these animals was sacrificed to obtain samples. All were bled sparingly from the tail and saved, except two which died during bleeding. Most animals contributed at least two, and some as many as five samples at different stages after treatment. All of the irradiated recipients were between 8 and 15 weeks of age and no animal was used which weighed less than 20 g. at the time of treatment. Each animal was individually identified and was weighed weekly and examined daily.

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*These abbreviations will be used for the described experimental groups through the remainder of this section.

b. Preparation of cell suspensions

Bone marrow donors were between 8 and 18 weeks of age. The animals were first exsanguinated, then the femora excised. The bone marrow plug was washed out by a stream of Locke's solution from a syringe, and the cells suspended by gentle aspiration first through a 22 gauge, then a 27 gauge hypodermic needle. A sample of the suspension was removed and diluted in a red blood cell pipette with a solution of crystal violet in 1% acetic acid, and nucleated cells were counted in a hemocytometer. Individual doses of 20 to 45 x 10^6 nucleated cells were given to homologously treated animals, 10 to 15 x 10^6 to isologously treated recipients.

For fetal liver cells, 16 to 19-day fetuses were used, the stage determined by timed matings. The fetuses were decapitated and the livers removed into Locke's solution. After removal of all detectable extraneous tissue, the livers were gently pressed through a coarse sieve, with care to avoid excessive maceration of connective tissue. The free cells were washed through the sieve and further suspended by aspiration through a 22 gauge needle. These suspensions were passed through a very fine wire mesh, and finally aspirated through a 30 gauge needle. Samples were taken and nucleated cells counted as before. Doses of 25 to 30 x 10^6 cells were given to homologous recipients, 15 to 20 x 10^6 to isologous recipients.

c. Radiation conditions

The animals were radiated in groups of 12 in a Lucite holding device placed on a revolving turntable, positioned 54.6 cm. from the radiation source. Irradiation was with a Westinghouse

250KV, 20 ma. machine, at 240 KV and 15 ma. with 1 mm. added aluminum filtration. The rate of irradiation in air was 150 r./min.; the total dose to all CBA recipients, except one group, was 750 r. One group received 850 r. Several groups of radiation controls received doses from 550 to 850 r. The radiation dose to all BAF_1 recipients was 850 r. The animals to be treated with bone marrow or fetal liver cells were injected in a tail vein within four hours after irradiation. Cell concentrations were calculated so that it was never necessary to inject more than 0.5 ml. for a complete dose.

d. Collection of samples

Samples for test were collected at weekly intervals from the tail as described in Section B. 1. b., p. 18, except that the animals were warmed only a few minutes before bleeding, and samples of never more than 0.30 to 0.35 ml. were collected. Of this, 0.15 ml. was collected into isotonic citrate solution for hemoglobin determinations, and the remainder into siliconed 1 ml. centrifuge tubes for serum. The animals to be sampled were selected at random each week, except that as a rule animals were not bled oftener than alternate weeks. Occasionally a particularly strong animal was bled sparingly on successive weeks, if additional samples were deemed necessary.

e. Hemoglobin determinations

The hemoglobin solubility method of Popp and Cosgrove (66) was employed to determine the progress of erythrocyte repopulation in chimeras. Determinations were made on both homologously and isologously treated animals. Red cells collected in isotonic citrate were washed once and lysed with approximately ten volumes of distilled water. Hemoglobin concentrations of the lysates were determined

by the acid hematin method, read at 540 mμ on a Klett colorimeter. The hemoglobins were converted to the carbonmonoxy form, and a volume containing 6.8 mg. of hemoglobin added to 4.3 ml. of 3.5 M phosphate buffer. This was subsequently adjusted with distilled water to a final volume of 5.0 ml. of the phosphate buffer at 3.01 M, pH 6.8. Each sample was thoroughly mixed and sealed, allowed to incubate 18 to 22 hours at room temperature, then filtered through No. 1 filter paper to remove precipitated hemoglobin, and the optical density determined at 540 mμ on the Klett colorimeter. Preparation of a standard curve from mixtures of Type I and Type II hemoglobins and the inclusion of several standards each time determinations were made permitted estimation of the composition of an unknown sample within a range of 20%.

f. Electrophoretic and serological determinations

The methods were as described in Sections B. l. c., pp. 19-24, and C. 1. c., pp. 65-67, for classification of variant Trf, Pre and Ss phenotypes.

3. Results

These studies were designed primarily to test qualitatively for the presence of donor proteins of the three marker types, to determine whether transplants of the specific serum protein-synthesizing cells are established. However, the amounts of donor type protein present were estimated in a semi-quantitative way as a rough indication of extent of transplant establishment, and as a basis for evaluation of the significance of observations on homologously treated animals relative to the controls, because non-specific effects resulted in some ambiguity. To standardize these estimates, mixtures of normal sera were subjected to the same classification procedures used for the test samples. Minimum detectable levels of donor type protein and minimum levels consistently detectable without error were determined. Isologously treated controls and radiation controls were also tested to determine to what extent ambiguity could occur. The following levels were established: minimum detectable concentrations of Trf-b in Trf-a, 10%; Ss-h in Ss-1, 12.5%; Pre-a in Pre-o, 12.5 %; minimum levels repeatably detectable, Trf-b in Trf-a, 15%, Ss-h in Ss-1, 20%, Pre-a in Pre-o,20%. Samples from homologously and isologously treated animals and radiation controls were always intermixed and tested at the same times, identified only by code number until classification had been completed. Each sample was assigned an estimated value for concentration of donor type protein, with the results of the standardizations as reference.

The results from studies of the Trf and Ss serum markers are summarized in Table 11, with data on the weights, survival and rate of red cell repopulation of the CBA chimeras in each treated group

Table 11

Results from irradiated CBA mice treated with CBA or B/6 bone marrow or fetal liver

Treatment*	Week	Ave Wt.	% Surv.	Hemoglobin No.	Hemoglobin % Repop.	Trf Total	Trf No. with Trf-b	Trf Est. % Trf-b	Ss Total	Ss No. with Ss-h	Ss Est. % Ss-h
HFL	0	21.7	-	-	-	-	-	-	-	-	-
	1	19.1	100	-	-	-	-	-	-	-	-
	2	19.5	85	3	25	5	0	-	5	1	35
	3	20.0	85	5	32	5	0	-	5	2	30,35
	4	20.1	77	6	66	6	0	-	6	0	-
	6	19.4	77	9	93**	9	6	6-15	9	0	-
	8	19.3	62	7	95**	7	1	15	7	0	-
	12	21.5	38	4	97**	4	0	-	4	0	-
IFL	0	21.3	-	-	-	-	-	-	-	-	-
	1	17.0	100	-	-	-	-	-	-	-	-
	2	20.1	63	3	0	3	0	-	3	0	-
	3	20.3	50	2	0	2	0	-	2	0	-
	4	20.7	38	2	0	2	0	-	2	0	-
	6	19.3	38	-	-	3	0	-	3	0	-

*Abbreviations of treated groups are as listed on p. 110.

**Not including one individual which at 6 weeks had regressed to CBA type.

Table 11 (Continued)

Treatment*	Week	Ave Wt.	% Surv.	Hemoglobin No.	Hemoglobin % Repop.	Trf Total	Trf No. with Trf-b	Trf Est. % Trf-b	Ss Total	Ss No. with Ss-h	Ss Est. % Ss-h
HBM	0	23.7	-	-	-	-	-	-	-	-	-
	1	20.8	100	-	-	4	0	-	3	0	-
	2	20.9	81	12	14	10	3	3-15	11	5	20,20,25,30,35
	3	19.5	81	6	34	6	1	25	7	0	-
	4	17.8	50	6	42	5	3	15,15,50	6	0	-
	5	17.8	13	-	-	1	0	-	1	0	-
	6	-	0	-	-	-	-	-	-	-	-
IBM	0	22.5	-	-	-	-	-	-	-	-	-
	1	20.6	100	-	-	-	-	-	-	-	-
	2	20.9	50	3	0	3	0	-	3	0	-
	3	20.7	50	2	0	3	0	-	3	0	-
	4	22.2	50	2	0	2	0	-	2	0	-
	5	22.1	50	-	-	2	0	-	2	0	-

*Abbreviations of treated groups are as listed on p. 110.

included as general parameters. Because the numbers of animals in the various groups are rather small, the quantitative values should not be regarded as having much absolute significance, but rather as an expression of qualitative observations.

The data suggest that donor type transferrin does appear in some radiation chimeras transitorily, in animals treated with either fetal liver or bone marrow. In the HFL group, this occurred during the period of secondary disease, as indicated by mortality and weight changes. Although the recorded values are at the minimum level for reliable detection, the results at six weeks are probably significant in view of the number of observations at this time. It is clear that this is not a permanent effect. Survivors to eight and twelve weeks having only donor type hemoglobin have no detectable level of donor type transferrin. The single individual still having detectable Trf-b at eight weeks also had a detectable level at six weeks.

In the HBM group, Trf-b is detected somewhat earlier, from two to four weeks after treatment. Since secondary disease develops earlier in this group and no animals have survived longer than six weeks after treatment, no information is available on long-term survivors. Two individuals in this group had rather high levels of Trf-b. Both exhibited symptoms of severe secondary disease at the time of sampling and both died within a week. It is of interest that both received relatively high doses of B/6 bone marrow, 45×10^6 cells. Clearly the presence of detectable levels of donor type transferrins is neither necessary nor permanent in either group, and the observed levels have no relationship to the levels of donor type red cells. However, it is not known how extensive or how persistent levels below 15% may be.

The results with the Ss marker were similar to those with the transferrins. With Ss the recorded levels are higher, but the level for reliable detection of Ss-h is also higher. This is due to the quantitative nature of this distinction, and the effects of non-specific factors to be discussed later. The time of appearance of donor contributions to the Ss level is the same in both HFL and HBM groups, and the recorded levels are high enough to be regarded as significant relative to rather extensive observations of isologous controls and radiation controls.

In the HFL group two of the three individuals showing donor Ss contributions died by seven weeks. The third survived but at six weeks had regressed to CBA red cell type. In the HBM group a significant number had high Ss levels at two weeks. All of these animals died within two weeks after these samples were taken.

There is no correlation between levels of donor type Trf and Ss. For the HFL group, $r = -0.17$, for the HBM group, $r = 0$. For the HFL group, r would have had to be smaller than -0.34 to be significantly negative at the 5% level.

Although the B/6 into CBA combination is not well-suited for detection of Pre changes, the samples from both HFL and HBM individuals were carefully inspected for differences in Pre, after electrophoretic separation at pH 8.6 to classify for the transferrin trait. In no instance could a detectable decrease in the normal level of Pre-a be detected, and in general it appeared that the concentration might be increased somewhat. This was studied further in the C into BAF_1 combination in which the recipients were Pre-o and the donors Pre-a. For this part of the study only bone marrow-treated animals were available.

Nine homologously treated animals were tested at 7 to 12 days after treatment, and 12 at eight weeks. For comparison, ten isologously treated and ten radiation controls were tested at 7 to 12 days, and six isologously treated animals at eight weeks. The results with all 47 samples were uniformly and convincingly negative. Although animals were not available at intermediate stages, the fact that no trace of Pre-a could be found demonstrates that no permanent transplant of Pre-a-producing cells is established and suggests that there is no transitory effect, at least up to two weeks, when the effect was observed with Trf and Ss in bone marrow treated animals. It should be emphasized, of course, that this is a different strain combination. The particular animals used in this study were not tested for hemoglobin types, but it is well established from rather extensive studies of this combination (239) that at two weeks low levels of donor hemoglobin are found, and that by five weeks there is essentially only donor type hemoglobin; no transplant regressions have been observed. At approximately four weeks, a relatively mild secondary disease develops, which most of the homologously treated animals survive.

4. Discussion

The general effects of radiation on Trf and Ss patterns have been discussed in Sections B. 2. b., pp. 27-34, and C. 2. b., pp. 72-73. The effect on Trf is to shift the relative proportions of major and minor components. The strain combination used was intended to permit the most sensitive possible detection of donor type Trf-b, which is electrophoretically slower than Trf-a. However, if the same shift occurs with respect to donor type Trf-b, as might perhaps be expected, then much of the donor type transferrin might be in the Trf-b' minor form which overlaps Trf-a and would be undetectable. This effect would lead to underestimation of the amount of donor type transferrin actually present, at least at low levels.

Radiation causes approximately a two-fold increase in Ss material in both Ss-h and Ss-1 types. From the relative quantities of Ss in Ss-h and Ss-1 sera, estimated in Section C. 2. d., p. 85 , one would expect a two-fold increase to result in a band position in controls equivalent to that obtained with a mixture of 10% Ss-h and 90% Ss-1. This is approximately what was observed. The highest level in an isologous control corresponded to a mixture containing 15% Ss-h serum. As a consequence of this increase after radiation, it would appear that the recorded Ss-h levels in homologously treated animals, while significantly different from the controls, would nevertheless be somewhat overestimated.

It is obvious that these serum traits are not of value as general markers for repopulation in homologous chimeras as the hemoglobins have been, whether treatment is with bone marrow or fetal liver. The results do suggest that some cells synthesizing Trf and Ss are destroyed by radiation, and that in some instances

donor cells of these types may form temporary transplants until the animal recovers the ability to synthesize its own protein types. In general, it would appear that the cells synthesizing these components are either less radiosensitive or are located in more protected sites, e. g. the liver, than the erythropoietic cells. The fact that donor protein is found in animals protected with either bone marrow or fetal liver indicates that at least some of the serum protein-synthesizing cell types producing these proteins are located in the bone marrow, as well as the liver.

The failure to find Trf-b in long-term HFL survivors is consistent with observations noted in a personal communication by Cohen (240), who studied the sera of fetal liver-treated animals of the same strain combination, at 200 days after treatment only, and found at this time that animals still carrying donor type skin grafts and having donor type hemoglobin had no detectable Trf-b.

The observations on the Ss trait may have additional significance in view of the genetic association of Ss with the H-2 complex. This has been discussed in Section C. 3., pp. 96-103.

5. Summary

1. Three serum protein variant systems in the mouse have been used as markers in an investigation of the serum protein types of radiation chimeras homologously and isologously treated with fetal liver and bone marrow cells.

2. The transitory appearance of donor type transferrin or Ss component has been observed in a few individuals between two and six weeks after treatment, but the serum types of long-term survivors receiving fetal liver are those of the host within the limits of detection, even though the erythrocytes are entirely donor type. No donor type Pre-a could be detected in Pre-o recipients at two or eight weeks after treatment.

3. These results suggest that although some cells producing these serum proteins are probably destroyed by radiation, enough are either less radiosensitive or more protected than erythropoietic cells to permit the chimera to regain ability to synthesize its own serum components. It also appears probable that cell types producing transferrin and the Ss component occur to some extent in the bone marrow, as well as the fetal liver.

II. MOLECULAR HYBRIDIZATION OF SHEEP HEMOGLOBINS

A. Introduction

Molecular hybridization between genetically variant hemoglobin types has been of value in the study of the human hemoglobins, from both the chemical and genetic points of view. The technique has been a valuable chemical tool in the study of gross structure, molecular associations, compatibilities and interactions of hemoglobins (33, 241-245) and other proteins (76). With it important contributions have been made to our understanding of the genetic control of the human abnormal hemoglobins. Many abnormal hemoglobins have been classified as to the molecular subunit in which genetic change has occurred (32, 242, 244, 246-248), and chemical evidence supporting the concept that two genetic loci determine the structure of adult hemoglobin A has been provided (32).

Application of the technique to hemoglobin variants of other species has not been reported. Hemoglobins of other species have been used, but only in connection with studies of human hemoglobins (249-251). Our understanding of the genetic mechanisms controlling hemoglobin specificity in mammals in general could be extended, and the general applicability of findings with human hemoglobins tested, by chemical studies of hemoglobin variants in other mammalian species. The hemoglobin variants of the domestic sheep are promising material for such studies (51-58). The animal is large enough to supply more than adequate amounts of hemoglobin, yet small enough for housing in ordinary animal quarters and for easy handling and treatment. The general physical and chemical differences between the two hemoglobin types (54, 57, 58), and the inheritance of the difference have been rather well-studied (51-54).

As will be shown, several aspects of the difference make it one of particular interest.

The possibility of molecular hybridization was first suggested by observations by Field and O'Brien (252) that the sedimentation coefficient for human hemoglobin at acid pH was significantly decreased relative to that at neutral pH. This was extended by Hasserodt and Vinograd (253), with similar results, to alkaline pH. These observations suggested that the hemoglobin molecule was dissociating, as a result of pH changes, into the smaller subunits which Perutz et al. (16) had shown by X-ray diffraction to exist in the hemoglobin molecule. It was known from the work of Rhinesmith et al. (17, 18) that the hemoglobin molecule consisted of at least two different kinds of chains, termed α and β, and the work of Ingram (19) indicated that the hemoglobin S or C defect occurred in only one of the two kinds of chains. These observations suggested that differing human hemoglobins might be recombined by mixing and dissociating at low pH, then allowing them to reassociate at neutral pH to form "hybrid" types. This work was prompted by the observation that, although they must probably be synthesized separately, the differing subunits always associate in vivo in heterozygotes to form only two detectable types, with no evidence of the several possible hybrid types which could be formed by different combinations of the different kinds of chains (254).

The first hybridization study was reported by Itano and Singer in 1958 (241). Assuming that dissociation at acid pH was into symmetrical half-molecules, they postulated that recombination between hemoglobins A and S, which differ by a charge of two, should

result in a new, intermediate species with a charge of one. This was not found. The conclusion was that either dissociation is symmetrical, and incompatibility exists between symmetrical half-molecules from different molecular species, or that the dissociation is asymmetric and A and S have an identical subunit, such that if exchange does occur it merely regenerates the original two types. The second possibility was tested (242) by labeling one of the hemoglobin types, either by oxidation to methemoglobin, or by ^{14}C-glycine. It was then possible to show exchange between HbA and HbS or HbC, indicating that the second hypothesis was correct. This reaction may be represented by the following equation:

$$\underset{HbA^{**}}{\alpha_2^{A^*}\beta_2^{A^*}} + \underset{HbS}{\alpha_2^{A}\beta_2^{S}} \rightleftharpoons \alpha_2^{A^*} + \beta_2^{A^*} + \alpha_2^{A} + \beta_2^{S} \rightleftharpoons \underset{HbA^{**}}{\alpha_2^{A^*}\beta_2^{A^*}} + \underset{HbS^{*}}{\alpha_2^{A^*}\beta_2^{S}} + \underset{HbA^{*}}{\alpha_2^{A}\beta_2^{A^*}} + \underset{HbS}{\alpha_2^{A}\beta_2^{S}}$$

*Denotes radioactively labeled pair of chains.

This gives a theoretical exchange of 25% of the activity.

The technique was also used by Vinograd et al. (244) to identify the β chain as the subunit in which the HbS defect occurs, and by Jones et al. (33, 245) to determine the gross molecular structures of HbH and HbF. Studies at alkaline pH have made further contributions to understanding of the mechanism of dissociation and the quaternary structure of the molecule (243).

Later experiments (246) on hybridization between HbI and HbA or HbS substantiated the evidence by Ingram (255), and Murayama and Ingram (28), that the defect in HbI was in the α-chain. Another experiment gave similar results for Ho-2 hemoglobin (32). It has also been shown that while human hemoglobin can be hybridized with canine hemoglobin (249, 250) or rabbit hemoglobin (251) to provide useful tools for the study of human hemoglobins, it will

not hybridize with bovine hemoglobin (249).

In earlier studies column chromatography or moving boundary electrophoresis were used for separation of the different hemoglobin types. Recently, extensive studies to identify the defective chains of a large number of abnormal hemoglobins have been greatly facilitated by the use of starch gel electrophoresis for separation of the hemoglobins (247, 248).

The sheep hemoglobin variants were first observed by Harris et al. in 1955 (51). The two hemoglobin species, called A and B, differ in electrophoretic mobility, with A having the higher mobility at pH 8.6. Genetic evidence from breeding tests and population studies is consistent with an hypothesis of control by a pair of codominant alleles (52, 53, 55, 56). A similar observation, probably of the same types, was reported somewhat later by van der Helm et al. (54) in Dutch sheep. They called the types I and II. I is probably the same as B, II the same as A. These workers have reported further genetic data, as well as some physical-chemical studies defining differences between the two hemoglobin types (54, 58).

It was found in these studies that the two hemoglobin types behave in chromatography with Amberlite IRC-50 in the same way as they do in electrophoretic separation at pH 8.6; the II (A) hemoglobin is faster than I (B). (Hereafter the assumption will be made that II = A and I = B, and only the symbols A and B will be used.) B is soluble in 2.8 M phosphate buffer while A is not. When treated with 0.1 N NaOH for 10 minutes, B is 48% denatured, while A is only 34% denatured. Analysis of amino acid compositions

indicated that relative to B, A contains significantly more threonine, serine, and glutamic acid, but less glycine, alanine, and aspartic acid. Studies of the oxygen affinity showed that A has a higher oxygen dissociation curve than B (57), and further surveys showed the occurrence of this type more frequently at higher altitudes, as previously noted by Evans et al. (53, 56). No significant difference was found in rate of incorporation of ^{59}Fe into the two types of hemoglobin in a heterozygote. In several heterozygous animals suffering from anemia due to Strongylus larval infections it was found that as the hemoglobin levels of the animals dropped, the ratios of B to A increased.

In 1948, Porter and Sanger (256) determined the N-terminal amino acids of a number of mammalian hemoglobins. They found for sheep hemoglobin, valine and methionine. At that time the genetic difference in sheep hemoglobins had not been observed, so it is not known which hemoglobin type was studied. This result has been confirmed by Ozawa and Sataki (257), again with hemoglobin of unknown type. These workers also determined the second members of the N-terminal sequences and reported val-leu and met-gly. These two independent and identical results plus comparable results on cattle and goats (256-258), which are in the same taxonomic family, leave little doubt that the sheep hemoglobin molecule consists of at least two different kinds of chains.

The most interesting aspect of the sheep hemoglobin variants is the large difference between the two types in amino acid compositions (54). Most evidence with respect to human hemoglobin differences (259) and the proteins studied in E. coli tryptophan

synthetase mutants (1) indicates that a given mutation changes only a single amino acid. In the sheep a large number of amino acids have apparently been changed. The evidence is now persuasive that in humans synthesis of the major adult hemoglobin component is controlled by two loci (32), each determining the specificity of one of the two different chains. In the sheep the evidence for control of the observed difference by a single locus is convincing. It is of interest to inquire whether, in the sheep, this large structural change involves only one chain or both chains. Experience with the human hemoglobins suggests that this might be tested by a hybridization experiment. An exchange to the extent predicted by random recombination with no new species formed, would provide presumptive evidence that the change involves only one chain. This report presents the result of such an experiment, and a number of studies accessory to it.

B. Materials and Methods

1. Animals

Four sheep were used in the study, two of hemoglobin type A and two of type B. One animal of each type was used for unlabeled samples (I am indebted to Dr. C. Sato for allowing me to collect samples from these animals), the other two were used for the preparation of radioactively labeled hemoglobin samples described in a subsequent section. Hemoglobin type was determined by starch gel electrophoresis. No direct comparison has been possible, but the assumption is reasonable that these are the types designated A and B by Evans et al. (52), and II and I by van der Helm et al. (54). The samples were obtained from sheep representing breeds included in the study by Harris et al. (53) and show the same relative electrophoretic mobilities.

2. Hemoglobin solutions

Unlabeled solutions of hemoglobin were prepared by the method of Drabkin (250), but without crystallization. The sheep were bled by puncture of the jugular vein, and the blood collected in isotonic citrate-saline. The cells were washed three times in ten to twenty volumes of isotonic saline. Carbon monoxide was carefully bubbled through the cell suspensions and the cells were then lysed by addition of an equal volume of distilled water and 0.4 volume of toluene. After thorough mixing the lysates were placed in a refrigerator overnight. They were then centrifuged 15 min. at about 1,500 rpm. and the upper toluene and stroma layers removed. The clear hemoglobin solution was pipetted off the lower layer of stroma and centrifuged again in a Spinco Model L preparative

ultracentrifuge for ½ hour at 30,000 rpm. The supernatant hemoglobin solution was dialyzed under carbon monoxide against several changes of Developer 6c or 6f (described later) in the cold. After dialysis the samples were diluted with developer to a final concentration of 50 mg. per ml. and stored under carbon monoxide in serum bottles at 4°C.

3. ^{14}C-labeled hemoglobin samples

Radioactively-labeled samples were prepared by incubating reticulocytes from sheep of known hemoglobin type with the desired labeled amino acid, according to the procedure of Borsook et al. (261), as modified by Vinograd and Hutchinson (243). To stimulate the necessary high reticulocyte level, the type B sheep was made anemic by continued bleeding over a period of one week, the type A sheep by administration of phenylhydrazine for about one week. (I am indebted to Drs. P. Tso and Joan Wallace for making available to me cells from these animals.) Two samples of each type were prepared, one labeled with ^{14}C-valine, the other with ^{14}C-methionine. After incubation the cells were washed several times and hemoglobin solutions prepared as described above. Preparations of both types had specific activities of about 2,500 cpm./mg. with ^{14}C-valine and about 500 cpm./mg. with ^{14}C-methionine.

4. Ferriheme-labeled hemoglobin samples

Methemoglobin samples were prepared as described by Itano and Robinson (262), from fresh solutions of carbonmonoxyhemoglobin in phosphate buffer, μ = 0.25, pH 6.8. The hemoglobin was diluted to 10 mg./ml. in phosphate buffer, μ = 0.01, pH 6.6 and converted to methemoglobin by oxidation with an excess of potassium ferricyanide. The ferricyanide was removed by extensive dialysis

against the phosphate buffer used for dilution, under a nitrogen atmosphere. The removal of ferricyanide required dialysis for 48 hours against at least six changes of buffer. Samples were used immediately at the conclusion of dialysis.

5. Chromatography

The hemoglobins were separated by ion exchange chromatography on 200-250 mesh Amberlite IRC-50 as described by Allen et al. (263). The 1 x 35 cm. columns were maintained at 6°C. by a circulating alcohol-water mixture in water jackets. Samples of 15 to 20 mg. in 1 to 2 ml. volumes were routinely chromatographed on these columns. Before each run, the pH of the effluent was checked to verify that it was the same as that of the buffer being employed. If columns were left inactive for some time, they were always re-equilibrated by the passage of at least 1 liter of developer before further chromatography. Eluates from the column were collected on a fraction collector equipped with an ice bath, in 2 ml. fractions. To collect the major hemoglobin components in a minimum volume of developer, after elution of the leading minor components the columns were warmed to 28°C. for 30 minutes, after which most of the material on the column moved off with little or no retardation. After warming, it was necessary to re-equilibrate the column by passage of at least one liter of developer.

Two buffers were used as developers. For chromatography of hemoglobin type A to remove minor components and partially purify the major component Developer 6c was used. For purification of type B and for the separation of mixtures of types A and B, Developer 6f was used.

Developer	Reagent	g/l.	pH
6c	$NaH_2PO_4 \cdot H_2O$	5.52	6.60
	Na_2HPO_4	0.71	
	KCN	0.65	
6f	$NaH_2PO_4 \cdot H_2O$	5.06	6.70
	Na_2HPO_4	0.95	
	KCN	0.65	

6. Starch gel electrophoresis

The equipment and general method have been described in Section I, pp. 19-24. In these experiments the hemoglobins were separated at pH 6.6, in phosphate buffer at μ = 0.01, for 3 hours at 9 V/cm. The samples were introduced into slots 1 x 6.5 x 9 mm., with four slots across the width of the gel, and four or five rows of slots per 30 cm. tray. The samples were used at total hemoglobin concentrations of 5 to 10 mg./ml.

7. Sedimentation velocity studies

Studies of sedimentation velocity at various levels of hydrogen ion concentration were carried out in the Spinco Model E analytical ultracentrifuge. Samples were run in Kel-F cells at a nominal speed of 56,100 rpm. for 2 to 3 hours at constant 20°C. Hemoglobins A and B were run simultaneously at each pH in a double cell rotor, using a 1° wedge. For particular runs, small differences in sedimentation coefficient between the two species should therefore be significant. The samples, at a concentration of 10 mg./ml., were dialyzed for 24 hours at 4°C. against a buffer at the appropriate pH. The pH of the last dialysate and of the hemoglobin samples after the run were determined for every sample. The buffers used were of the following compositions:

pH	Composition
4.7	0.1 M sodium acetate 0.15 M sodium chloride glacial acetic acid to pH 4.7
7.1	0.0286 M K_2HPO_4 0.0143 M KH_2PO_4
10.0-11.0	0.18 M sodium chloride 0.075 M NaOH 0.08-0.055 M glycine
11.25	0.13 M sodium chloride 0.0185 M $Na_3PO_4 \cdot 12H_2O$ 0.033 M $Na_2HPO_4 \cdot 7H_2O$

8. Hybridization

The hybridization procedure was as described by Vinograd and Hutchinson (243). For alkaline hybridization, 10 mg. HbA and 10 mg. HbB were mixed in a total volume of 2 ml. and put in dialysis bags. Control samples of 10 mg. of each type in a volume of 1 ml. were also put in separate dialysis bags and dialyzed, with the hybridization mixture, against the glycine-NaOH buffer, pH 11.0, μ = 0.25, described for the sedimentation velocity studies, for 18 hours at 4°C. The mixtures and controls were then dialyzed against Developer 6f for 24 hours, at which time the pH of the dialysate was determined to ascertain that it was that of the normal buffer. The hybridization mixtures were chromatographed with Developer 6f. Two ml. fractions were collected, their concentrations determined on a Beckman DU Spectrophotometer at 522 mμ, and plated for determination of radioactivity. One-half ml. of each sample or a dilution of it, containing not more than 0.5 mg./ml., was plated. Previous studies had shown that under these conditions correction for self absorption is unnecessary (243). The

planchets were counted on a Nuclear-Chicago gas-flow Geiger counter, Model D-47, with a "micro-mil" window, to at least 2,500 counts, and specific activities calculated. The control samples were mixed after final dialysis, chromatographed on the same column after re-equilibration, and read and counted as above.

Hybridization at pH 4.7 was conducted in the same way, except that the samples were dialyzed against the acetate buffer, pH 4.7, μ = 0.25, described for sedimentation velocity studies. During dialysis back to neutral pH, a great deal of precipitate usually formed in the solutions. This was removed by centrifugation before chromatographing.

Treatment for hybridizations with methemoglobins was conducted in the same way, at pH 4.7, except that the sodium chloride was eliminated from the buffer. The mixtures were separated by starch gel electrophoresis instead of chromatography. Dialysis of the methemoglobin samples was always carried out under an atmosphere of nitrogen. Extensive precipitation, particularly of methemoglobin, but of both types in the mixtures, occurred in these experiments during dialysis from pH 4.7 to pH 6.6. In one experiment an equal volume of pH 4.7 buffer was added directly to each sample to give a final molarity of acetate of 0.05, left for 1 hour in the refrigerator, then dialyzed back to neutral pH. In this instance only negligible precipitation occurred.

9. Nomenclature and abbreviations

To simplify reference to the various forms in which the hemoglobins may exist and the two different molecular species of sheep hemoglobins, the following abbreviations will be employed:

HbA and HbB refer to the two different sheep hemoglobin types in general without specification as to the form in which they occur.

MHb will be used as a general designation for methemoglobin or ferrihemoglobin.

MHbA and MHbB refer to the specific methemoglobin forms.

HbCO will be used as a general term for the carbonmonoxy form of hemoglobin.

HbCO-A and HbCO-B refer to the specific carbonmonoxyhemoglobins.

HbCO-A* and HbCO-B* refer to the radioactively labeled carbonmonoxyhemoglobins.

C. Results

1. Dissociation studies

Experiments were first performed to ascertain whether sheep hemoglobins dissociate to an extent sufficient to permit hybridization. This was done by determining sedimentation velocities at various levels of hydrogen ion concentration. The dissociation behaviors of both hemoglobin types were determined using a double cell rotor in the Spinco Model E analytical ultracentrifuge, which made possible simultaneous determinations and the detection with increased sensitivity of small differences in behavior between the two types.

The sedimentation coefficients obtained for sheep HbCO-A and HbCO-B at nine different pH's are shown in Table 12. For comparison, the values for human carbonmonoxyhemoglobin A determined in previous studies (252, 253) are included. The relationships are more clearly visualized when the sedimentation coefficients are plotted against pH, as shown in Figure 10. Again as a reference, the curve for human carbonmonoxyhemoglobin A is included. Several aspects of these results are of interest in that they suggest further differences between the two sheep hemoglobins. HbB seems to be more easily dissociated than HbA. This might be explained at acid pH by the fact that, as will be shown later, HbB has about four more positive charges than HbA, but HbB dissociates at a somewhat lower alkaline pH as well. Although both sheep types begin to dissociate at a lower alkaline pH than does human hemoglobin A, it appears that if a second dissociation into quarter-molecules, as postulated by Vinograd and Hutchinson (243) for

human hemoglobins, occurs at all, it does not occur as easily as the first step. The curve has a long inflection from pH 10.5 to 11.25.

Table 12

Sedimentation coefficients of sheep carbonmonoxyhemoglobins at various levels of hydrogen ion concentration (1% hemoglobin solutions)

	$s_{20,w}$		
pH	Sheep HbA	Sheep HbB	Human HbA*
4.7	3.4	3.2	3.5
7.1	4.3	4.3	-
10.0	4.0	4.0	4.2
10.25	3.7	3.5	-
10.5	2.9	2.7	3.6
10.75	2.6	2.6	2.7
10.9	2.6	2.5	2.55
11.2	2.6	2.6	2.55
11.25	2.5	2.5	-
11.6	-	-	2.0

*Values taken from Field and O'Brien (252), and Hasserodt and Vinograd (253).

The sheep hemoglobins follow, in a general way, the dissociation curve of human hemoglobin, suggesting that the gross dissociation behavior of sheep hemoglobins is not very different from that of human hemoglobin. Therefore attempts at hybridization under the conditions used for human hemoglobins might have some chance of success. If the two sheep hemoglobins have a relationship similar to that between human hemoglobins A and S, it should be possible to show molecular hybridization by the exchange of a radioactive label between the two types. This possibility was tested in several experiments utilizing chromatographic separation of the hemoglobin

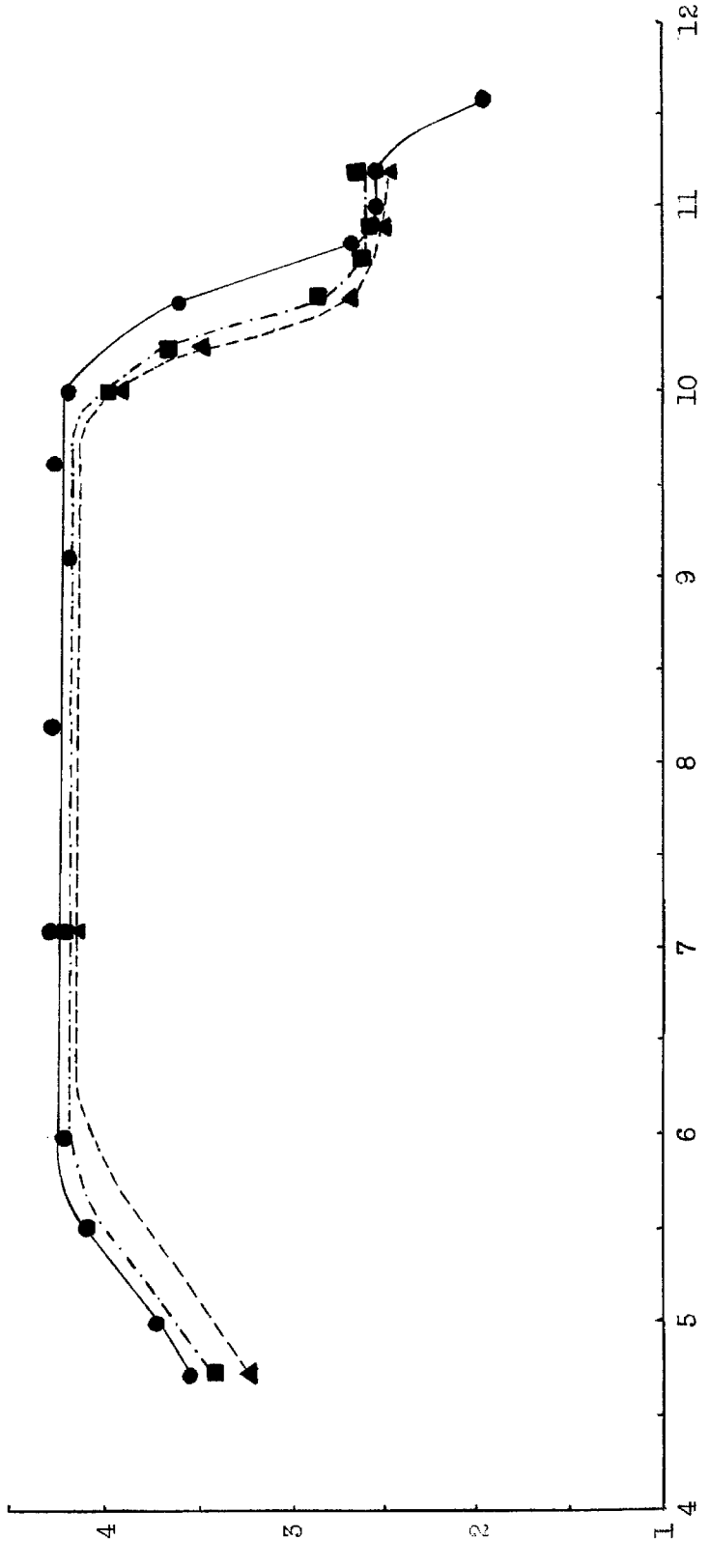

Figure 10. Sedimentation coefficients of sheep HbCO-A and HbCO-B at various levels of hydrogen ion concentration. The curve for human hemoglobin is taken from other studies (See text).
■-·-·-■ Sheep HbCO-A, ▲-----▲ Sheep HbCO-B, ●——● Human HbCO-A.
(All determinations at 1% hemoglobin concentration)

types after treatment.

2. Chromatography of sheep hemoglobins

The chromatographic method described by Allen et al. (263) was used, but the developers described for the human hemoglobins are not satisfactory for sheep hemoglobins. Both hemoglobins move off the columns with little or no retardation on these developers. It is necessary to lower the pH of the developer to pH 6.6 or 6.7 before the sheep hemoglobins can be adsorbed to the resin.

Typical chromatograms of HbA on Developer 6c, and of HbB on Developer 6f are shown in Figures 11 and 12, with the typical patterns of specific activities obtained with ^{14}C-valine labelled material. The variability of the specific activities suggests significant heterogeneity in the lysates. It is known that a number of minor components are present in lysates of human red cells (263), and the same is apparently true for sheep. The fastest minors, which move through the columns without adsorption and elute at the solvent front, constitute as much as 20% of the total sheep hemoglobin in some samples. The presence of these minors and the variability of specific activities necessitated preliminary purification of the major hemoglobins before hybridization in order to eliminate complexities due to reactions of minor components. This was done by chromatographing a number of samples of each type, and retaining for hybridization experiments small fractions contained in two or three tubes collected from the peak of the major component and showing a constant specific activity. The material collected from the columns after preliminary purification is rather dilute, so it was concentrated as described by Vinograd and

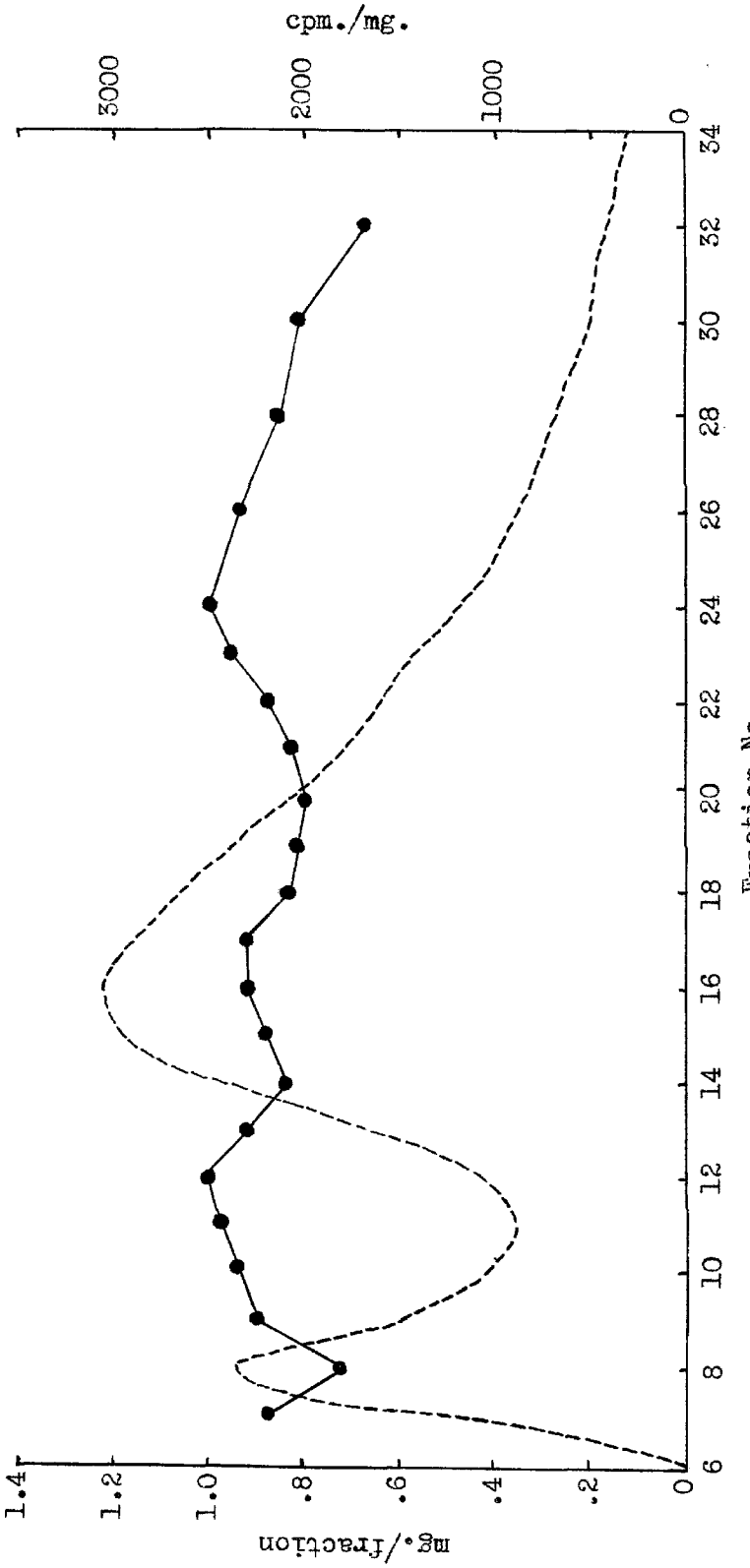

Figure 11. Chromatogram and specific activities of ^{14}C-valine labeled sheep hemoglobin A on IRC-50 with Developer 6c.
------ Concentration, ●——● Specific activity.

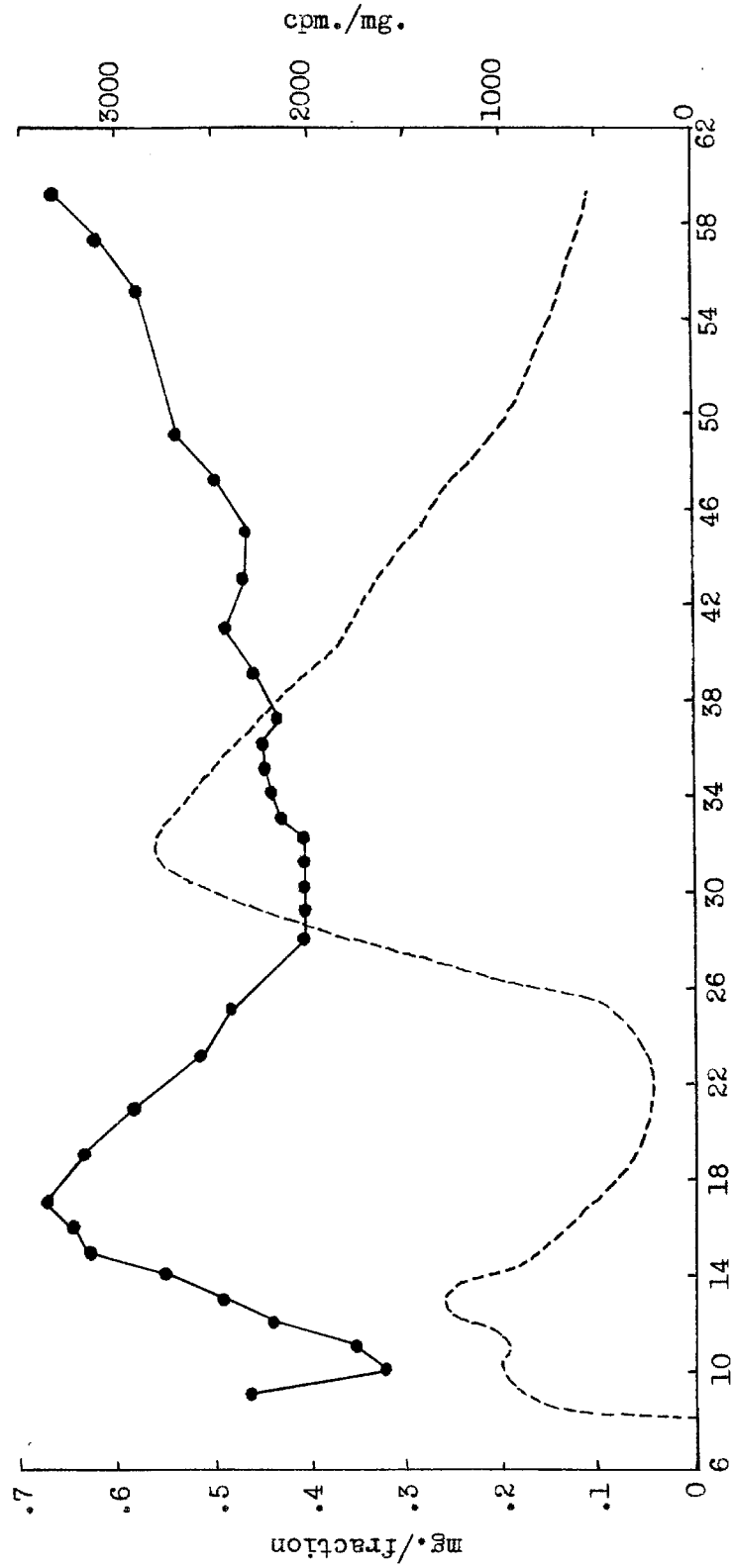

Figure 12. Chromatogram and specific activities of ^{14}C-valine labeled sheep hemoglobin B on IRC-50 with Developer 6f.
------ Concentration, ●——● Specific activity.

Hutchinson (243), by centrifugation in a Spinco Model L preparative ultracentrifuge for 25 hours at 36,000 rpm.

3. Hybridization experiments with radioactive labels

Since the sedimentation coefficients at pH 11.0 indicate essentially complete dissociation into half-molecules, the first experiment was carried out at this pH. If dissociation at this pH is asymmetric, and the two sheep types differ in a single chain, then essentially complete hybridization would be expected, with an exchange of 25% of the radioactivity.

Figure 13 shows the results of an experiment in which purified HbCO-B* was mixed with purified unlabeled HbCO-A. The quantitative results are summarized in Table 13 with those of the other experiments. Due to an error in preparation of developer, the chromatogram of the control may not be directly comparable with the

Table 13

Summary of hybridization experiments with radioactive label

		HbA		HbB		
Experiment	pH	Total cpm.	cpm./ mg.	Total cpm.	cpm./ mg.	% Hybr.
1. Control	11.0	1,900	380	14,400	2,090	
Hybridization	11.0	2,500	230	15,900	1,960	0
2. Control	4.7	1,300	120	13,950	1,860	
Hybridization	4.7	2,450	220	16,000	1,770	20%
3. Control	4.7	24,700	1,820	3,450	230	
Hybridization	4.7	23,300	1,660	4,300	310	26%

experimental, but without the control for comparison it is clear that the specific activity of HbA is significantly less than the 525 cpm./mg. expected from complete dissociation and random

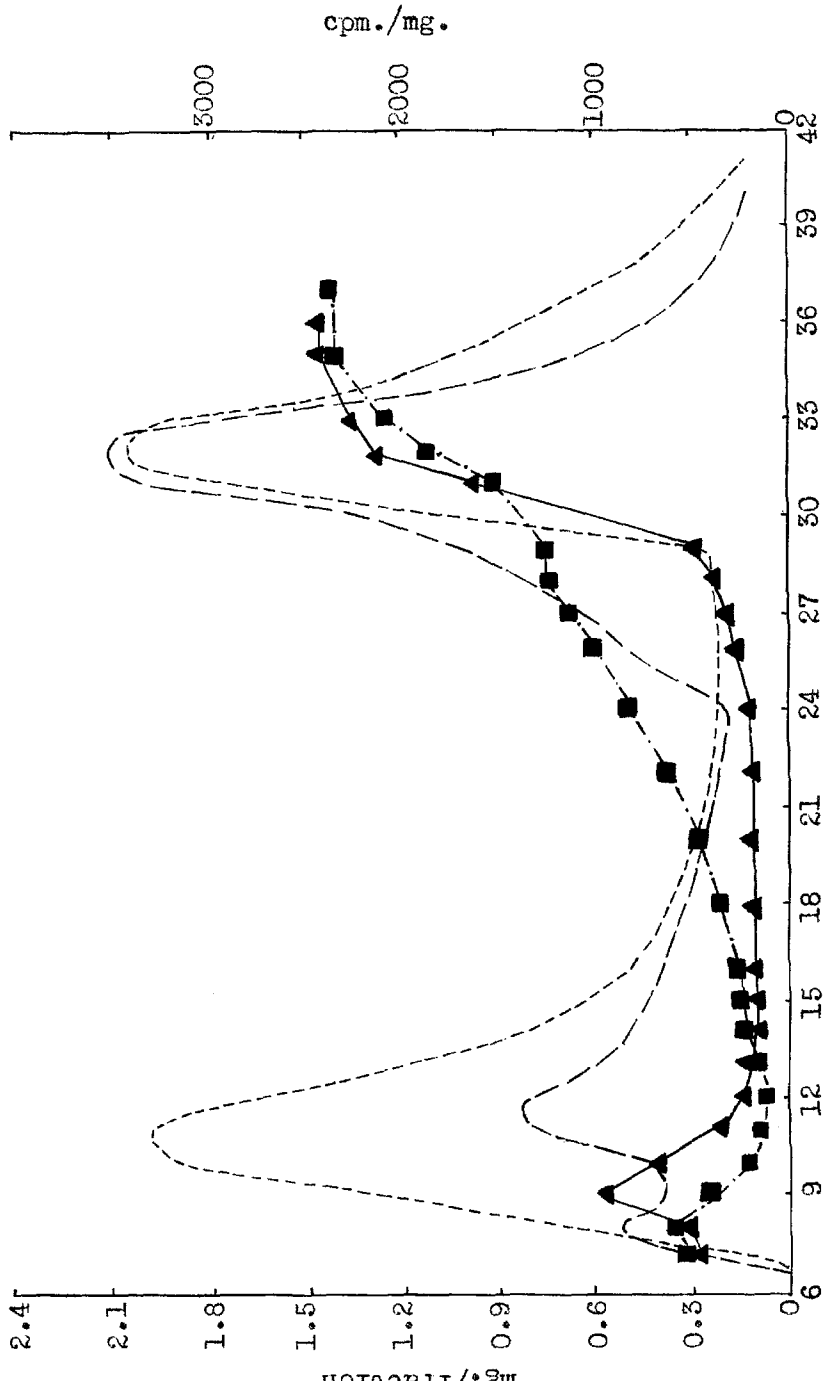

Figure 13. Chromatograms and specific activities of hybridization mixture and control from experiment with HbCO-A and HbCO-B* at pH 11.0.
■----■ Hybridization specific activity, ▲——▲ Control specific activity,
-----Hybridization chromatogram, ———Control chromatogram.

reassociation. The first few tubes always show a high specific activity, probably due to the presence of labeled non-heme protein, so their values are not relevant. At the peak concentration of HbA the specific activities are only about 150 cpm./mg., and the specific activity of the entire HbA fraction is only about 230 cpm./mg., while the control values are significantly higher. In the HbB region, where the chromatograms appear to be more directly comparable, there is no evidence of loss of activity in the amount which would be expected. About 130 cpm./mg. less are found in the hybrid than in the control. This may indicate some exchange, but random exchange has not occurred.

One reason for this lack of exchange may be that dissociation is symmetrical at this pH, with incompatibility between symmetrical halves preventing exchange, as suggested by Vinograd and Hutchinson (243) for human hemoglobins. The other possibility is that dissociation is asymmetric, but with differences in both subunits such that the different pairs of chains are not completely cross-compatible. In experiments with human hemoglobins hybridization was obtained at pH 11. The explanation was that dissociation at this pH went to quarter-molecules, and with sufficient time, even at a low rate of dissociation into quarter-molecules, random exchange could take place. In the present instance, there is no evidence for the operation of such a mechanism. However, at this pH sheep hemoglobin may dissociate into quarter-molecules only very slowly, if at all, as indicated by the long inflection in the dissociation curve relative to that of human hemoglobin A.

Because further experiments at pH 11 seemed unlikely to yield more information about genetic relationships between the two

hemoglobins, the next experiments were carried out at pH 4.7, where hybridization of the human hemoglobins proceeds in a seemingly straightforward way. Figure 14 shows the chromatogram of hybrid and control samples and their specific activities after hybridization at pH 4.7. The samples were again purified before hybridization, and the labeled material was HbB. The relative specific activities suggest that some exchange has taken place, and a quantitative analysis of the results (Table 13) supports this conclusion. With completely random dissociation and reassociation involving a common subunit, an exchange of about 465 cpm./mg. would be expected. In this case only about 100 counts in excess of the control have been gained by the HbA component and only about 90 counts have been lost by the HbB component. The average of the specific activity gained by HbA and that lost by HbB as a percentage of the 465 cpm./mg. predicted on theoretical grounds yields an estimate of 20% hybridization. It thus appears that a significant exchange has occurred, but it fell far short of that expected from complete dissociation and random reassociation.

The consistent presence of material of high specific activity, whether fast minors or non-heme protein, at the solvent front, leads to some ambiguity in interpretation of results such as those from the two previous experiments. Exchange of activity must be detected above the background of the non-specific activity present even in purified samples. A second experiment was carried out using HbCO-A* and unlabeled HbCO-B in an attempt to eliminate these ambiguities. In this experiment, hybridization was attempted with the unpurified heterogeneous lysates, to test the possibility that the low exchange in the previous experiment was due to selection

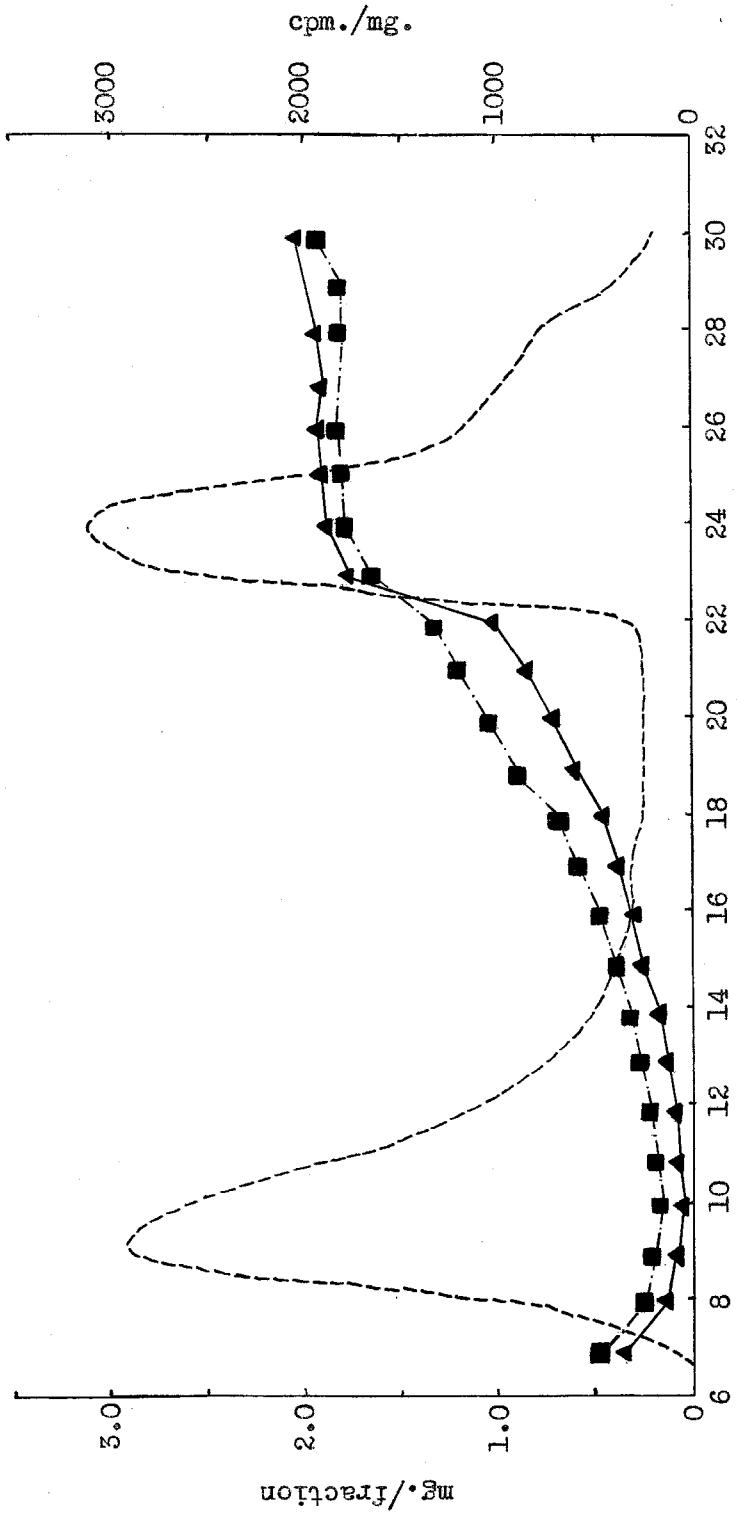

Figure 14. Chromatogram and specific activities of hybridization mixture and control from experiment with HbCO-A and HbCO-B* at pH 4.7.
■---■ Hybridization specific activity, ▲—▲ Control specific activity,
------ Chromatogram of hybridization mixture and control.

of fractions other than the true major components. Since the majority of the activity in this instance would be at the front and in the first major peak, with only a trailing of activity in the B region of the control, an increase in specific activity of HbB in the experimental mixture should be more easily detected.

The results of this experiment are shown in Figure 15, and summarized in Table 13. A decrease in specific activity of HbA of about 160 cpm./mg. and an increase in specific activity of HbB of about 80 cpm./mg. was noted. This result adds materially to the significance of the result in the first hybridization experiment at pH 4.7, because the calculated percent of hybridization this time was 26%. Thus the finding is repeatable; under these conditions hybridization occurs, although complete exchange is somehow prevented.

Three possible explanations may be offered for failure of complete exchange in these experiments. 1) The conditions for hybridization may not be adequate for sheep hemoglobins. Despite the lower sedimentation coefficients at this pH, dissociation may not be sufficiently complete, or other undefined procedural effects may prevent random exchange of subunits which could otherwise take place. 2) The molecule may dissociate as indicated by the sedimentation studies, but into symmetrical halves, rather than the asymmetric subunits which, by analogy with the human hemoglobins, are probably necessary for exchange. (In this connection it should be pointed out that in none of the hybridization studies of human hemoglobins has there been evidence for hybridization between symmetrical halves at either acid or alkaline pH, to yield, for example, $\alpha_2^A \beta^A \beta^S$.) Similarly, no such hybrid types are found in

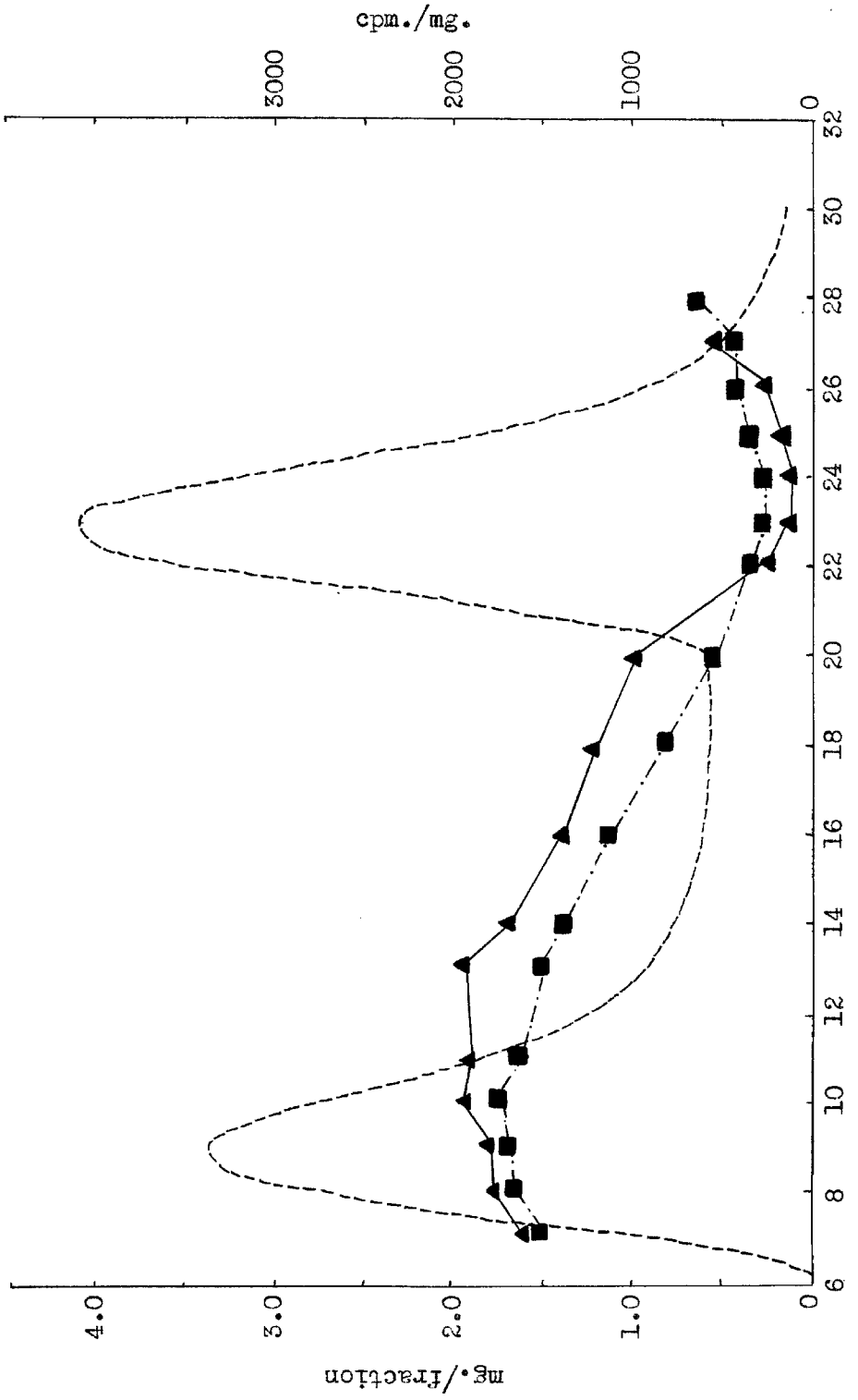

Figure 15. Chromatogram and specific activities of hybridization mixture and control from experiment with HbCO-A* and HbCO-B at pH 4.7.
■-·-■ Hybridization specific activity, ▲——▲ Control specific activity,
------ Chromatogram of hybridization mixture and control.

vivo, even though double heterozygotes do exhibit "hybrid" hemoglobins consisting of a pair of aberrant chains of each type, for example $\alpha_2^{Ho\text{-}2}\beta_2^{S}$ (32). 3) The sheep hemoglobins may differ in both subunits to an extent such that a subunit of one molecular species is only slightly compatible with the complementary subunit of the other, and reassociations favor the complementary subunit of the same species. The first explanation, inadequacy of hybridization conditions, can be tested by a different kind of hybridization experiment which should be independent of the second and third possible difficulties.

4. Hybridization experiments with ferriheme labels

These experiments, designed to test primarily the adequacy of the hybridization procedure, are in principle the same as those described by Singer and Itano (242). They employ the ferriheme group of methemoglobin both as a marker and as a device for altering the electrophoretic mobility of the hemoglobin molecule. MHb and HbCO of the same molecular species were mixed and carried through the hybridization procedure as previously performed, making only those changes necessary because of the unstable nature of the MHb. Since the ferriheme has one more positive charge than the ferroheme, making a total difference per molecule of four charges, the MHb can be easily separated from HbCO by starch gel electrophoresis. If dissociation and random reassociation can occur under these conditions, then a hybrid species which is half MHb should be formed, migrating at a rate midway between the MHb and HbCO species. This hybridization should take place regardless of the direction of the dissociation, i. e., whether the molecule splits symmetrically or

asymmetrically, because the globin chains of the two forms are now identical, and there should be no incompatibility between them.

Five experiments of this type, differing only in time at pH 4.7, gave essentially the same result each time for both HbA and HbB. Controls consisting of MHb and HbCO separately before and after acid treatment, and mixtures of MHb and HbCO after acid treatment and without acid treatment were always included. In each experiment, hybridization between types was also tested, again using the ferriheme as a marker. Thus MHbA with HbCO-B and HbCO-A with MHbB were tested in the same way as the mixtures described above. All four hybridization mixtures and their controls were taken simultaneously through all the steps of the procedure, from acid treatment in the same container through separation on the same starch gels.

The results of two of these experiments after separation by starch gel electrophoresis at pH 6.6 are shown in Figure 16, Appendix I, p. 184. The apparent degree of MHb character was noted for each band before staining. The results show clearly that extensive hybridization has occurred between HbCO-A and MHbA and between HbCO-B and MHbB. The intermediate species in each case seems to be present in quantities near those expected with complete dissociation and random reassociation, and this component appeared to have a half-methemoglobin character. In neither case are similar bands seen in the control mixtures in significant amounts, and none could be detected in the separate MHb or HbCO samples, either before or after acid treatment. It is clear that extensive exchange can occur under the hybridization conditions employed, if the half-molecular subunits are compatible. Thus it seems possible to eliminate the first explanation for incomplete hybridization between HbA

and HbB with radioactive labels, that of inadequacy of hybridization conditions.

The results from mixtures of HbA and HbB (Figure 16) treated concurrently with those just discussed suggest that a significant degree of exchange has occurred here as well. HbCO-B and MHbA have nearly identical mobilities, indicating a charge difference of about four between HbCO-A and HbCO-B on the acid side of the isoelectric point. In control mixtures, HbCO-B and MHbA form a single band; in the hybridization mixture two new bands appear, one faster by about two charges, the other slower by about two charges. In the hybridization mixture of HbCO-A and MHbB, which are separated by about eight charges, two new bands also appear having approximately two and six positive charges more than HbCO-A. It appears that in these experiments at least as much exchange has occurred as in the experiments with radioactive labels, and possibly somewhat more.

As a possible alternative to hybridization, one might postulate oxidation-reduction reactions to explain the appearance of new bands in the four kinds of hybridization mixtures just discussed. If oxidation of sheep HbCO and reduction of sheep MHb were very facile reactions, and the most stable condition of the hemoglobin molecule under these conditions were one in which two of the four heme groups were ferrihemes and two were ferrohemes, then the reaction in the hybridization mixtures might represent an exchange of electrons rather than an exchange of subunits. Itano and Robinson (262) have studied the intermediate species formed by partial oxidation of human carbonmonoxyhemoglobin A, and have shown that this reaction proceeds by steps, with oxidation of one heme at a time at random without influence by the oxidation state of the

other hemes in the molecule. There is no evidence that the half-oxidized molecule is more stable. A partial oxidation experiment was done with sheep hemoglobins A and B to test for possible preferential formation of a half-oxidized form. Hemoglobin solutions at a concentration of 15 mg./ml. were treated for one hour with levels of ferricyanide calculated to provide 1/2, 1/8 and 1/80 the total needed for complete oxidation of the hemes, assuming a molecular weight of 17,000 per heme and one mole of ferricyanide per mole of heme oxidized. The solutions were then dialyzed against phosphate buffer under nitrogen for 36 hours to remove the ferricyanide or ferrocyanide and separated by starch gel electrophoresis as usual. The result is shown in Figure 17, Appendix I, p. 185.

While the different partial oxidation states detected by Itano and Robinson (262) by moving boundary electrophoresis are not so sharply resolved on the starch gel, it is clear from this experiment that there is no preferential half-oxidized state for the sheep hemoglobin molecule at pH 6.6. In the mixture treated with 1/2 the calculated amount of ferricyanide, a significant amount of fully oxidized material, with the same electrophoretic mobility as a fully oxidized control, is found. A smaller amount of intermediate, half-oxidized material is evident. This is true for both HbA and HbB. In the mixtures treated with smaller amounts of ferricyanide the exact nature of the products is more difficult to determine. There are significant amounts of the half-oxidized material, but the broad diffuse boundaries of these bands indicate overlapping with 1/4- and 3/4-oxidized materials. In all cases large amounts of unoxidized HbCO remain, verified by comparison with a control.

While less clean than might be desired, the results of these

separations of partially oxidized hemoglobins by starch gel electrophoresis in no way support the idea that half-oxidized sheep hemoglobin is unusually stable, at least at pH 6.6. It may still be argued that the mechanism may be different at pH 4.7. This cannot be tested in such a way as to distinguish it from hybridization, but several other factors argue against it. First, the greater hybrid formation in mixtures of MHb and HbCO of the same type, as compared with mixtures of the two different types, would require that the MHb forms of HbA and HbB be good oxidizing reagents for HbCO of the same type, but not for HbCO of the other type. There is no apparent basis for such an effect. Furthermore, the earlier experiments using radioactive labels, which can involve no significant oxidation-reduction phenomena, showed similar amounts of exchange between HbA and HbB. Consequently, while it cannot be conclusively disproven, such a mechanism must be considered extremely improbable.

D. Discussion

The two sheep hemoglobins differ by about four charges, and oxidation adds four positive charges to each. Making the single rather well-founded assumption that each hemoglobin consists of two pairs of different chains, only the following model fits the observations from the ferriheme labeling experiments: One pair of chains does not differ significantly in charge between the two hemoglobin types. For convenience this half-molecule will be designated α_2 for both types, but this in no way requires that it be identical in the two types. The consequence of this assumption is that the other half-molecule must differ between the two types by four charges. These two electrophoretically different subunits will be designated β^{A}_{2} and β^{B}_{2}. For further convenience in discussion of the hybridization results, the subunits are assigned arbitrary charges as indicated below. The equations to follow represent the four kinds of hybridization experiments performed using the ferriheme label.

Charge designations of hemoglobin species and dissociation products:*

HbCO-A = $\alpha_2 \beta_2^A = 0$; $\alpha_2 = 0$, $\beta_2^A = 0$

HbCO-B = $\alpha_2 \beta_2^B = +4$; $\alpha_2 = 0$, $\beta_2^B = +4$

MHbA = $\alpha_2^M \beta_2^{AM} = +4$; $\alpha_2^M = +2$, $\beta_2^{AM} = +2$

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*These assignments of charge do not indicate absolute charge, but are relative values consistent with the facts and assumptions discussed above. Likewise, the assigned values for α_2 relative to β_2 subunits have no absolute significance; e. g., for HbCO-A = 0, assignments of $\alpha_2 = 0$, $\beta_2 = 0$ give exactly the same predictions as $\alpha_2 = -2$, $\beta_2 = +2$.

MHbB = $\alpha_2^M \beta_2^{BM} = +8;\ \alpha_2^M = +2,\ \beta_2^{BM} = +6$

Hybridization experiments:

1. HbCO-A + MHbA

$$\underset{(0)}{\alpha_2\beta_2^A} + \underset{(+4)}{\alpha_2^M\beta_2^{AM}} \rightleftharpoons \underset{(0)}{\alpha_2} + \underset{(0)}{\beta_2^A} + \underset{(+2)}{\alpha_2^M} + \underset{(+2)}{\beta_2^{AM}} \rightleftharpoons \underset{(0)}{\alpha_2\beta_2^A} + \underset{(+2)}{\alpha_2\beta_2^{AM}} + \underset{(+2)}{\alpha_2^M\beta_2^A} + \underset{(+4)}{\alpha_2^M\beta_2^{AM}}$$

or

$$\underset{(0)}{\alpha_2\beta_2^A} + \underset{(+4)}{\alpha_2^M\beta_2^{AM}} \rightleftharpoons \underset{(0)}{2\alpha\beta^A} + \underset{(+2)}{2\alpha^M\beta^{AM}} \rightleftharpoons \underset{(0)}{\alpha_2\beta_2^A} + \underset{(+2)}{2\alpha\alpha^M\beta^A\beta^{AM}} + \underset{(+4)}{\alpha_2^M\beta_2^{AM}}$$

2. HbCO-B + MHbB

$$\underset{(+4)}{\alpha_2\beta_2^B} + \underset{(+8)}{\alpha_2^M\beta_2^{BM}} \rightleftharpoons \underset{(0)}{\alpha_2} + \underset{(+4)}{\beta_2^B} + \underset{(+2)}{\alpha_2^M} + \underset{(+4)}{\beta_2^{BM}} \rightleftharpoons \underset{(+4)}{\alpha_2\beta_2^B} + \underset{(+6)}{\alpha_2\beta_2^{BM}} + \underset{(+6)}{\alpha_2^M\beta_2^B} + \underset{(+8)}{\alpha_2^M\beta_2^{BM}}$$

or

$$\underset{(+4)}{\alpha_2\beta_2^B} + \underset{(+8)}{\alpha_2^M\beta_2^{BM}} \rightleftharpoons \underset{(+2)}{2\alpha\beta^B} + \underset{(+4)}{2\alpha^M\beta^{BM}} \rightleftharpoons \underset{(+4)}{\alpha_2\beta_2^B} + \underset{(+6)}{2\alpha\alpha^M\beta^B\beta^{BM}} + \underset{(+8)}{\alpha_2^M\beta_2^{BM}}$$

3. HbCO-A + MHbB

$$\underset{(0)}{\alpha_2\beta_2^A} + \underset{(+8)}{\alpha_2^M\beta_2^{BM}} \rightleftharpoons \underset{(0)}{\alpha_2} + \underset{(0)}{\beta_2^A} + \underset{(+2)}{\alpha_2^M} + \underset{(+6)}{\beta_2^{BM}} \rightleftharpoons \underset{(0)}{\alpha_2\beta_2^A} + \underset{(+6)}{\alpha_2\beta_2^{BM}} + \underset{(+2)}{\alpha_2^M\beta_2^A} + \underset{(+8)}{\alpha_2^M\beta_2^{BM}}$$

4. HbCO-B + MHbA

$$\underset{(+4)}{\alpha_2\beta_2^B} + \underset{(+4)}{\alpha_2^M\beta_2^{AM}} \rightleftharpoons \underset{(0)}{\alpha_2} + \underset{(+4)}{\beta_2^B} + \underset{(+2)}{\alpha_2^M} + \underset{(+2)}{\beta_2^{AM}} \rightleftharpoons \underset{(+4)}{\alpha_2\beta_2^B} + \underset{(+2)}{\alpha_2\beta_2^{AM}} + \underset{(+6)}{\alpha_2^M\beta_2^B} + \underset{(+4)}{\alpha_2^M\beta_2^{AM}}$$

A comparison of the components found in the four kinds of hybridization is shown in Figure 18, Appendix I, p. 185. The species which have been designated as 0, +2, +4, +6 and +8 have the same mobilities in the different mixtures. This supports the charge designations of the +2 and +6 species resulting from the hybridization experiments numbered 3 and 4 above, because these are comparable in mobility with the +2 and +6 species produced in the experiments numbered 1 and 2 above.

The quantities of hybrids formed in the four different hybridization mixtures suggest that hybridizations between MHb and HbCO of the same type are more facile and more complete reactions than those between MHb of one type and HbCO of the other. If dissociation of the sheep hemoglobin molecule at pH 4.7 is principally

symmetrical, exchange between MHb and HbCO of the same type should not be inhibited, but exchange between HbA and HbB might be. Formation of hybrids between types would then be dependent upon a much lower rate of coincident asymmetric dissociation, or upon further dissociation into quarter molecules. In either case the rate of formation of hybrids could be much slower, even if the two hemoglobin types have a completely cross-compatible asymmetric half-molecule. Having reasonably excluded inadequate hybridization conditions as an explanation for incomplete exchange between types, the only apparent alternative is that the two sheep hemoglobin types differ in both subunits, and that the asymmetric half-molecules are only partially cross-compatible. On the basis of these experiments, this explanation seems as good on chemical grounds as that of symmetrical dissociation. However, it entails some genetic complications which should also be considered.

The simplest interpretation of available evidence concerning the chemical nature and the genetic control of the two variant sheep hemoglobins is one derived by complete extrapolation of current concepts of the human hemoglobins. This assumes two genetic loci or cistrons, without specification in this case whether linked or unlinked, each determining one polypeptide chain. At one locus a pair of alleles would determine the difference between HbA and HbB by the substitution in the specified polypeptide chain of a number of different amino acids, resulting in a difference in surface charge of about four at acid pH. The other chain may then be considered the same in both hemoglobin types at least electrophoretically and to the extent that it can associate compatibly with either of the two variant types of chains. In hybridization experiments this "common"

chain would be exchanged between the two types, but it must now be assumed that less exchange occurs than predicted because upon dissociation the molecule splits predominantly into symmetrical half-molecules which are not cross-compatible. This general model fits the observations in the hybridization experiments, and presents no difficulties in genetic interpretation. Such incomplete exchanges are not unprecedented in hybridization studies. They are observed with the human hemoglobins in cases in which the genetic and chemical evidence strongly supports the presence of a common subunit (32, 241-243, 247-248).

If incomplete exchange in these experiments is due to incompatibility between asymmetric halves of the molecule, then present concepts of the genetic determination of protein structure would require that the genetic difference between HbA and HbB involve alternative alleles at each of two loci determining the two different polypeptide chains. If it is further assumed, consistent with the evidence for human hemoglobins, that the two loci are separable, it becomes difficult to understand how such a situation could have escaped detection in the genetic studies of the sheep hemoglobins. If significant incompatibility <u>in vivo</u> as well as <u>in vitro</u> were to exist between the chains in such a situation, then aberrant ratios should have been detected in crosses permitting segregation of these alleles. Certain segregants might die because of inability to form a functional hemoglobin, or particular alleles might not be expressed because of the absence of compatible partners. For example, under these conditions a cross between two double heterozygotes should give a ratio of 5 HbA: 4HbAB: 5HbB, instead of the observed 1:2:1. If segregants with incompatible chains did not die and all

of their alleles were expressed, then they should be detected as different and unpredicted phenotypes, e. g. with separate α_2 and β_2 half-molecules, or perhaps α_4 and β_4, which would probably have electrophoretic mobilities different from HbA or HbB. Neither case is supported by available evidence.

A more reasonable alternative is to assume that the chains are sufficiently compatible in vivo, but less so in vitro, i.e. that the alternatives at one locus are essentially isoalleles, recognizable at present only by the hybridization technique. There would then be four hemoglobin types, two electrophoretically HbA and two HbB, depending upon the nature of the second chain. This would predict that if many hybridization experiments were done with different pairs of sheep, some pairs would give the result obtained in these studies, some the theoretical exchange and some up to 200% of theoretical, the frequencies of such pairs depending upon the gene frequencies of the isoalleles. This seems unlikely and unnecessarily complex, but it is possible. The existence of such a possibility without evidence to refute it serves to demonstrate how little is really known about the genetically determined diversity of sheep hemoglobin in particular and proteins in general, and to emphasize the danger in minimizing the fact that numerous kinds of alterations may occur in protein structure which are not reflected in altered electrophoretic behavior.

Another possibility is that the two loci or cistrons are adjacent. There could then be differences in both cistrons resulting in differences in both chains and partial incompatibility in hybridization experiments, but the trait could still segregate as a single gene effect. It would seem necessary to assume that in such a

situation some mechanism entirely excludes recombination between cistrons, or very strongly selects against recombinants, even in the heterozygous condition. Without such a mechanism, equilibrium would become established between linkages in coupling and repulsion phases, as with any other linked loci in a random mating population. The same difficulties would then arise as with unlinked loci.

A final possibility to be considered is that only a single locus, controlling a single polypeptide chain, is involved in determination of specificity of the sheep hemoglobins. This could occur if the four chains of each hemoglobin type were identical, e. g. HbA = α_4, HbB = β_4. As previously noted, two kinds of N-terminal amino acids have been found in approximately equal quantities, suggesting two kinds of chains. However, if the animals tested were heterozygous, the N-terminal valines could conceivably have come from one type and the N-terminal methionines from the other. It seems unlikely that this would happen in two independent studies, and the finding of the same N-terminals in other members of the Bovidae (256-258) makes it still more unlikely. Furthermore, if the four chains were identical in each type, then hybridization should give rise to a new intermediate species detectable either chromatographically or electrophoretically. No such material was found. This possibility is therefore discounted.

These hybridization studies have yielded observations which should be useful in future studies of the sheep hemoglobins, and have raised some interesting questions. One rather firm conclusion may be drawn. Most, if not all, of the electrophoretic difference between the two hemoglobins resides in one of the two polypeptide chains, while the other chain is electrophoretically similar, if

not identical, in both types. This is presumptive evidence that only one chain is involved in the difference, and only one locus or cistron involved in its determination. However, a firm conclusion in this regard must await a detailed chemical analysis of the globins, and perhaps additional genetic studies. It seems unlikely that the hybridization technique can contribute much more at this time to the solution of the problem of genetic determination of sheep hemoglobin types.

Some other applications of the hybridization technique to the sheep hemoglobins are indicated. Particularly in view of a report that sheep fetal hemoglobin dissociates into four subunits upon dilution (264), it would be of interest to hybridize sheep adult and fetal hemoglobins, as Jones et al. (5) have done with the human types. The close taxonomic relationship of sheep with cattle and goats, and the identity and apparent uniqueness of their N-terminal methionine, should encourage attempts to hybridize hemoglobins among these related species as a means of defining their phylogenetic relationships, particularly in the case of cattle which also have two electrophoretically variant types (46-50).

E. Summary

1. The hybridization behavior of the two electrophoretically variant sheep hemoglobins, which have significantly different amino acid compositions, has been studied to obtain further information on the nature of the structural differences between them.

2. Sedimentation velocity experiments show significant decreases in sedimentation coefficients of both types at pH 4.7 and above pH 10.0, indicating dissociation of the molecules into subunits. The two hemoglobin types display slightly different dissociation behaviors.

3. Hybridization experiments employing hemoglobin labeled either with ^{14}C-valine or by oxidation to methemoglobin yield exchanges between variant types to the extent of 20-25% of values predicted on theoretical grounds. The adequacy of hybridization conditions has been verified by an experiment in which methemoglobin and carbonmonoxyhemoglobin forms of the same variant species were hybridized.

4. The principal electrophoretic difference between the two types, amounting to approximately four additional positive charges in HbB at acid pH, has been shown to reside in only one of the two subunits. The two types do not differ detectably in electrophoretic mobility of the second polypeptide chain.

5. The incomplete hybridization probably results from either an inadequate degree of asymmetric dissociation or a partial incompatibility between asymmetric half-molecules due to differences in both chains.

6. Consideration of the genetic implications of these two

interpretations suggests that the former is more consistent with genetic evidence from studies of both sheep and man, but the latter is also possible if certain additional assumptions are made.

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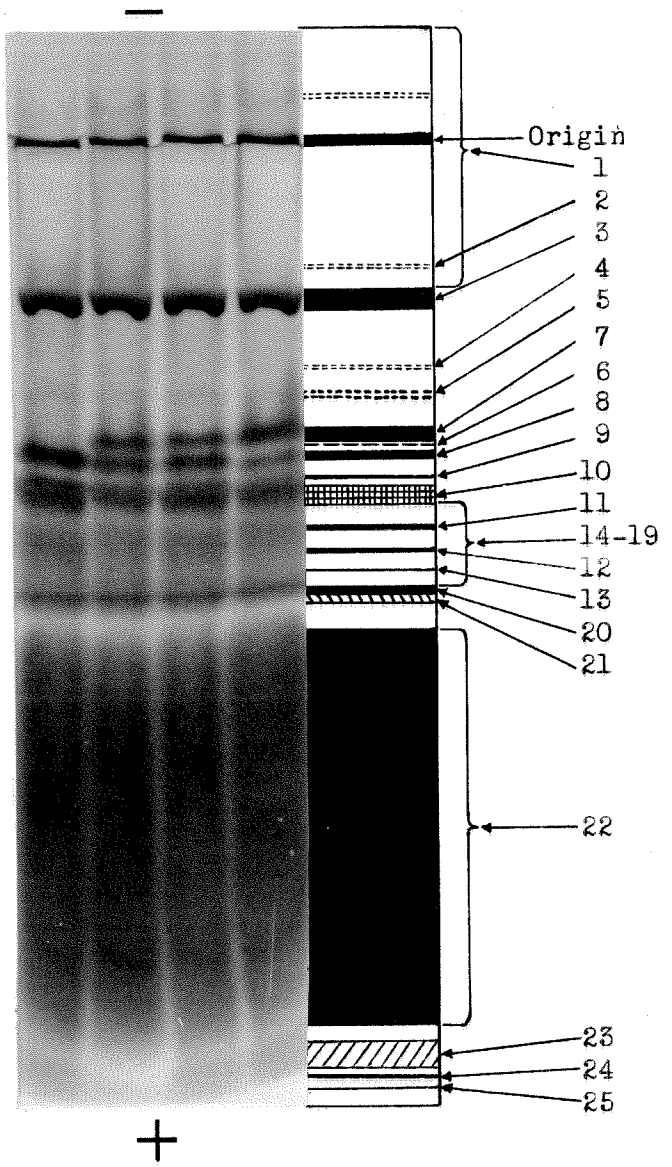
IV. APPENDIX I, FIGURES



1. Gamma-globulins
2. F
3. S α_2-macroglobulin
4. Haptoglobin
5. Haptoglobin
6. Esterase
7. Transferrin
8. Transferrin
9. Transferrin
10. Hemoglobin-haptoglobin
11. E
12. D
13. C
14. Esterase
15. Ceruloplasmin
16. Aminopeptidase
17. Alkaline phosphatase
18. Cholinesterase
19. Hemoglobin-haptoglobin
20. F α_2-globulin
21. Hemoglobin-haptoglobin
22. Albumin
23. Prealbumin
    (Esterase, Cytochrome oxidase)
24. Prealbumin-B
25. Prealbumin-A
    (Esterase, Cytochrome oxidase)

Figure 1. Typical starch gel electrophoretic pattern of adult mouse serum.

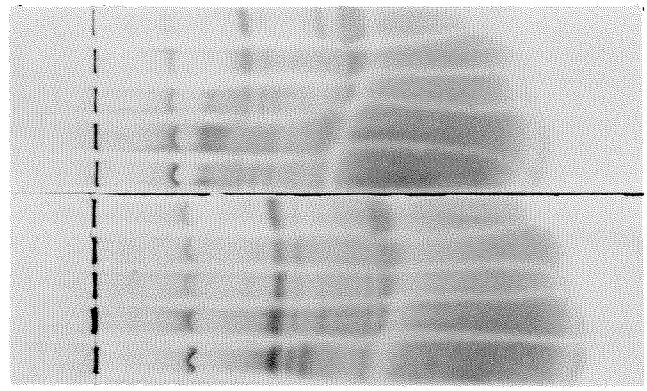


Figure 2. Changes in serum electrophoretic patterns during postnatal development. Top to bottom: B/6 -- 18-day fetus, 4 days of age, 11 days, 16 days, 25 days; CBA -- 18-day fetus, 1 day of age, 3 days, 8 days, 20 days. All samples were pooled or individual males, except fetuses, which were not sexed.

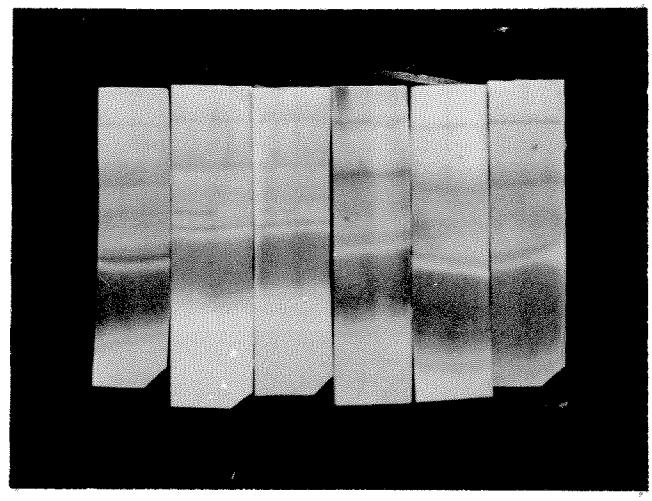


Figure 3. A sex difference in serum patterns during postnatal development. Each strip has an individual B/6 female on the left, an individual B/6 male on the right. Samples were taken at ages 7, 8, 9, 10, 11 and 13 days from left to right. Note the striking difference at 8 and 9 days.

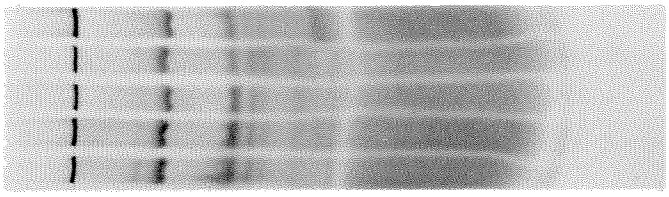


Figure 4. Electrophoretic patterns of B/6 females during and following pregnancy. Top to bottom: non-pregnant female, 10 days pregnant, 18 days pregnant, at parturition, 3 days post-partum. (The intense band behind the Fα_2-globulin in the top sample is an artifact frequently observed after prolonged storage of the serum.)

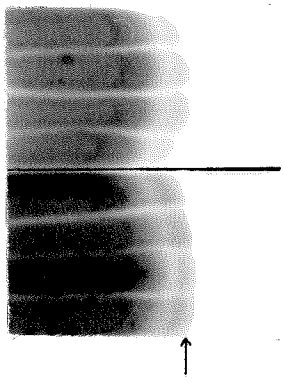
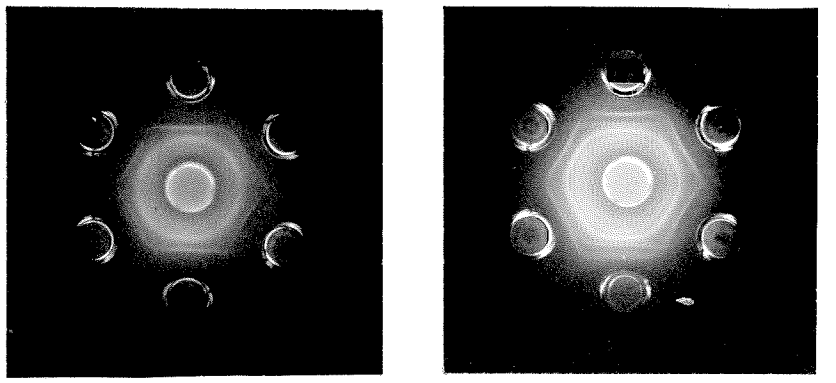
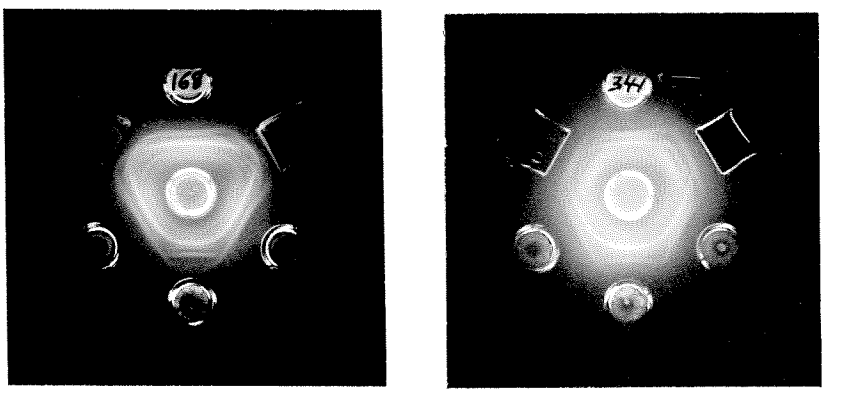


Figure 5. Prealbumin phenotypes. Shown are samples from eight CBA x B/6 second generation males. Phenotypes from top to bottom are: 1). Pre-ao, 2). Pre-a, 3). Pre-ao, 4). Pre-o, 5). Pre-ao, 6). Pre-o, 7). Pre-a, 8). Pre-a. The Pre-a component is indicated by the arrow at the bottom of the gel.



(a) (b)

Figure 6. Immunodiffusion phenotypes. Center wells in both plates contain undiluted AMG. Samples in outer wells are mouse sera diluted $\frac{1}{4}$. Clockwise, starting at top, these are: (a). B/6, (B/6 x CBA)F_1, CBA, B/6, (B/6 x CBA)F_1, CBA; (b). D/2, (D/2 x CBA)F_1, CBA, D/2, (D/2 x CBA)F_1, CBA. The band of interest swings close to the outer wells containing CBA samples, and is in intermediate positions opposite other samples.



(a) (b)

Figure 7. Comparative immunoelectrophoresis. Center wells contain AMG. Clockwise from top the samples are: (a). B/6 serum at $\frac{1}{4}$, starch gel section from anode side of origin with B/6 serum, B/6 serum at $\frac{1}{4}$, CBA serum at $\frac{1}{4}$, B/6 serum at $\frac{1}{4}$, starch gel section from anode side of origin with CBA serum; (b). B/6 serum at $\frac{1}{4}$, agar gel section from α_1-globulin region with B/6 serum, B/6 serum at $\frac{1}{4}$, CBA serum at $\frac{1}{4}$, B/6 serum at $\frac{1}{4}$, agar gel section from α_1-globulin region with CBA serum. The Ss band is identified by the characteristic reaction with CBA serum in the bottom wells.

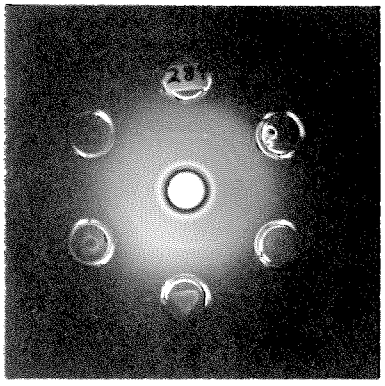


Figure 8. Inhibition analysis. The agar in this plate contains CBA serum at a concentration of 1/32. The center well contains AMG. Clockwise from top, test sera (diluted to $\frac{1}{4}$) are: A/He, B/6, CBA, D/2, C, Flx. Note slight bending of precipitin band at wells containing CBA and Flx (Ss-1) sera.

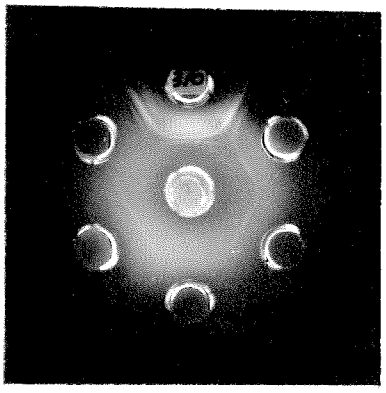
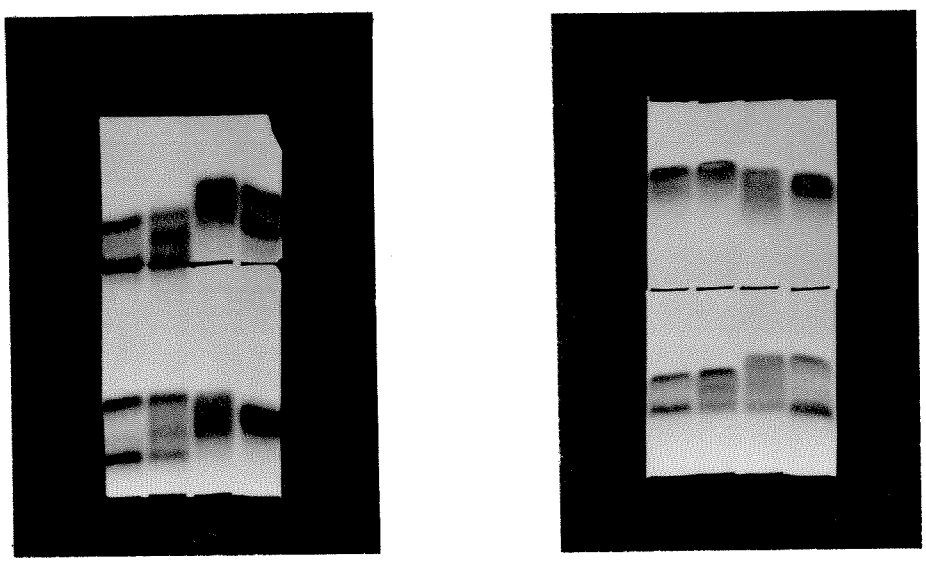


Figure 9. Analysis of AMG preabsorbed with 1/3 volume CBA serum. Preabsorbed AMG is in center well. Test samples clockwise from top are: unabsorbed AMG, A/He serum at $\frac{1}{4}$, B/6 serum at $\frac{1}{4}$, CBA serum at $\frac{1}{2}$, C serum at $\frac{1}{4}$, Flx serum at $\frac{1}{4}$. Note that single band between center well and A/He well is continuous with Ss band formed between A/He well and unabsorbed AMG at top. Note also reaction of excess mouse serum antigens in center well with unabsorbed AMG at top.



(a) (b)

Figure 16. Starch gel electrophoresis results of hybridizations between carbonmonoxy- and methemoglobins. C = control mixture, H = hybridization mixture. Samples left to right are: (a) Top, (HbCO-A + MHbA) C, (HbCO-A + MHbA) H, (HbCO-B + MHbB) H, (HbCO-B + MHbB) C; Bottom, (HbCO-A + MHbB) C, (HbCO-A + MHbB) H, (HbCO-B + MHbA) H, (HbCO-B + MHbA) C. (b) Top, (HbCO-B + MHbB) C, (HbCO-B + MHbB) H, (HbCO-B + MHbA) H, (HbCO-B + MHbA) C; Bottom, (HbCO-A + MHbA) C, (HbCO-A + MHbA) H, (HbCO-A + MHbB) H, (HbCO-A + MHbB) C.

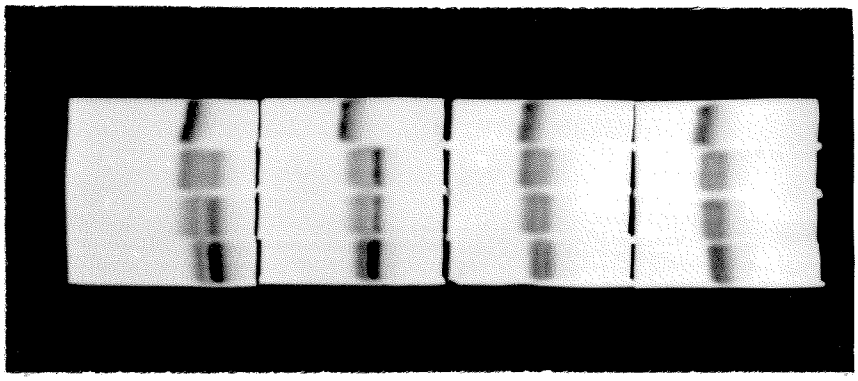


Figure 17. Starch gel electrophoresis of partially oxidized sheep hemoglobins. From top to bottom starting at the left, the hemoglobin types and the levels of ferricyanide added as a fraction of the amount calculated to give complete oxidation are: HbA - excess, 1/2, 1/8, untreated; HbA - excess, 1/8, 1/80, untreated; HbB - excess, 1/2, 1/8, untreated; HbB - excess, 1/8, 1/80, untreated.

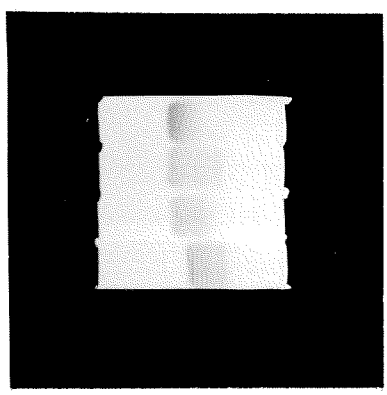


Figure 18. Comparison of the products of the four hybridization combinations. Top to bottom: (HbCO-B + MHbB), (HbCO-A + MHbB), (HbCO-B + MHbA), (HbCO-A + MHbA). Compare also adjacent hybridization mixtures in Figure 16.

V. APPENDIX II, MANUSCRIPT SUBMITTED TO *GENETICAL RESEARCH* FOR PUBLICATION AS A SHORT NOTE

A Revised Nomenclature for the Mouse Transferrin Locus

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In independent investigations, reported almost simultaneously (Cohen, 1960; Shreffler, 1960), the authors have described a protein variant system in the serum of mice, involving an electrophoretic difference in the iron-binding beta-globulin component, transferrin, and have shown the difference to be genetically determined by a pair of co-dominant alleles at a single locus. Both reports introduced the same gene symbol, Trf, for this locus, but in one, letter designations were used (Cohen, 1960), making the allele symbols Trf^{A} and Trf^{B}, and the three phenotypes TrfA, TrfAB and TrfB, while in the other, number designations were used (Shreffler, 1960), with the corresponding symbols Trf^{1}, Trf^{2}, Trf-1, Trf-1.2 and Trf-2. To avoid confusion in future references to this locus due to the two differing systems of nomenclature, the authors have agreed upon the nomenclature proposed in this communication.

Well-established precedents for both letter and number systems in designating haemoglobin and serum protein phenotypes and the alleles controlling them may be found in work with this and other species. In the only pertinent protein variant system in the mouse, the haemoglobin system, number designations have been used (Russell & Gerald, 1958). With all of the well-studied transferrin variant systems in other species, letter designations have been used (for reference, see Cohen, 1960; Shreffler, 1960). In order to be consistent with the established nomenclature for

transferrin polymorphisms in other species, and with a view to simplifying the possible addition of new alleles, we have decided to use letter designations for the mouse transferrin variants.

Furthermore, because of the nature of the system; the lack of any criterion more compelling than frequency among inbred lines for assigning a type allele; and the possibility of additional alleles, it seems advisable to base the nomenclature upon the rules for multiple allelic systems in the mouse. These rules, recently set forth by the Committee on Standardized Genetic Nomenclature for Mice (1959) state that, "To differentiate members of an allelic series letters, numbers, or letters and numbers may be used as superscripts. Where letters are used they should be lower case and placed before the numbers." Therefore the revised allelic designations that we now propose for the transferrin locus have lower case superscripts, and in accordance with this convention, the designations for phenotypes are also written in lower case. The proposed symbols are thus $\underline{Trf}^{a}$ and $\underline{Trf}^{b}$ for the two alleles, and Trf-a, Trf-ab and Trf-b for the three known phenotypes. The two previous and the newly proposed designations are summarized and compared in Table I. In addition to use as phenotypic symbols, the symbols Trf-a and Trf-b are valuable as designations for the two differing proteins themselves. The convention introduced by Shreffler (1960), in designating the minor transferrin components seems useful also. Thus the minor components previously designated as Trf-1' and Trf-2', would now become Trf-a' and Trf-b'. The still faster and fainter minor components detected by Cohen (1960) would be Trf-a" and Trf-b".

Table I

Summary of revised and former nomenclature systems

Designations	Revised	Cohen (1960)	Shreffler (1960)
(i) of Alleles			
In CBA lines	$\underline{Trf}^{a}$	$\underline{Trf}^{A}$	$\underline{Trf}^{1}$
In other lines	$\underline{Trf}^{b}$	$\underline{Trf}^{B}$	$\underline{Trf}^{2}$
(ii) of Genotypes and the corresponding Phenotypes			
$\underline{Trf}^{a}/\underline{Trf}^{a}$	Trf-a	TrfA	Trf-1
$\underline{Trf}^{a}/\underline{Trf}^{b}$	Trf-ab	TrfAB	Trf-1•2
$\underline{Trf}^{b}/\underline{Trf}^{b}$	Trf-b	TrfB	Trf-2

If new electrophoretic types of transferrins are found, these should be designated in order of discovery. If the same letter should happen to be assigned to two different types, these might be distinguished by prefacing the duplicated letter with another, designating, for example, the laboratory which reported it. Thus, $\underline{Trf}^{hc}$ and $\underline{Trf}^{jc}$, Trf-hc and Trf-jc could be used for two different hypothetical variants found at Harwell and Jackson laboratories, and both initially designated Trf-c. If variant types should be recognized upon other criteria than electrophoretic mobility, these may be simply handled by adding number subscripts for the new phenotypes to the letter designating the electrophoretic mobility of the protein, and number suffixes to the allelic superscript. For example, if Trf-b should be found divisible into two groups on the basis of antigenic differences, the appropriate phenotypic symbols should be Trf-b_1 and Trf-b_2, and the allelic symbols, $\underline{Trf}^{b1}$ and $\underline{Trf}^{b2}$. Such usage is in

accordance with the rules previously cited, and has been the basis for our choice of letter symbols for this system.

For the information of those who may wish to use the transferrin system, we have listed in Table II the lines which we have screened to date, and the alleles found in them.

Table II

Inbred lines and sublines classified for transferrin type

Line	Allele	Line	Allele
A/Jax	b	A/Fa	b
A/HeJax	b	A/Lab	b
AKR/Jax	b	A/2GLab	b
BALB/cJax	b	AKR/Lab	b
CBA/Jax	a	BALB/cLab	b
C3H/ ? *	b	C3H/HeLab	b
C57BL/6Jax	b	CBA/Fa	a
C57BL/10Gn-lu	b	CBA/Lab	a
C57Br/cd/Jax	b	C57BL/Fa	b
DBA/1Jax	b	C57BL/Lab	b
DBA/2Jax	b	C57BL/6Lab	b
RF/Jax	b	C57BR/cdLab	b
WB/Re	b	C57L/Lab	b
WC/Re	b	CE/Lab	b
		KL/Fa	b
		RIII/Fa	b
		Ju/Fa	b

*Commercially obtained, subline not known.

References

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RUSSELL, E.S. & GERALD, P.S. (1958). Inherited electrophoretic haemoglobin patterns among 20 inbred strains of mice. Science, 128, 1569-1570.

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