STUDIES ON HEMOGLOBINS AND THE ERYTHROCYTE MEMBRANE IN RELATION TO INFECTIOUS PARASITEMIA

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CHAPTER I

INTRODUCTION

Hemoglobins are the major constituent of the red blood cell and have a role in transporting oxygen to tissues of the animal body. Each hemoglobin molecule consists of four polypeptide chains of which two pairs of peptides are normally identical; for example, in hemoglobin A there are 2α and 2β chains plus a heme group for each polypeptide chain (1). Abnormalities within hemoglobin molecules are due to amino acid substitutions in one type of polypeptide chain. These changes can cause anemia. A well known example is sickle cell anemia in which valine is substituted for a glutamic acid residue in each β-chain of human hemoglobin A (2,3). This amino acid substitution causes aggregation of sickle cell hemoblobins (Hb S), a decrease of Hb S solubility and, finally, failure to transport oxygen.

It was found that individuals having Hb S and newborn infants have a greater resistance to malaria with reference to normal adults. Sickle cell and fetal hemoglobins are believed to be the cause of this resistance to malaria (4). There is a possibility that passive immunity transferred to the newborn infant from an immune mother may be a factor in the resistance of newborn infant to malaria (5). There has been no direct evidence concerning the presence of malaria antibiodies and human serum proteins have a half life of only about 10 days (6).

More than fifty years ago, the small punctiform body which appeared

in the erythrocytes of cattle suffering from an acute infectious anemia was named Anaplasma marginale (7). Anaplasmosis is a disease resulting from infection with A. marginale organism. The anemia caused by this blood parasite results in decrease of milk production and growth rate of cattle and often death. The economic loss due to this disease in United States and other countries is severe. Vaccines have been developed for this disease, but are not completely effective.

In constrast to adult cattle which are quite susceptible to anaplasmosis, newborn calves are resistant to this disease (8,9). The nature of this resistance is unknown and needs to be clarified to completely understand the mechanism of reproduction and life cycle of the organism. The bovine reticuloendothelial system is not fully developed, nor functional, at birth (10,11). It has been suggested that the erythrocyte environment plays a significant role in the host-parasite equilibrium in anaplasmosis (12). The transfer of passive immunity to the calf from an immune dam does not fully explain the resistance of calf to anaplasmosis (8,13). The presence of fetal hemoglobin in the young may also play a role in the resistance to anaplasmosis. There is a report that hemoglobin types of cattle may play a role in resistance to trypanosomiasis (14). Allison (15) stated that hemoglobin types are obviously most likely to affect parasites of red cells such as Plasmodium, Babesia, Theileria, and anaplasmosis. In babesiosis, newborn puppies are highly susceptible while young calves are relatively resistant to the infection (16). As newborn animals are usually resistant to this type of parasitemia and have fetal hemoglobins, the presence of absence of fetal hemoglobin in newborn puppies needs to be established. The possibility that fetal hemoglobin in newborn calves is the reason

for neonatal resistance to anaplasmosis is the primary objective of this study.

This dissertation will report the results of research on variation of fetal and adult hemoglobins in young calves during maturation, hemorrhage, exchange transfusion, and A. marginale infection. Hemoglobin types of animals, absence of fetal hemoglobin in newborn puppy, which is quite susceptible to Babesia, and erythrocyte membrane protein difference among the calf, cow and A. marginale infected cow also will be reported.

CHAPTER II

LITERATURE REVIEW

Erythrocyte

The erythrocytes in circulating blood are mature cells of the erythrocyte series. These mature cells are nonnucleated biconcave discs that stain a pink or pinkish gray color with Wright's stain. The bovine erythrocytes have a mean diameter of about 5.5 microns and the majority range from 5.0 to 7.0 microns. Mean values of erythrocyte number, packed cell volume (PCV), and hemoglobin concentration of adult cattle blood are 7.0 million per mm^3 whole blood, 35.0 per cent, and 11.0 gm % respectively (17).

Characteristics of erythrocytes such as function, energy metabolism, composition, formation and life span will be reviewed. The major function of erythrocytes is to carry oxygen from the lungs to all cells of the body and carbon dioxide to the lung from these cells. Hemoglobin makes up 34% of the wet weight of the erythrocyte while stroma makes 2 to 5% (18). The stroma can function in limited metabolism such as glucose transport. Details on membrane structure and function will be reviewed in the membrane section.

Energy production in erythrocytes is primarily by the hexose monophosphate pathway (aerobic) and the Embden-Meyerhoff glycolytic pathway (anaerobic). In the presence of oxygen most of the cells of the body use the effective aerobic breakdown of glucose to CO_2 and H_2O as a

source of energy. Thus, transport of glucose into the red blood cell is necessary. Murphy (19) reported that 89% of the glucose is utilized via the anaerobic glycolytic pathway. The hexose monophosphate pathway yields 25% of the potential energy of the cell in the form of NADPH. This energy is used in glutathione reduction and in protecting the cell against various oxidant compounds. The energy produced from the Embden-Meyerhoff pathway in the form of ATP is used primarily to preserve cellular integrity by maintaining the concentration of chemical gradients across the membrane (20).

Among many other enzymes in erythrocytes, the role of catalase, glutathione peroxidase, acetylcholinesterase, carbonic anhydrase, glucose-6-phosphate dehydrogenase and methemoglobin reductase are rather well described (21).

Erythropoiesis occurs in bone marrow. Miesher (22) has suggested that erythropoiesis is controlled by the oxygen tension in the bone marrow and this is supported by other research (23). This support includes the facts: (a) that polycythemia occurs in the bone marrow, and hypoxemia (lowered O_2 in the blood) induces erythropoiesis, e.g., living at high altitudes, and, conversely, (b) that erythropoiesis may be depressed at high levels of oxygen intake in (i) hemolytic anemia, (ii) pernicious anemia, and even (iii) normal subjects. Many other reports support the modern view that the influence of oxygen tension is indirect and is mediated via a chemical or humoral factor called erythropoietin (23).

The maturation stages of erythrocytes are rubriblast, prorubricyte, rubricyte, metarubricyte, reticulocyte, and then the mature red cell. In the maturation process the nucleus and cytoplasm become smaller and

nucleus moves out from the cell prior to reticulocyte step. Prorubriblast and rubricyte can function in mitotic division and can synthesize RNA. Hemoglobin begins to appear at the stage of rubricyte (24).

The life span of human erythrocytes is 120 days (25). The red cell life span of 3 months old calves has been found to be 54 days by means of radioactive iron (26). Carbon^{14} studies have revealed 160 days life span of matured cow red cell (27). Brown and Eadie (28) reported red cell life span of dog as 110 days as determined by means of radioactive iron.

General Structure and Function of Hemoglobin

Mammalian hemoglobin consists of four heme prosthetic groups combined with four polypeptide chains and each polypeptide chain is composed of helical and nonhelical segments similar to whale myoglobin. The heme is a ferrous iron porphyrin and is common to all types of hemoglobin and myoglobin (29,30). Hemoglobin is an allosteric protein with oxygen as its substrate and modifier (31). The phylogenetic differences in adult hemoglobins and the differences between embryonic, fetal, and adult forms within one species are all the result of changes in the amino acid composition of the globin moiety (32). The center of the hemoglobin molecule is occupied by a cavity filled with water. Of the 4 different contacts between subunits, the ones between unlike subunits are chiefly nonpolar while those between like subunits, if present, can only be polar (30). Polar residues are excluded from the interior of the α and β subunits. Larger nonpolar side-chains generally lie either in the interior or in surface crevices of the subunits or at the boundaries between like subunits. Nonpolar side-chains protruding into the

surrounding water are rare. As in myoglobin, the hemes lie in non-polar pockets of the globin chains. Excluding the covalent bond between iron and histidine, there are about sixty possible interactions between atoms of the globin chains coming to within 4 A of atoms of the hemes; most are nonpolar (30).

The ability of the ferrous iron in hemoglobin to combine reversibly with molecular oxygen is due to its non-polar surroundings. The cooperative effects (nonpolar) depend on the structural changes undergone by the hemoglobin molecule on combination with ligands. Dissociation of hemoglobin molecule into dimers occur symmetrically, so that $(\alpha\beta)_2$ splits into $2\alpha\beta$, and the tetramer probably breaks at the contacts $\alpha_1\beta_2$ and $\alpha_2\beta_1$ (33).

$$\alpha_2\beta_2 \underset{\text{PCMB}}{\overset{\text{pH 6.0}}{\rightleftharpoons}} 2\alpha\beta \underset{\text{PCMB}}{\overset{\text{pH 6.0}}{\rightleftharpoons}} 2\alpha + 2\beta$$

Dissociation is favored by high concentrations of an electrolyte, such as NaCl, which are known to weaken polar interactions and to strengthen nonpolar bonds. The result of increasing the neutral electrolyte concentration would therefore be expected to weaken the polar contacts between like subunits and to strengthen the predominantly non-polar bonds between unlike subunits (30). Dissociation of the component chains of human and animal hemoglobins has been achieved by use of 6M urea during separation and isolation of hemoglobin peptide chains with IRC-50 column chromatography and/or electrophoresis (34,35).

The α and β-chains of mammalian hemoglobins each have a pattern of sites which are invariably occupied by the same residues. These include nearly all heme contacts and most contacts between subunits. It has

been suggested that mutations at these sites would be likely to affect respiratory function (36). The function of hemoglobin and myoglobin is such that they do not take part in the metabolic oxidation processes of the body directly. Myoglobin is an oxygen store house and hemoglobin is the carrier which brings along the oxygen for this metabolism and helps to remove the CO_2 which has been formed (37).

Hemoglobin Heterogenity of Man and Animal

The screening of human blood samples in many parts of the world has led to the discovery of nearly a hundred different mutant hemoglobins (38,39). In cattle, three normal hemoglobin types are known, which are Hb A, B, and F (39,40,42). Presence of two fetal hemoglobins in newborn water buffalo was reported (43). The presence of an embryonic hemoglobin in Fleckvich breed has been reported (44). Hemoglobin B homozygotes are found only in Jersey, Guernsey, South Devon, Zebu (White Fulani) and Zebu cattle. Hemoglobin AB heterozygotes are found in Jersey, Guernsey, South Devon, Brown Swiss, Brittany Shorthorn, Santa Gertrudis, Flamande, Limousine, Charolais, Parthenaise, Salers and Aubrac, Garronaise, and Tarentaise cattle. Hemoglobin A homozygotes are found in Ayrshire, Holstein, Hereford, Wesh Black, Friesian, Shorthorn, Aberdeen Angus, North Devon, Red Poll, Sussex, Dexter, and Galloway and all the cattle breeds mentioned above except Brittany Shorthorn (14,40, 45). There was no report on the hemoglobin type of Scottish Highland cattle in United States. A third type, Hb C, has been found in Brahman and offsprings from Brahman and Hereford (45). Efremov and Braend (41) reported a cattle hemoglobin variant, Hb D, in Muturu cattle.

In sheep, hemoglobin type of A, B, C, D and F are known. Of these

Hb C is abnormal (46,47,48,49). It is known that there is one adult canine hemoglobin (50). LeCrone (51) reported fetal hemoglobin absence in newborn Beagle dogs on the basis of results obtained by agar-gel electrophoresis, alkali denaturation, blood smears and column chromatography. In the horse, the presence of two electrophoretically distinct hemoglobins have been reported (52,53) but they are not named at this time. Kilmartin (54) reported a horse hemoglobin variant. The presence of an equine fetal hemoglobin has not been reported.

Composition and Amino Acid Sequences of Bovine Hemoglobins

Bovine hemoglobin A and B are presumably the products of allelic genes (40,52). The composition of each bovine hemoglobin is as follows: $\alpha_2\beta_2^A$ for Hb A, $\alpha_2\beta_2^B$ for Hb B, and $\alpha_2\gamma_2$ for Hb F. Amino acid sequences of bovine hemoglobin chains, α, β^A, β^B and γ, had been determined by Schroeder and his collaborators (55,56,57). The amino acid sequences of α chain is same for Hb A, B and F while β^A, β^B, and γ differ. Epstein and Motulsky (58) stated that the producer genes for α and β chains of human hemoglobin are not linked. This may be true for the bovine also.

Hemoglobin Synthesis and Gene Control

The synthesis of hemoglobin is under gene control, heme and globin groups are synthesized separately and joined together to form the complete hemoglobin molecule. There are some reports that the heme group may coordinate as well as stimulate the synthesis and assembly of the hemoglobin molecule (59,60). The formation of the heme group occurs as shown in the following scheme (61):

Acetate $\xrightarrow{\text{TCA cycle}}$ Succinyl CoA $\xrightarrow{\text{Glycine}}$ α-Amino-β-ketoadipic acid $\xrightarrow{-CO_2}$ δ-Amino levulinic acid $\xrightarrow{\text{2 molecules condense}}$ Porphobilinogen $\xrightarrow{\text{4 molecules condense}}$ Uroporphyrinigen III $\xrightarrow{-4\ CO_2}$ Coproporphyrinogen III $\xrightarrow{-2\ CO_2,\ -4\ H}$ Protoporphyrinogen IX $\xrightarrow{-6\ H}$ Protoporphyrin IX $\xrightarrow{Fe^{++}}$ Heme

Globin is synthesized via the well studied protein biosynthetic pathway. Hemoglobin molecules begin to appear in the red blood cell at the stage of the rubricyte, during erythrocyte maturation in bone marrow (24). The rate of globin synthesis in human reticulocytes has been reported by Karpatkin (62). After two hours incubation with C^{14}-leucine, reticulocytes from individuals having sickle cell anemia, blood loss anemia and sickle cell thalassemia, synthesized 2.84, 0.509 and 0.298 μ moles x 10^{-13} globin per reticulocyte respectively.

Since the amino acid sequences of α, β, γ, and δ subunits of the human hemoglobin types are structurally different, it has been postulated that separate genes control the synthesis of each polypeptide chain (63). The synthesis of Hb A ($\alpha_2 \beta_2^A$) would be under the control of two different genetic loci. Each of these genes determine the primary structure of a specific polypeptide chain. Inheritance of an abnormal gene of the α type resulted in all possible combinations with non-α chains, therefore, it was proposed that the genetic loci controlling the synthesis of α and β chains are not closely linked on the same chromosome. It may be possible that loci are either some distance apart on the same chromosome or even on different chromosomes (64). In contrast to this postulate, Pearson and Moore (65) suggested a close linkage between the β and the δ types. During early stages of devel-

opment the synthesis of embryonic ϵ chain is succeeded by that of the γ chain of fetal hemoglobin. After birth the γ chain is soon almost completely replaced by the β chain for human and bovine hemoglobins and also the δ chain for human hemoglobin (44,66). The production of the δ chain during adult life occurs at the rather fixed rate of about 1/40 of that of the β chain. It seems, therefore, that genetic mechanisms control the rates of chain synthesis. In 1955, Edington and Lehman (63) described the hereditary persistance of Hb F (PHF). The anomaly, as it is expressed in the heterozygous Negro carriers, is characterized by (1) the absence of any severe hematological and clinical manifestation, (2) the presence of 20 to 30% of Hb F during adult life, (3) a decreased level of Hb A and (4) an approximately equal distribution of Hb F in the red blood cells. In combination with other chain abnormalities, there are Hb S-PHF, Hb C-PHF, β-thalassemia-PHF, α-thalassemia-PHF, and δ-thalassemia-PHF. In occasional patients with acquired hemotologic disorders, e.g., aplastic anemia or leukemia, the synthesis of γ chain is apparently resumed, and some erythrocytes containing Hb F appear in the circulation (67). Adults in whom Hb F synthesis persists or is renewed are frequently anemic. It is probable that the anemic person has the benefit of the presence of Hb F which has a higher oxygen binding capacity than Hb A. There are reports that erythrocytes with greater proportions of Hb F have longer survival times (68). Studies of patients heterozygous for both Hb S and PHF suggested that Hb F, in proportions of 25% uniformly distributed in all erythrocytes, protects against sickling and hemolysis (68). The switching mechanism of γ to β chain synthesis and the control mechanism of γ chain synthesis persistance are not known. One may

apply the theory of Jacob and Monod (69), Britten and Davidson (79), or Tomkins et al. (71), on the regulation of γ and β chain synthesis. With the Jacob and Monod theory, it is probable that the structural gene for the γ chain is under the control of a linked operator gene. This gene combines with repressor RNA which is synthesized by a separately located regulator gene and the combination of the operator with the repressor RNA prevents the operator from activating the structural gene. When the controller gene, by virtue of an assumed mutational change, fails to become activated, the synthesis of the γ chain can be continued. According to the Britten and Davidson theory (79), one can postulate the switching mechanism or persistance of Hb F as follows: If one or more factors (signals) are produced by cell maturation, anemic, or genetic abnormality condition, the factor(s) can bind to the sensor gene which causes transmission of a signal to the integrator gene, activator RNA, receptor gene, and producer gene, all in sequence. The producer gene produces β or γ peptide chains. With loss or absence of the initial factor(s), no peptide synthesis would be observed. Applying the theory of Tomkins et al. (71), if one or more factors are produced by cell maturation, anemia or a genetic abnormality condition as above, the factor(s) may bind to the repressor protein which is the product of regulator gene and bind the m-RNA produced by the structural genes. The inactivation and removal of the repressor protein allows synthesis of β or γ chain depending on the case. Absence or loss of the factors somehow causes inactivation of the peptide synthesis.

As only a few hemoglobin types are observed out of all possible polypeptide chain combination, e.g., α_4, β_4, $\alpha_2\beta_2$, $\alpha_2\delta_2$, δ_4, $\alpha_3\beta_1$, etc., there must be a control mechanism(s) for polypeptide chain

assembly. The details of these mechanisms are not known. Baglioni and Compana (72) proposed that the α chains, released from polyribosomes upon completion, become associated with a β chain attached to polyribosomes before the synthesis is complete, whereafter both are released as an $\alpha\beta$ dimer. In vitro experiments suggest that from a mixture of different dimers various types of hemoglobin tetramers can be formed. All are in rapid reversible equilibrium with their dimeric subunits (73).

There are more than a hundred different mutant hemoglobins in man (38,39). These mutant hemoglobins have amino acid substitutions in their polypeptide chain(s) which resulted from mutation of nucleotide base sequence of respective structural gene. Amino acid substitution near to the heme group or contacting points between peptide chains causes pathologic conditions.

Physical and Chemical Differences Between Adult and Fetal Hemoglobins

Differences of physical and chemical characteristics of fetal and adult hemoglobins of man and animals have been investigated by a number of workers. Mammalian hemoglobins have a molecular weight of 65,000 and each of the four polypeptide chains consists of about 140 amino acids. There is no appreciable difference in size among these hemoglobins (74). Fetal hemoglobins differ from adult hemoglobins in their rate of denaturation by alkali (75,76), electrophoretic mobility (41,49,77,78), amino acid composition (55,56,57,79), oxygen dissociation curves (80,81, 82), solubility (83,84,85), crystallography (86,87,88,89), immunological specificity (90,91), monomolecular films (92), ultraviolet absorption spectrum (77), and oxidation to methemoglobin (93,94). Some of the

comparisons were made with bovine hemoglobins, others with human, sheep and goat hemoglobins. The primary sequence of γ chain of bovine Hb F differs from the β chain of bovine Hb A by 23 amino acids, and the α chain is the same for both (55,56,57).

It has long been known that human fetal blood shows a higher oxygen affinity than the maternal blood (95). The difference of oxygen affinity between fetal blood and maternal blood was attributed to the presence of fetal hemoglobin in the fetal blood (82). However, Allen et al. (96) suggested that some environmental factors, rather than the hemoglobin molecule themselves, play a determinate role in the human fetal-maternal difference in their oxygen equilibrium.

Hemoglobin Types in a Single Cell

Application of immunological methods have determined the presence of Hb S and Hb A, Hb A and Hb F in a single human red blood cell (97). The same situation may occur in the bovine red cell for Hb A and Hb F. Bangham (40) reported codominance of bovine Hb A and Hb B based on the observation on mating between Hb A and Hb B type cattle, which produced offsprings with 1Hb A:2Hb AB:1Hb B ratio. As codominance refers to active allelic genes in DNA and the DNA's in every single cell of one animal are the same, it is probable that Hb A and Hb B are present in the same cell.

Malaria in Carriers of the Sickle-Cell Trait and in Newborn Children

The growth of malaria parasites in cells containing different hemoglobin types has attracted a great deal of attention. The subject is of interest for two main reasons: it supplies information on the behavior

and metabolism of the parasite and its relationship to host cells; further, it is the key to a remarkable case of natural selection in man, that affecting the sickle-cell gene in many parts of the world.

When highly susceptible subjects are considered, the available evidence indicates that sickle-cell trait carriers are as easily infected with P. falciparum, and show parasites as frequently, as subjects without the trait. However, groups of trait carriers consistently have lower parasite count than groups of subjects without the trait. It was concluded that in trait carriers the infection continues for the normal length of time but the presence of sickle-cell hemoglobin limits the multiplication of tropozoites so that the degree of parasitemia remains low (4). It was found some years ago (98,99,100) that the malarial parasites were capable of injesting the host cell cytoplasm by phagotrophy. These findings were subsequently confirmed by numerous investigators (101,102). In this manner the large hemoglobin moiety can be incorporated into the cytoplasm of the plasmodium. Moulder (103) stated that hemoglobin S has a solubility only about one-fiftieth that of the normal hemoglobins and the great increase in the viscosity of the cytoplasm of host red cells containing hemoglobin S could easily interfere with the phagotrophy of host material by the parasites. Thus, individuals with hemoglobin S in their red blood cells are more resistant to malaria. Such studies have been limited by the inability to culture such hemotropic parasites in vitro.

Allison (104) pointed out that there was an apparent correlation between the increase in concentration of normal adult hemoglobin and the malaria infection rate in newborn Luo childred. Gilles (105) also reported a striking correlation of this type in Gambian infants. The

two changes may, of course, be simultaneous but independent; nevertheless the possibility remains that cells containing fetal hemoglobin may provide an unsuitable medium for the development of human plasmodia. If fetal cells even partially suppress the multiplication of the parasites any passively acquired immunity from the mother would have a better chance of success. On this hypothesis, the replacement by adult cells after the third month would provide an internal environment more in keeping with the metabolic requirements of the parasite. It seems that the hypothesis could be most adequately tested by an in vitro culture technique in which the growth of plasmodia in fetal and adult cells could be compared (4). At this time, it is questionable the plasmodium could be cultured at the level for multiplication.

Trypanosomiasis and Bovine Hemoglobin

The possibility that other blood parasites might be influenced by inherited hemoglobin types in erythrocytes remains to be studied. In 1958, Bangham and Blumberg (14) reported that bovine hemoglobin B is absent from the Muturu and N'Dama breeds of Nigerian cattle which are more resistant to trypanosomiasis than Zebu cattle in which the frequency of hemoglobin B is relatively high.

Lehmann (106) found a much higher incidence of AB hemoglobin heterozygotes in Indian Gir cattle than would be expected from the random mating hypothesis. Therefore, it is thought that the heterozygous condition must be at a considerable advantage, and the advantage may be related to blood parasites.

Fetal Hemoglobin Change in Newborn Infant and Calf

Persistence of fetal hemoglobin in the erythrocytes of normal children had been studied by Chernoff and Singer (107). They reported that Hb F is from 55-85% in the newborn infant while it is less than 1% in the normal adult. Other researchers have reviewed fetal hemoglobin disintegration and their findings have been summarized (66,76,108).

Grimes et al. (42) studied fetal hemoglobin persistence in newborn dairy calves by paper electrophoresis and subsequent densitometric scanning. At birth, Hb F composed from 41 to 100% of the total hemoglobin. The amount of Hb F diminished rapidly and was replaced entirely by Hb A in 14 bull calves and 4 heifer calves at the average ages of 65 to 97 days respectively. They also stated that the disappearance of hemoglobin F from the blood of 5 calves (Guernsey, Jersey, and Brown Swiss) was obscured by the simultaneous appearance of Hb B, which had the same electrophoretic mobility as hemoglobin F on paper at pH 9.0. Proof for the presence of Hb B was obtained by an electrophoresis below pH 7.4.

In 1964, Muta (109) examined the changes in bovine fetal hemoglobin as a function of the age of the calf over the period of 6 hours to 35 days. He detected two major components (each about 30% of the total) and five minor components on cellulose-acetate, agar-gel, and CM-cellulose media from newborn calf blood. The major and minor components decreased with increasing calf age. The presence of two major hemoglobin components does not coincide with the results of Huisman et al. (110), Muller (111), and Grimes et al. (42) who observed only a single component. Muta's observations need to be reexamined and clarified.

Schmid and Thein (44) studied bovine embryo hemoglobin of the Fleckvich breed of Russia. In bovine embryos up to a crown/rump length of 1 cm, the blood contains only embryonic hemoglobin (Hb E) which can be differentiated from Hb A and Hb B by starch gel electrophoresis. In embryos between 1 and 2 cm in the length; both Hb E and Hb F were demonstrated. In two embryos with length of 5.5 and 13.5 cm and in a newborn calf, a hitherto unknown hemoglobin band in the region of Hb B was found which was considered to be a special form of Hb F. This hemoglobin band was named Hb F-Munich and persisted in that calf until 8 days after birth.

Hemoglobin F disintegration in a Black-brindled breed was studied using paper electrophoresis in veronal buffer (112). Fetal hemoglobin concentration was 75.5% at birth, 41.3% at 1 month, 2.9% at 3 months and none was detected after 110 days of age.

Ranjekan and Barnabas (43) studied Hb F disintegration in the water buffalo. There are two fetal hemoglobins in water buffalo referred to as Hb F_1 and Hb F_2. Hemoglobin F_1 is the major fetal hemoglobin and F_2 is the minor. These have identical γ chains and differ in α chain. Ranjekan and Barnabas (43) reported that a γ chain containing hemoglobin persisted in calf blood for more than 28 weeks of age.

Methods of Hemoglobin Identification and Quantitation

Electrophoresis

Electrophoresis, probably the oldest form of migratory analysis, is certainly the most gentle one towards the substances it separates. Zone electrophoresis in a supporting medium has developed to such a state that the theory of electrophoresis can no longer afford to remain centered

around the free, frontal type. In zone electrophoresis, proteins are separated according to molecular weight and charge in an electrical field. In 1949, Pauling (113) separated human Hemoglobin S from Hemoglobin A by electrophoresis. Since Tiselius' pioneering work on paper electrophoresis, this technique was widely used for protein separation (114). Starch (115) and polyacrylamide (116) gels owe much of their separating ability in protein electrophoresis to molecular sieve effects. The above two gel-electrophoretic methods serve as excellent qualitative identification of proteins, including hemoglobins. Both methods are also used for preparative purposes by direct elution of "squashed" segments of the strip (111,115,117).

Agar gel electrophoresis has also been applied to the separation of major and minor hemoglobins with various buffer systems (118,119). This technique separates hemoglobins but more zone spreading is obtained than with starch gel, polyacrylamide gel or cellulose acetate membrane.

Electrophoresis of hemoglobin on cellulose acetate membranes was first described by Kohn (120), who observed good separation of the major hemoglobin components in 2 hours but failed to resolve and demonstrate a minor component, human hemoglobin A_2. Graham and Grunbaum (121) reported a separation of minor hemoglobin components on cellulose acetate membrane in 1 to $1\frac{1}{2}$ hours using a discontinuous buffer system of Tris-EDTA-borate, pH 9.1, at the anode and barbital buffer, pH 8.6, at the cathode. They also reported that cleared cellulose acetate membrane, after hemoglobin electrophoresis, gives a quantitative determination of each hemoglobin component by scanning the density of protein bands with a recording densitometer (Beckman Analytrol).

Various buffer systems have been used for hemoglobin electrophore-

sis; phosphate, veronal, Tris-citric acid, sodium hydroxide-boric acid, Tris-glycine, Tris-HCl, Tris-EDTA-borate and others. Among these, Tris-EDTA-borate system at pH 8.6 has been reported as the buffer giving better resolution than any others (122).

Column Chromatography

The work of Boardman and Partridge (123) on separation of pairs of animal carboxyhemoglobins on Amberlite IRC-50 cationic exchange resin was extended by Prins and Huisman (124,125) to human hemoglobins. Hemoglobin F could be separated by elution in the first fractions at variable concentrations of sodium ions, or the hemoglobins separated as zones in a flat cuvette could subsequently be photographed as they are chromoproteins. Then a densitometric tracing was prepared from the negative (124). Since two horse hemoglobins were separated by ion exchange chromatography on carboxymethyl cellulose (128), a method developed by Peterson and Sober (127), Diethylaminoethyl (DEAE) cellulose was used as a chromatographic medium for the separation of human and animal hemoglobins with a gradient of dilute phosphate buffer (128,129). An improved separation was achieved in DEAE-sephadex column using Tris-HCl buffers of higher molarities and a single pH gradient (129).

Isoelectric Focusing

Isoelectric focusing is an analytical method developed by Svensson (130), in which ampholytes, such as proteins and peptides, can be focused at their respective isoelectric points in a pH gradient influenced by an electric field. Electrofocusing, isoelectric fractionation, isoelectric separation, stationary electrolysis, isoelectric condensa-

tion, and isoelectric analysis are synonyms.

In 1954, Kolin (131) prevented convective disturbances in a liquid column by using a density gradient consisting of a sucrose solution with gradually decreasing sucrose concentration from the bottom to the top of the column. Seven years later, Svensson (130) derived the theoretical foundation of isoelectric focusing.

Finally, in 1966, Vesterberg and Svennson (132) developed a method of synthesizing a series of ampholites, polyaminopolycarboxylic compounds, whereby they became suitable for producing a natural, equilibrated pH gradient.

Two main applications of isoelectric focusing in biochemistry are: first, the analytical separation of high molecular weight ampholytes, specifically proteins, according to their isoelectric points; second, characterization of proteins by their isoelectric points. The precision and reproducibility of the determination is about 0.01 pH unit, at least in the pH region of 5-8 (133).

Clear focusing of various hemoglobins according to their isoelectric points have been demonstrated (133).

Alkali Denaturation

In 1866, Körber found that the hemoglobin of fetal and cord blood is more alkali resistant than placental hemoglobin. After Körber's finding, an alkali denaturation method was used for qualitative and quantitative tests for human fetal hemoglobin in a mixture of other forms of hemoglobin. When a solution of oxyhemoglobin is made highly alkaline, the hemoglobin molecules are converted to a denatured form designated by different investigators as denatured globin hemichrome,

globin parahematin, and denatured globin ferrihemochromogen (134). The denaturation process is accompanied by change in color and by loss of solubility at a neutral pH. However, this technique does not distinguish the normal and abnormal adult hemoglobins from each other (135).

The differences in the resistance of various hemoglobins against alkali denaturation are referred to in different degrees of complementation of a nonpolar "anti-heme" pattern in the globin surface; higher resistance to alkali indicating a higher degree of spatial adjustment of globin to the rigid planar prophin structure of the heme molecule. The denaturation reaction is first order with respect to hemoglobin concentration (136). Human hemoglobin F is more alkali resistant than any other human adult hemoglobin and this higher resistance to alkali has been attributed to the γ-chains of Hb F (70). The alkali denaturation rate of human hemoglobin A is about 100-fold faster than Hb F while the reverse situation is observed with adult and fetal bovine hemoglobins (75,76). Quantitatively, the two bovine hemoglobins differ in rate of denaturation by about a factor of two (75).

Peptide Mapping

After Pauling's (113) first demonstration of electrophoretic separation of hemoglobin A and S, Ingram (3) in England first tried peptide mapping of human hemoglobin A and S and found that one of glutamic acid residues of β-chain of hemoglobin A was substituted by valine in hemoglobin S. In peptide mapping, free sulfhydryl groups are generally blocked first by aminoethylation or performic acid treatment. Then the protein is digested by protease at 37^{o} C and pH 8-9 yielding peptides (137,138). Trypsin is the most popular enzyme for this purpose because

of its high specificity in hydrolyzing peptide bonds, specifically the carbonyl end of arginine and lysine.

Peptides obtained are subjected to electrophoresis in a volatile buffer, then ascending or descending chromatography in a second dimension is done using a volatile solvent. The resultant chromatogram is dipped in acetone-ninhydrin mixture to develop color at 80^{o} C (138).

This technique has been used widely to identify hemoglobin variants and other proteins. Peptides on chromatogram may be eluted with 0.01 N-ammonia solution to determine amino acid sequence and composition (138).

Hybridization and Chain Separation

Hemoglobin hybridization is based on dissociation of hemoglobin polypeptide chains and recombination of these polypeptide chains. A mixture of two hemoglobins is exposed to pH 4.7 for 72 hours to dissociate them into their subunits. After adjustment of the solution to a slight alkaline pH, the polypeptide chains associate to form hemoglobin molecules and this recombination is a random process which can give all possible combinations of polypeptide chains. These can be detected by electrophoresis (139). Jones et al. (140) showed the appearance of Hb A in the hybridization of Hb F_2 with Hb H:

$$\underset{\text{Hb F}}{3\alpha_2\gamma_2} + \underset{\text{Hb H}}{3\beta_4^A} \longrightarrow \underset{\text{Hb A}}{2\alpha_2\beta_2^A} + \underset{\text{Hb F}}{\alpha_2\gamma_2} + 4\beta_2^A + 2\gamma_2$$

This hybridization indicates that Hb F_2 has 2 α subunits identical to those of Hb A.

The technique of hybridization has been used to identify hemoglobin

variants. Huehns and Shooter (139) hybridized Hb A_2 with Hb G_{Br} and found that Hb G_{Br} has a mutation in the α-chain.

Individual polypeptide chains of hemoglobin molecules were separated by various techniques to determine which polypeptide chain had different amino acid substitution (30,34,35).

Hemorrhage and Hemoglobin Type Change

Sheep

Two allelic adult hemoglobins, A and B, as well as fetal hemoglobin are found in sheep (46,47). When sheep carrying the gene for Hb A were made anemic by hemorrhage or by parasitism, Haemonchus contortus, the concentration of Hb A declined and another hemoglobin appeared (141). Blunt and Evans (142) suggested that this might be a new hemoglobin and van Vliet and Huisman (48) called it hemoglobin C. When blood loss ceased Hb C gradually disappeared, and Hb A returned to its former level. When sheep with both Hb A and B were bled, only the concentration of Hb A declined on the appearance of Hb C. Sheep with Hb B only, produced no Hb C with hemorrhage (48,143). Results of structural analysis of Hb A, B, C and F indicated that the non-α chains of these hemoglobin types were different, although their α-chains were similar if not identical. The structure of the β-chain of Hb C was distinctly different from that of the γ-chain of Hb F (144). Blunt (145) reported that Hb C denatures faster than the normal Hb A with alkali and is present almost entirely in reticulocyte. Beale et al. (144) stated that Hb C is present in normal sheep with Hb A or AB as a minor component, and that Hb C synthesis in anemic sheep is due to an increase in rate of synthesis rather than to the switching of a new gene. The functional attributes of Hb C is

not known.

Goats

A phenomenon similar to that occurring in sheep has been observed in the goat by Huisman and his collaborators (146). During severe anemia from blood loss, the β-chain of the hemoglobin of the adult goat (β-A) is replaced by another type of β-chain (β-C). Since two adult hemoglobin types (A and B) differing in their α-chains may be present in the adult goat, both hemoglobin types apparently disappear during anemia and are replaced by two new hemoglobin types. The β-C chain of the goat differs from the β-A chain of the goat by a minimal number of 18 amino acid residues and from the β-C chain of the sheep by perhaps only one amino acid.

Cattle

Bell and Huisman (147) studied hemoglobin types in a mature cow, heterozygous for the Hb A and B (Guernsey), during severe blood loss anemia. No new hemoglobin component was detected, and the relative amount of Hb A and B remained unaltered. They also reported that Hb A is 36-45% (mean, 41.5%) and Hb B is 55-64% (mean, 58.5%) in Guernsey cattle with Hb A and B types as determined by DEAE-cellulose chromatography.

Deer

During the severe anemia due to hemorrhage of a deer with two distinct hemoglobin types, Hb-I and Hb-II, the animal failed to demonstrate any change either in hemoglobin type or in the proportions of hemo-

globins already present (148). Sickling of the erythrocytes, which were observed by supravital stains, was absent during the period of anemia.

Erythrocyte Enzyme Activity Differences Between Young and Old Animal

The influence of age on the activities of erythrocytic enzymes of the glycolytic and hexose monophosphate pathways as well as the activities of glutathione reductase and glutamate-oxalacetate transaminase were determined (149). The comparison was done using less than 48 hours old calves and cattle over 6 years of age. A significant decrease in enzyme activities with increasing age were found for all enzyme activities except glutamate-oxalacetate transaminase. The change in enzyme activity may be due to isozymes, enzyme concentration or cessation of production of unique fetal enzymes.

Anaplasmosis

Anaplasmosis is a disease resulting from infection of red blood cells by a blood parasite, Anaplasma marginale. This blood parasite was first observed more than 50 years ago and causes severe economic loss to the cattle industry of the United States and other countries. An anemia is caused by the infection and is accompanied by weakness, fever, normal urine, constipation, weight loss, decreased red cell count and hemoglobin level (150,151). Death occurs in many cases but recovery is not infrequent, the recovered animals probably remaining lifelong carriers of the infection (152). Even though there is massive red cell loss due to the infection, no hemoglobinuria is observed in anaplasmosis (8). Adult cattle are easily infected by the A. marginale organism, and as many as 70% of the red cells are destroyed in less than 10 days. Calves

infected at birth develop a prolonged parasitemia of low intensity unless splenectomized prior to the exposure (89). Normally the infected red cells are rapidly removed by reticuloendothelial (RES) system of spleen (153). Summers (154) reported that there is a marked increase of phagocytic cells of RES during the acute phase of the disease. Brock (155) observed no A. marginale in reticulocytes, basophilic cells or cells with basophilic stippling. The incubation period after inoculation varies from 15 to 45 days in cattle. When the tick transmits the A. marginale body, the incubation period may be prolonged up to 3 months (156).

Four other forms of anaplasma organism have been observed: A. centrale, A. ovis, Paranaplasma caudata, and Paranaplasma discoides (157). Recognition of the 2 latter species has yet to be confirmed.

Ultra-Structure and Classification of A. Marginale

The Anaplasma marginale body is a round form, 0.3 to 1.0 micron in diameter, dense, homogeneous, and bluish-purple in color. It contains two to eight subunits known as initial bodies (158). The initial body is a round or oval structure, 300 to 400 nanometers in diameter and is enclosed in a double membrane. Electron microscopy shows a separation of A. marginale from the cytoplasm of the red cell by a limiting or matrix membrane (159,160). The initial structure of these bodies consist of dense aggregates of fine granular material embedded in an electron-lucid plasma. Initial bodies are surrounded by an electron dense structure plus the double plasma membrane. It was suggested that the membrane portion was supplied by RBC and matrix by anaplasma organism (161). The envelope of the organism was found to be relatively

resistant to heat or sonic oscillation (162).

The classification of A. marginale is difficult and has caused controversy. Foote et al. (163) studies A. marginale using the electron microscope and first concluded that their findings supported the virus concept, but subsequent studies suggested the anaplasma body to be neither protozoan nor viral but probably rickettsial (160).

Development in the Bovine Host

The arthropod vector, the tick, mediates the transfer of A. marginale organism from cattle or wild ruminant. The latter usually, wild deer, do not develop severe clinical disease (164). The tick and the horsefly transmit A. marginale from cow to cow (165).

When mature deer RBC were transfused into an infected cow, the initial body of A. marginale penetrated into deer RBC within 12 to 16 hours (166). It has been suggested that the organism grows in the host by binary fission, matures, initial bodies apparently emerge from host cell and may attack new red blood cells (166).

One theory proposes that initial body is attached to the host cell inner and outside membrane in early stage of the development, within 6 days following infection (166). In mixed population stages, which is 10 days after infection, constant increase in number of A. marginale bodies was observed. In the vigorous growth and transfer stage, which is 15 to 19 days after inoculation, multiple infections of individual RBC were commonly observed. Apparent transfer of initial and marginal bodies to adjacent RBC was also observed. A massive multiplication stage occurs 19 to 20 days after infection. During this time and for a subsequent 2-week period of recovery, A. marginale bodies predominate in

the red cell population (166).

Metabolism and Properties of A. marginale Organism

The detailed metabolism of A. marginale organism for its growth is not known. Some of the findings which describe the reported research on the A. marginale organism will be discussed. The marginale bodies purified by Rogers and Dimopoullos (167) contained protein, nucleic acids, phospholipids, and sterols, but no detectable carbohydrate (168). Total lipids from purified marginale body preparations were separated by thin-layer chromatography contained large quantities of phospholipid with smaller amounts of sterols, free fatty acids, tryglycerides, and sterol esters. As determined by gas-liquid chromatography, the major fatty acids in the various lipid fractions included both long chain saturated and unsaturated acids with myristic, palmitic, palmitoleic, steric, oleic, and linoleic acids predominating. Moderate amounts of other fatty acids ranging from C 12:0 to C 24:1 were also found (169).

It was found that a decrease in the concentration of RBC phospholipids (170) and a change in the net erythrocytic surface charge (171) occurred in bovine anaplasmosis. The decrease was accounted for by the declining concentration of lecithins and cephaline (172).

The metabolic integrity of anaplasma infected RBC was assessed by Mann (173) who compared the following properties of normal and light to heavily infected RBC: GSH and ATP content, glycolytic rate, and activities of representative enzymes of the Embden-Meyerhoff and pentose shunt pathways. The metabolic rate and enzyme activities of infected cells rose as the intensity of infection rose, but the changes in RBC properties were largely attributable to relative differences in the mean age

of the comparative RBC sample. Phase-contrast microscopic examinations revealed that infected RBC underwent more rapid autohemolysis than normal RBC upon being deprived of glucose substrate. Mann (173) suggested that failure of the infected RBC to maintain its volume within critical limits may be due to either a breakdown of the cation transport mechanism, which is energy-dependent, or to a membrane permeability defect.

The incorporation of an external amino acid into anaplasma infected and normal RBC was studied by Mason and Ristic to establish the rate of protein synthesis of A. marginale. A consistent relationship was shown between the presence of infected RBC and the increased incorporation of radioactivity from 2-glycine-^{14}C (174).

There is an association of catalase with the marginale body which can be demonstrated by antisera tests (175). An increase of catalase activity was observed as the number of RBC containing A. marginale bodies increased. As the mature RBC does not have a protein synthesizing system it is believed that the organism synthesizes catalase. Antisera decreased catalase activity of partially purified marginale bodies. Generally, hydrogen peroxide is antibacterial and if A. marginale organisms do not favor H_2O_2 for their growth, the organism may have to secrete catalase to remove H_2O_2 in erythrocytes. Dimopollous (176) measured activities of lactate dehydrogenase (LDH), ATPase and acetylcholinesterase in partially purified A. marginale bodies. A low level of LDH was found while high activities of ATPase were observed. Since ATPase is linked to membrane transport, disturbances in ATPase activity during infection could impair the proper function of the erythrocyte by rendering it osmotically fragile and less able to adapt to stress. Decreased acetylcholinesterase activity in mature and infected

bovine RBC was reported (176).

The respiration rate of blood from a calf during the period of rapid rise of infection was approximately double that of normal blood. In protozoan blood infections, the rise in the host blood respiration rate was usually 10 or greater than that of the normal host (176). A comparative study of Anaplasma, Plasmodium, and Babesia demonstrated the ability of only the latter two organisms to dehemoglobinize erythrocytes (177).

Blood Transfusions During Patent Bovine Anaplasmosis

Smith and Ingram (11) reported that the newborn calf has immunologic immaturity. Williams and Jones (12) transfused infected and non-infected blood to splenectomized anaplasmosis carrier calves and found a significant increase in the number of parasitized erythrocytes. Blood containing a predominant population of immature RBC, neonatal calf blood, and heterologous blood failed to induce a parasitemic response. The above two observations could suggest that erythrocytes of newborn calf do not favor marginal body growth. Williams and Jones (12) also suggested that the RBC environment played a significant role in the host-parasite equilibrium in anaplasmosis.

Immunity

As mentioned earlier, deer and newborn calves do not develop any drastic clinical sign of the disease upon A. marginale infection (8,9, 164). This inherent property of certain susceptible animals is called natural resistance. The basis of this natural resistance in calves is not fully known, but may be related to certain erythrocytic factors

(178). Roby and Gates (179) reported that, based on observation of infectivity titration, the most susceptible animal to anaplasmosis is a splenectomized cow. Splenectomized calves and nonsplenectomized cows were less susceptible and the nonsplectomized calf was the least susceptible.

Surviving animals from anaplasma infection with treatment or with no treatment while keeping latent infection have an actively acquired immunity (157). Sterile immunity without previous infection can be acquired by exposing a susceptible, anaplasma free animal to the killed anaplasma organisms or their antigens. Although the vaccinated animals were resistant to clinical disease, they all became infected and were consequently carriers (180).

Babesiosis

Introduction and Classification

Babesiosis is a hemolytic disease produced by protozoan parasite belonging to the family of Babesiidae. Bovine babesiosis causes a considerable economic loss in the world, but little is known of the host-parasite interactions involved in cattle or in other animal species. All domestic animal species are susceptible to Babesia and some attention has been given to cattle, dogs, and horses for host-parasite interactions.

Neitz (181) recognized 17 distinct species of Babesia from various vertebrate hosts. These include Babesia bovis, B. bigemina, B. berbera and B. major from cattle; B. equi from horse, B. canis, B. vogeli and B. gibsoni from dog, and others. Babesia bigemina causes the Texas fever of cattle and was eliminated from the Southern states by control of the

arthropod vector, the tick. The genus Babesia consists of relatively large, pyriform, round or oval parasites which range in size about 1 to 4 microns in length.

There are common symptoms in the majority of Babesiosis cases. For example, fever, malaise and listlessness, mental depression, disinclination to move about, anorexia, or an unusual fussiness about certain items of food are observed in infected dogs. Icterus develops in advanced or neglected cases, and hemoglobinuria is usually a sign of parasite destruction of red blood corpuscles (182).

The taxonomic classification of Babesia parasites varies with different research workers of this area. The difficulty of the classification is because of incomplete knowledge of the parasite's metabolism and development.

Development in the Vertebrate Host

An arthropod vector, the tick, is required for transmission of the Babesia organism. The incubation period following tick transmission varies between 5 to 10 days, depending on the species of parasite involved. The cycle of development of the organism has been studied more extensively with Babesia canis than any others. Mostly, morphological studies have been conducted and very little research on biochemical changes and metabolism has been reported. Nuttall and Graham-Smith (183,184) reported the multiplication steps of B. canis in the host in the following order: pyriform parasite attacks the RBC, round form, enlarged amoeboid stage, round, pear shaped, minute round mass, and then 2 mature pyriform parasites. Multiplication in the red cell is asexual and usually by budding, giving rise to two or four daughter cells. The

formation of several parasites by multiple fission, endocellular schizogony, has been described for B. bigemina (185). The organisms grow not only in the red cell, but also in other tissues of the vertebrate host such as internal organs (181).

Metabolism of Babesia Organism

Detailed metabolism and nutritional requirements for Babesia organisms are unknown. There is a report that hemoglobin digestion occurs in Babesia and is more complete than in the plasmodidae (186). Therefore, one or more of proteases may be present in the Babesia organism and peptides or amino acids released by protease action could be used for the nutritional source. It has been known that a nucleus, DNA, cytoplasm, and reticulate mass are present in the Babesia organism (183,187,188). The presence of glycogen in the organism is not proven. Using the electron microscope, Rudzinska and Trager (186) observed that B. rodhaini engulfed pieces of erythrocytic cytoplasm by phagotrophy. Hoyte (189) stated that multiplication of B. bigemina occurs only within erythrocytes.

Animal Immunity and Resistance to Babesiosis

There is generally innate resistance for Babesia organisms (190). This innate resistance is not related with humoral antibiodies specific for organism.

Mahoney (191) reported that, in infected environments, the age incidence of B. argentina parasitemia rose from zero at birth to a maximum at 1 to 2 years of age and then declined. When the infection was carried with B. bigemina in an identical condition as above, the para-

sitemia was maximum at 6 to 12 months of age. This suggests that newborn calves have at least a 6 months resistance period to Babesia organisms. The duration of infection varies considerably with species of animal host and blood parasite. In contrast to newborn calves, newborn puppies are highly susceptible to the Babesiosis (16).

It has been known that the progeny of immune cows are less susceptible to infection with B. argentina, either by blood innoculation or by ticks, than calves of cow with no history of infection. Immunity to babesiosis usually persists while animals continue to carry small amounts of the Babesia organism (188). Antibodies appeared in the blood 11 to 15 days after the initiation of parasitemia upon B. caballi infection of a horse (192) and 11 to 34 days for B. canis infection (193). Hall (194) stated that calves up to about 8 weeks of age from cattle immune to B. argentina were more resistant to the disease than the calves of similar age from susceptible dams. He also found no transfer of infections organism to embryo from infected dam during pregnancy and suggested the presence of functional antibodies in the colostrum of immune cattle (194). Mahoney (195) observed a similar effect for a colostrum antibody.

Red Blood Cell Membrane

The red cell membrane is composed of 40 to 55 percent protein, 35 to 45 percent lipid, and 10 percent carbohydrate. The carbohydrates are covalently bound to protein or lipid (196,197,198). Sixty-five percent of the lipid is phospholipid, 23 percent is cholesterol, and 2 percent is cholesterol esters, glycerides, and free fatty acids. The remaining 10 percent is chiefly glycolipid. The free cholesterol is in dynamic

equilibrium with the cholesterol of the plasma (199,200).

Net movement of sodium or potassium across the cell membrane occurs by passive diffusion and by active transport. The direct and immediate energy source for active transport is ATP derived from glucose metabolism. Energy is released from ATP by the enzyme ATPase found in the membrane (201).

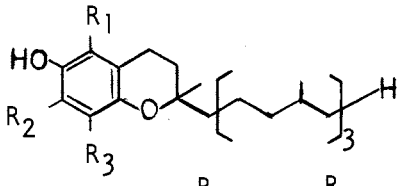
Structural organization of membranes has been studied by many workers and was reviewed recently (202). Bilipid layer, lipoprotein subunit and mosaic models have been proposed for the membrane organization based on several types of experimental evidence.

Since the membrane is the surface of red cell, one should not overlook the role of the membrane on blood parasite penetration. Differences in membrane composition may affect blood parasite penetration. There are some reports that red cells vary in their functional properties (203,204,205). Dimopollous (175) reported that there is a difference of ATPase, acetylcholinesterase and fatty acid composition between A. marginale infected and noninfected red cells.

Bovine erythrocyte ghost purification has been reported by Burger et al. (206). Total solubilization of the erythrocyte ghost proteins has been achieved by several methods including aqueous butanol, acidic and neutral detergents, urea, and acidic solutions of 2-chloroethanol (207). Polyacrylamide gel electrophoresis of membrane proteins solubilized by sodium dodecyl sulfate (SDS) has been reported (208,209).

Zacharius et al. (210) described a staining method for glycoproteins in polyacrylamide gel based on periodic oxidation of the sugar moiety to aldehyde groups and fuchsin-sulfite reaction with the reducing groups.

Vitamin E consists of a group of closely related vitamins abundant in vegetable oils. These compounds contain a hydroxyl-bearing aromatic ring system and isoprenoid side-chain. Eight of the 14 methyl derivatives of tocol [2-methyl-2(4',8',12,-trimethyltridecyl) chroman-6-ol] and tocotrienol [2-methyl-2(4',8',12',-trimethyltrideca-3',7',11'-trienyl) chroman-6-ol] are recognized as naturally occurring tocopherols (211). The most abundant are α-, β-, and γ-tocopherols. The general structure of tocopherol is:



	R_1	R_2	R_3
α-tocopherol	CH_3	CH_3	CH_3
β-tocopherol	CH_3	H	CH_3
γ-tocopherol	H	CH_3	CH_3

There is some evidence that tocopherols prevent the destructive, non-enzymatic attack of molecular oxygen on the double bonds of the polyunsaturated fatty acid components of tissue lipid (212). All of the tocopherols have been found to differ widely in both antioxidant activity (213) and vitamin E potency (214).

Slover et al. (212) described a method for the analysis of vitamin E. The individual tocopherols (both tocols and tocotrienols) have been identified and estimated as their trimethylsilyl ethers by a gas-liquid chromatography on SE-30, Apiezon L or OV-17 column. Walker and Kummerow (216) stated that vitamin E supplementation of a rat diet exerted no influence on the fatty acid composition of the phospholipid of erythrocytes.

CHAPTER III

HEMOGLOBIN TYPES AND VARIATION

Since certain hemoglobin types of man and bovine may influence resistance to hemotropic parasitisms such as resistance to malaria in sickle-cell anemia (4), bovine Hb AB and trypanosomiasis (14), and bovine Hb F and anaplasmosis (12), studies of bovine hemoglobin types in normal and A. marginale infected cattle may provide information which may facilitate disease control. The decrease of bovine Hb F as the calf matures may be related to the decrease of the newborn calf's resistance to anaplasmosis. Grimes et al. (42) reported postnatal loss of bovine Hb F in dairy calves on the basis of paper electrophoretic results and this pattern needs to be examined with a better analytical method. Blood exchange experiments of the newborn calf with compatible adult cattle blood may show the fate of the transfused red blood cells and the pattern of Hb F synthesis in newborn calves. Since the occurrance of fetal and new hemoglobins have been observed in anemic humans (4,63), sheep (141,142), and goats (146), hemoglobin types in anemic cattle are of interest and have been studied.

Experimental Procedure

Chemicals and Equipments

Acrylamide, N, N'methylenebisacrylamide, N, N, N', N'-tetramethylenediamine, ammonium persulfate, aniline blue black and riboflavin were

purchased from Canal Industrial Corp., Rockville, Maryland; Tris(Hydroxymethyl) aminomethane, 2-mercaptoethanol and carboxymethyl cellulose from Sigma Chemical Co., St. Louis, Missouri; urea from J. T. Baker Chemical Co.; toluene from Eastman; and cyanmethemoglobin standard from Hycel Inc., Houston, Texas.

A microzone model R-101 electrophoresis cell, 5.7 x 14.4 cm cellulose acetate electrophoresis membrane and veronal buffer were purchased from Beckman Instruments, Inc.; Supre-Heme buffer, later known as Tris-EDTA-borate, from Helena Laboratories, Taylor, Michigan; 7.2 x 17.2 cm cellulose acetate membrane and Gelman Rapid Electrophoresis Chamber from Gelman Instrument Company. All other chemical reagents used were of reagent grade or equivalent.

Animals and Blood Samples

Fresh blood samples were drawn by jugular venipuncture from animals. One percent heparin and 10% EDTA-solutions were used as anticoagulants.

Blood samples of Santa Gertrudis cattle were obtained from the animals at Winrock Farm, Ada Valley, Arkansas; Scottish Highland blood samples were supplied by Dr. David Harrington, Albuquerque, New Mexico.

The other cattle blood samples were obtained from animals at the Dairy Barn, Oklahoma State University, local farms at Stillwater and in this vicinity. The horse blood samples were supplied by Veterinary Research, Oklahoma State University.

Ten Holstein (7 female and 3 male) and five Ayrshire (4 female and 1 male) newborn calves were used for the study of bovine fetal hemoglobin decrease in neonatal calves. Blood samples were withdrawn from

these animals weekly or biweekly for a total period of 24 weeks from birth.

Two newborn Holstein calves, having hemoglobin type F, were exchange transfused with compatible blood (hemoglobin types A and B) from healthy adult Jersey cows. Blood compatibility was determined by the method of Hepler (217). Each calf received 7 liters of adult blood (estimated 2 x calf blood volume) by repeated withdrawal of 1 liter of calf blood and transfusion of 1 liter of adult blood. Exchange transfusion of another newborn Holstein calf having hemoglobin type F, with hemoglobin A type adult cow blood was attempted; clotting in the vein limited the exchange to 5 liters of blood. Blood samples were taken daily for 29 days post-transfusion, and the packed cell volume, red blood cell count, hemoglobin per cent and hemoglobin composition were determined.

A newborn Angus calf was hemorrhaged by withdrawal of 300 ml. blood 4 times during a period of 5 days, and blood samples were taken daily to establish changes in packed cell volume, red blood cell count, hemoglobin per cent and hemoglobin composition during the recovery from macrocytic hypochromic anemia. The blood sampling continued until the calf became 50 days of age.

Two six-months old Hereford cows were hemorrhaged by withdrawing 500 to 1500 ml. of blood 9 times during a period of 30 days. Blood samples were examined to determine if any hemoglobin type change occurred from 1 month pre-hemorrhage and post-hemorrhage periods. The exchange transfusions and hemorrhages were conducted and PCV, RBC, and Hb % were determined at the Veterinary Research Laboratories, Oklahoma State University, Stillwater, Oklahoma.

Methods

Isolation and Determination of Hemoglobin

Hemoglobin solutions were prepared by a modification of the method of Drabkin (218). The erythrocytes were washed 4 times with ice-cold 0.9% NaCl solution with subsequent centrifugation at 12,350 x g for 15 minutes at 4° C. After washing, packed erythrocytes were hemolyzed by the addition of an equal blood volume of deionized water with 0.4 volumes of toluene and subsequent incubation at 4° C for 18 to 24 hours. The hemolysate was then centrifuged at 12,350 x g for 15 minutes, and the hemoglobin layer was siphoned off. Hemoglobin concentration in whole blood was determined by the cyanmethemoglobin method (219) using cyanmethemoglobin standard supplied from the Hycel Incorporation.

Electrophoresis, Identification, and Densitometer Scanning of Hemoglobins

Disc Polyacylamide gel electrophoresis of hemoglobins followed the procedure described in the Canalco Model 6 System Instruction Manual at pH 8.1. Cellulose acetate electrophoresis[1] was done using Tris-EDTA-borate buffer, pH 8.6, at 4° C and 500 V for 40 minutes. The electropherograms were stained with a mixture of 0.5 gm Ponceau-S stain, 7.5 gm trichloroacetic acid and 7.5 gm sulfosalicylic acid in 250 ml distilled water. The membranes were washed in 5% acetic acid and methanol, cleared in 30% acetic acid in methanol, and the bands were quantitated

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[1] Beckman Microzone Electrophoresis System, Model R-101, Beckman, Instruments, Inc., Scientific and Process Instruments Division, Fullerton, California.

with a recording densitometer.[2] The electrophoretic results were compared with the published results (14,40,42,45) for identification.

Hemoglobin Purification and Chain Separation

Hemoglobin solutions obtained as previously described were treated with carbon monoxide and subjected to carboxymethyl cellulose column chromatography (220) and cellulose acetate electrophoresis for purification of hemoglobins present. The carboxymethyl cellulose column (2 x 44 cm) was prepared and used at 4° C with 0.01 M phosphate buffer pH 6.35. Hemoglobins were loaded on the column and 80 ml of 0.01 M phosphate buffer pH 6.35 was passed through the column. The column was developed with about 300 ml of 0.01 M phosphate buffer pH 6.35 and 0.01 M phosphate buffer pH 8.0 forming a pH gradient. The flow rate was 20 ml per hour. Volume of fractions 1 to 31 was 2.7 ml and 3.0 ml thereafter. The hemoglobins were separated in a Gelman rapid electrophoresis cell with Tris-EDTA-borate (TEB) on cellulose acetate. Each band was excised, eluted with the electrophoresis buffer, dialyzed against CO saturated deionized water and lyophilized.

Chain separation of hemoglobin molecules was achieved by incubating the proteins with 2M acetate buffer pH 4.7 for 72 hours or in TEB-6M urea, 2-mercaptoethanol (1%) buffer, pH 8.6, for 24 hours. Each hemoglobin (180 micrograms or more) was dissolved in 10 microliter of one of the above buffer and subjected to electrophoresis at 100 V and for 2 hours on cellulose acetate membranes with aluminum-lactate buffer-3M urea, pH 3.3 (221) for the pH 4.7 protein solution and TEB-6M urea,2-

[2]Beckman RB analytrol with scanning attachment, Beckman Instrument, Inc., SPINCO Division, Stanford Industrial Park, Palo Alto, California.

mercaptoethanol (1%) for the pH 8.6 protein solution. All of the above operations were conducted in a cold room where the temperature was maintained from 4 to 6° C.

Two dimensional electrophoresis for chain separation of hemoglobin molecules was attempted using 17.2 x 17.2 cm cellulose acetate membranes. The hemoglobin solution was applied on the membrane and in the first dimension electrophoresis was accomplished with TEB buffer. The membrane was blotted with filter paper and floated for five minutes on TEB-6M urea,2-mercaptoethanol buffer. The membrane was again blotted and in the second dimension electrophoresis was conducted in TEB-6M urea,2-mercaptoethanol buffer.

Results

Hemoglobin Types of Bovine, Equine and Canine

Figure 1 shows a typical electrophoretic separation of bovine hemoglobin in 6% polyacrylamide gel and on a cellulose acetate membrane at pH 8.1 and 8.6, respectively. In both systems newborn Holstein calf blood contained hemoglobin F and A. Hemoglobin F (Hb F) migrated faster than Hemoglobin A (Hb A) toward the anode at alkaline pH's. Hemoglobin B (Hb B) migrated faster under the same conditions, and clear separation from Hb F was demonstrated. Veronal, phosphate, citrate, Tris-HCl and Tris-EDTA-borate buffers were used for the separation of hemoglobins on cellulose acetate membranes and Tris buffer systems gave the only satisfactory results.

Blood samples from 88 newborn calves of Ayrshire, Holstein, Angus, Guernsey and Jersey breeds demonstrated the presence of hemoglobin F (range: 69 to 100%) along with one or two of the adult bovine hemo-

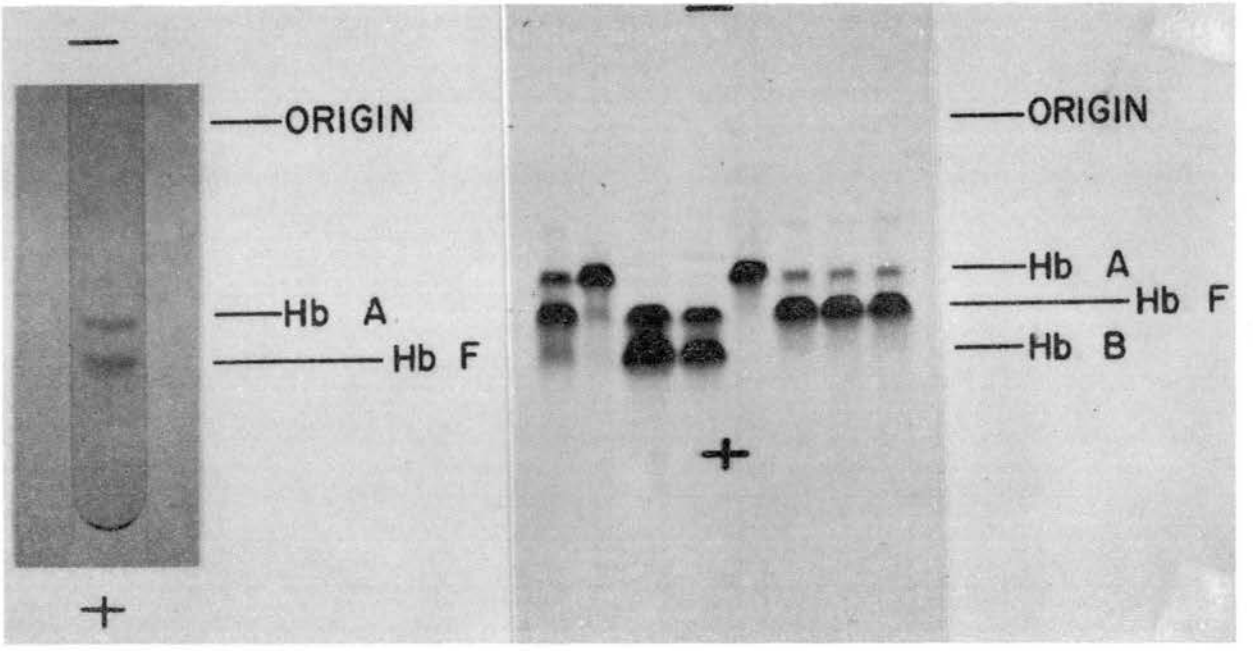


Figure 1. Electrophoretic Separation of Bovine Hemoglobins in a Polyacrylamide Gel Column (left) and on a Cellulose Acetate Membrane (right).

Polyacrylamide electrophoresis was conducted in a Hoefer electrophoresis cell. Electrophoretic conditions were 6% gel, Tris-glycine buffer, pH 8.1, 5 ma per gel and for 20 minutes at room temperature.

Cellulose acetate electrophoresis was conducted in Beckman Microzone Model R-101 cell. Electrophoretic conditions were Tris-EDTA-borate buffer, pH 8.6, 500 volt. and for 40 minutes at 4° C.

globins, A, B or AB. Blood samples from mature cattle—Holstein, Ayrshire, Angus, Hereford, Santa Gertrudis, Scottish Highland and Brahman—showed the presence of hemoglobin A, B or AB presence depending on breed and animal. No other hemoglobin types were observed. The total number of animals examined were 248, and the hemoglobin types of 226 individuals of known breeds are shown in Table 1. Holstein, Ayrshire, Angus, Hereford and Scottish Highland cattle showed the presence of hemoglobin A only. Among these, there has been no previous report of the hemoglobin type of Scottish Highland cattle. The hemoglobin types of Holstein, Ayrshire, Angus and Hereford are consistent with reports of others (40,42,45). Jersey and Brahman cattle show hemoglobin types of A, B, or AB as expected (14,40,45). Santa Gertrudis cattle showed hemoglobin types of A or AB, and no B homozygotes were found. This coincides with the report by Crockett (45).

Horse breeds of Shetland, American Quarter, Appaloosa, American Quarter x Percheron and mixed were examined for hemoglobin types. With the exception of the mixed, all of the horses demonstrated the presence of 2 electrophoretically distinct hemoglobins. These 2 hemoglobins migrated toward the anode at pH 8.6, and the electrophoretic mobilities were similar to those of bovine hemoglobin F and B. The presence of only one hemoglobin in the mixed breed horse was unexpected. The previous history of this animal is unknown. Hemoglobin solutions from several newborn horses were electrophoresed on cellulose acetate membranes to see if any fetal hemoglobin was present. Two hemoglobins of electrophoretic mobilities identical with those of adult horse hemoglobins were detected and no additional bands were observed.

Adult canine blood samples of several breeds (30 Beagle, 2 Scotty,

TABLE I

HEMOGLOBIN TYPES OF ADULT CATTLE

Breed	No. of animals examined	No. of Hb A/Hb A type individuals	No. of Hb A/Hb B type individuals	No. of Hb B/Hb B type individuals
Santa Gertrudis	103	89	14	3
Hereford	54	54		
Jersey	16	8	5	3
Holstein	15	15		
Angus	13	13		
Scottish Highland	11	11		
Ayrshire	7	7		
Brahman	7	3	2	2
Total	226	200	21	5

4 German Shepherd mixture, 1 Schnauzer, 1 Dalmatian, 1 Greyhound, and 1 English Setter) were analyzed for hemoglobin types. On cellulose acetate electrophoresis, only one hemoglobin type was detected from the above blood samples. Canine red cells had a higher lipid content and the hemoglobin denatured easily and upon aging blood samples gave multiple bands on electrophoresis.

Hemoglobin analysis of the newborn canine will be presented in Chapter IV.

Quantitative Variation of Bovine Fetal Hemoglobin During Maturation

Electrophoretic separation of bovine fetal hemoglobin from adult bovine hemoglobins (A and B) is shown on Figure 1.

Figure 2 shows a densitometer scan of the electrophoretically separated hemoglobin A and F on a cellulose acetate membrane from a 4 weeks old Holstein calf. Upper peaks represent the optical density of Ponceau-S stained protein bands, and the bottom peaks are the integration of these densities. Integration peaks were counted to calculate percentage of each protein. Figure 3 shows a typical pattern of Hb F decrease and Hb A increase during maturation of calves.

The variation of Hb F and A in the neonatal Holstein and Ayrshire calves is summarized in Table V and VI (Appendix). The amount of Hb F ranged from 68.7 to 97.1 per cent in the newborns with average of 89.2 per cent, and decreased with a concomitant increase of Hb A. After 21 to 23 weeks of birth, Hb F became less than one per cent and that of Hb A approached 100%. Figure 4 and 5 show the varying concentrations of Hb F and A in the calves after birth.

The lines on Figure 4 and 5 represent mean values of each hemo-

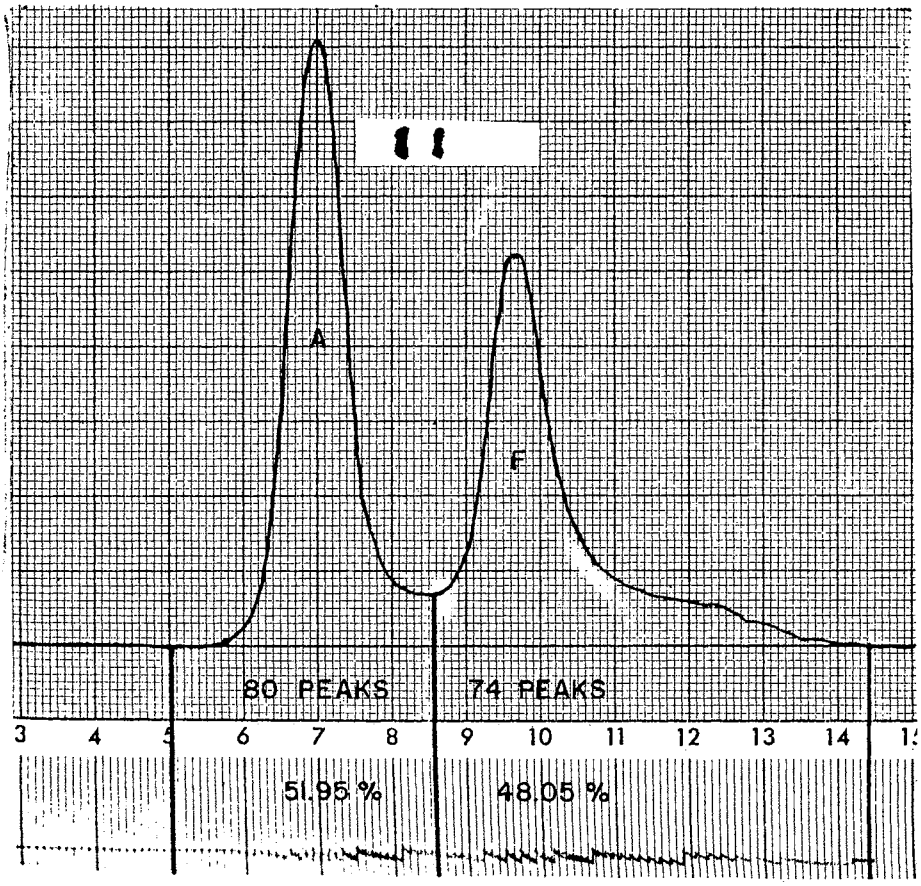


Figure 2. A Typical Example of a Beckman Model RB Analytrol Scan of a Cleared Cellulose Acetate Membrane After Electrophoretic Separation of Bovine Hemoglobin A and F.

Electrophoresis was conducted under the same conditions as in Figure 1. Top peaks correspond to densities of protein bands on the cleared cellulose acetate membrane. Bottom peaks are integration of top peaks, from which percentages of each hemoglobin were determined.

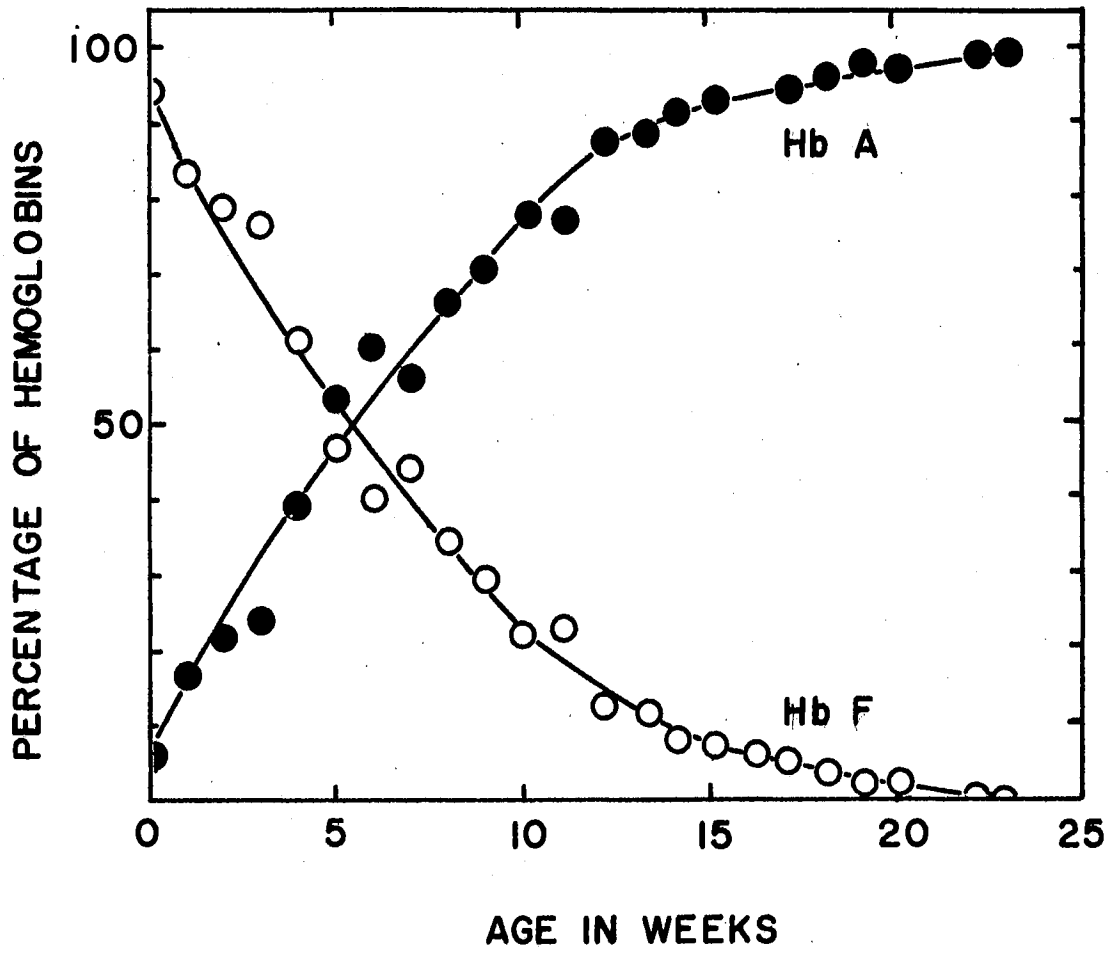


Figure 3. Adult and Fetal Hemoglobin Variation in a Newborn Calf.

Animal: Holstein calf, female No. 34. Percentages of each hemoglobin were determined as given in Figure 1 and 2.

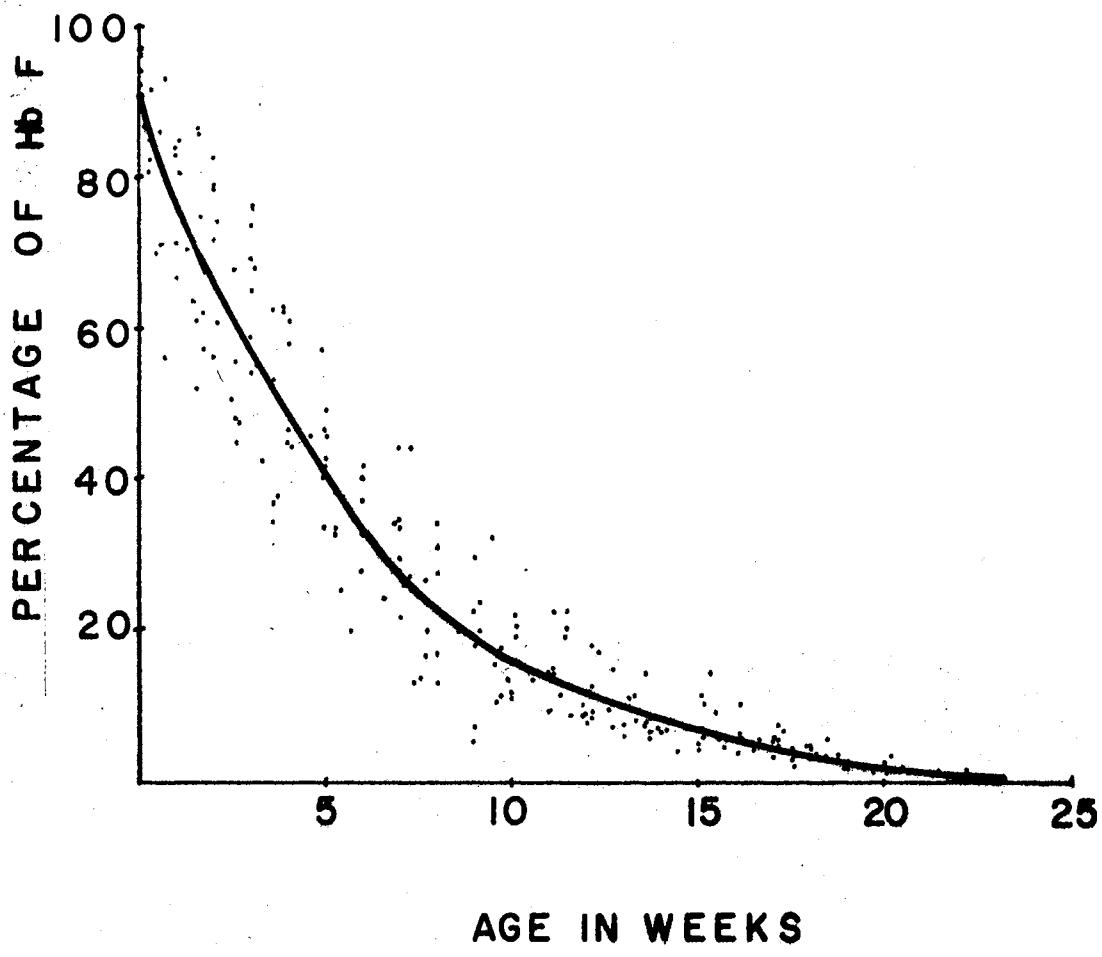


Figure 4. The Decreasing Pattern of Bovine Hemoglobin F in Holstein and Ayrshire Calves After Birth.

Percentage of bovine hemoglobin F was determined as in Figure 1 and 2. Ten Holstein and five Ayrshire calves were used for this study.

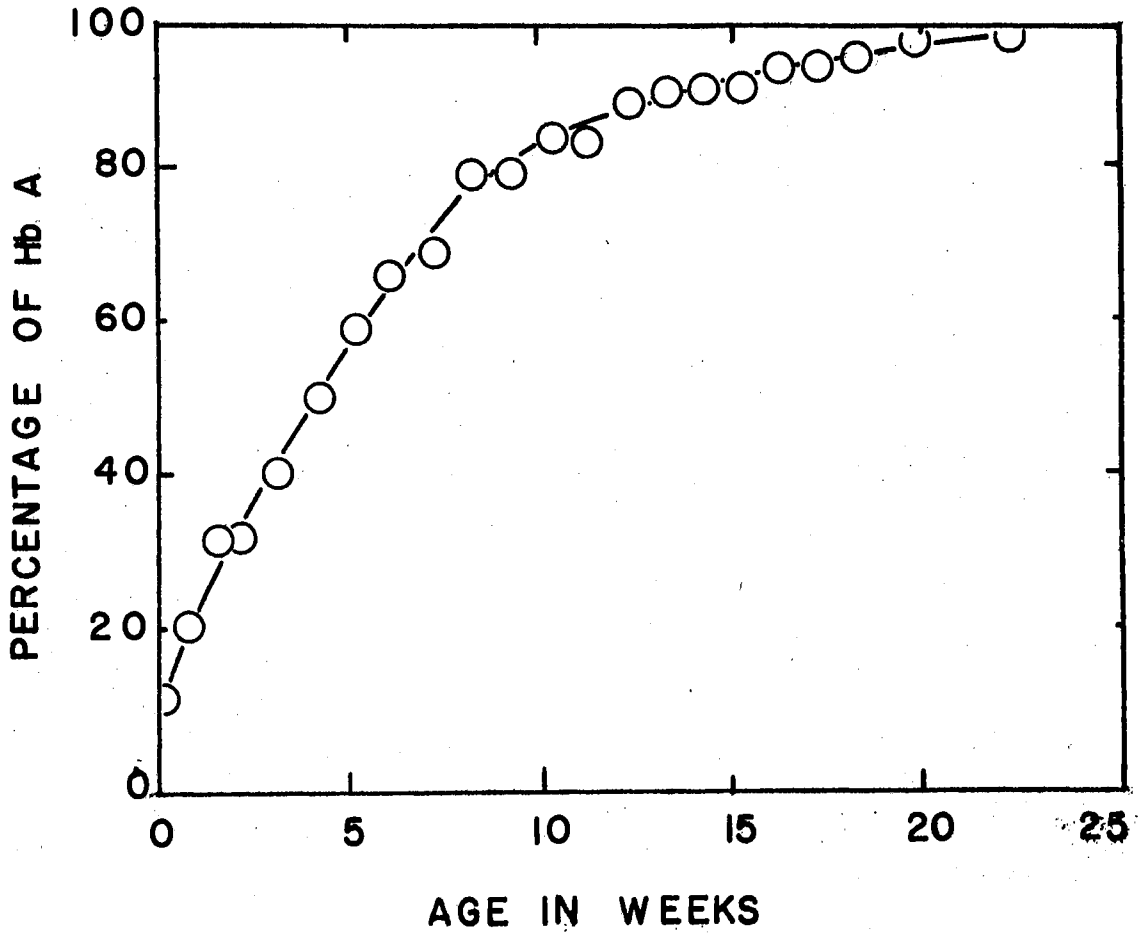


Figure 5. The Increasing Pattern of Bovine Hemoglobin A in Holstein and Ayrshire Calves After Birth.

Ten Holstein and five Ayrshire calves were used for this study and mean values were plotted. Percent of bovine hemoglobin A was determined as given in Figure 1 and 2.

globin per cent. The average log Hb F per cent was plotted against age in weeks and gave a rather straight line as seen in Figure 6. From these results a general equation was derived by statistical regression (Table VII in Appendix):

log Hb F% = 1.9969 - 0.0792A (A: age of calf in weeks)

variance = 0.0032

The decreasing pattern of Hb F in Holstein bull and heifer calves was essentially similar to the pattern shown on Figure 4.

The author observed a minor protein band in some Ayrshire and Holstein calf blood samples which migrated similar manner but a little faster than hemoglobin B on cellulose acetate electrophoresis at pH 8.6 and 4^{o} C (left margin of cellulose acetate, Figure 1). The protein was regarded, at first, as a new fetal hemoglobin and tentatively named as hemoglobin F_2.

Blood samples from 59 calves (Holstein, Guernsey and Jersey) were collected during a period of six months and 6 blood samples from different animals showed Hb F_2 on the cellulose acetate electrophoresis. Blood samples refrigerated immediately after the samples were taken had never shown this protein. With carboxymethyl cellulose column chromatography, hemoglobin F_2 was eluted in fractions 98 to 108 and were concentrated in fraction 103. These fractions were combined and lyophilized. A solution which contains only Hb F was treated similarly. Both hemoglobins gave identical electrophoretic mobilities on cellulose acetate membranes.

Electrophoretic mobilities of polypeptide chains (2 bands), obtained by incubation at pH 4.7, from isolated above coincided except that

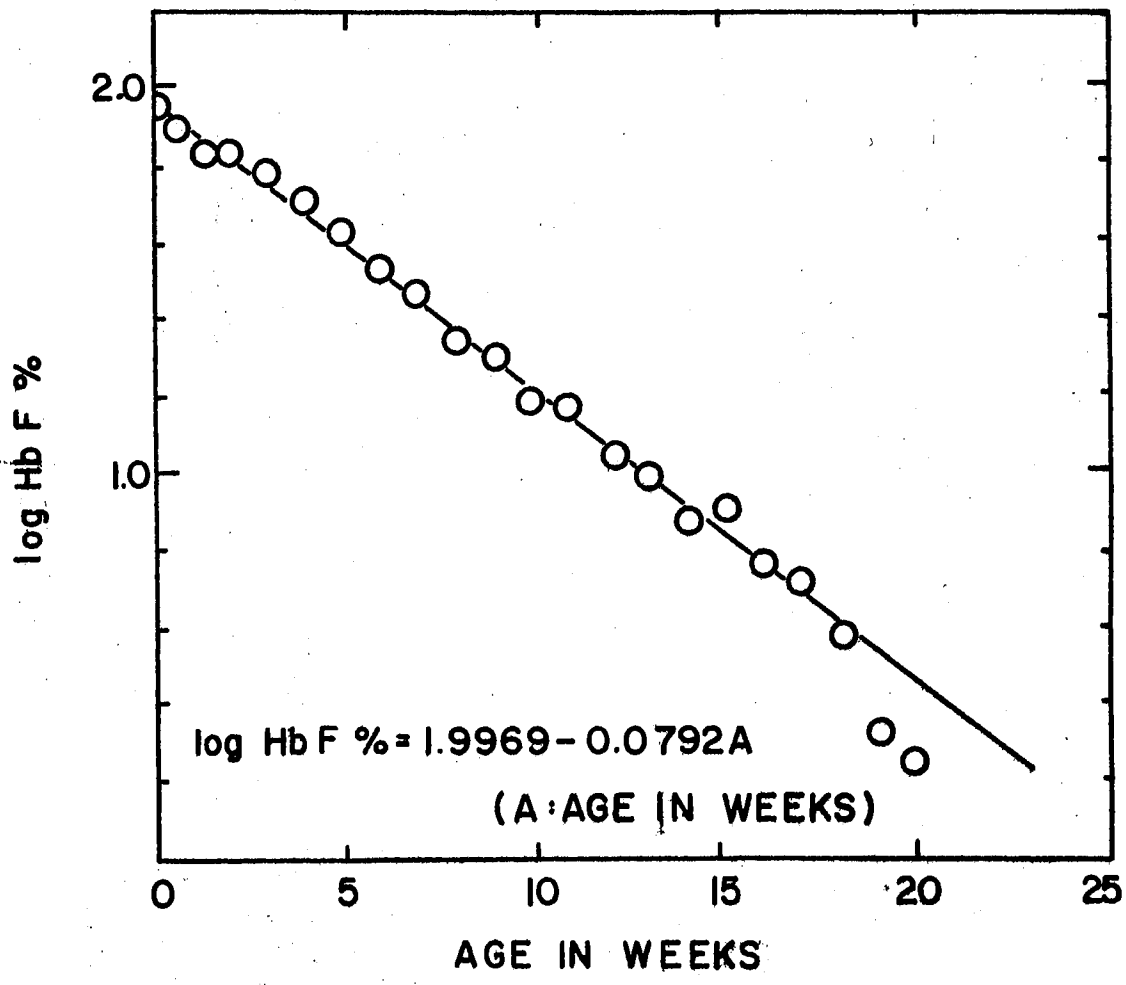


Figure 6. A Logarithmic Plot of Average Hemoglobin F Percent Against Calf Age.

See Figure 1 and 2 for description of separation and determination of bovine hemoglobin F. Derivation of the equation for bovine hemoglobin F decrease is described in Table VII (Appendix).

hemoglobin F_2 gave an additional slower moving protein band. Two dimensional electrophoresis did not give clear separation of the peptide chains.

The chain separation of hemoglobin F_2, F, A and B were achieved by incubating hemoglobins TEB-6M urea, 1% 2-mercaptoethanol. These protein solutions were subjected to electrophoresis in the incubation buffer. All of the protein solutions produced α-chains. Hemoglobin F_2 and F also showed γ-chains with the same electrophoretic mobility (Figure 7).

Hemoglobin solutions which contained some hemoglobin F_2 were frozen and thawed a few times and electrophresed after each treatment. The amount of hemoglobin F_2 was apparently increasing as the freezing and thawing progressed.

The above verified that the tentatively named hemoglobin F_2 is, in fact, denatured bovine hemoglobin F.

Exchange Transfusion and Hemoglobin Change

After complete exchange transfusion of 2 newborn Holstein calves (Hb F type) with adult Jersey blood (Hb A and B type), calf blood samples post-transfusion had equal proportions of Hb A and B. No Hb F was detected. Ten days after exchange transfusion, a marked drop in the PCV and RBC counts occurred in both calves. These hemolytic episodes were accompanied by rapid elimination of Hb B and the reappearance of Hb F. In fact Hb F concentration increased to 27.3% in one calf and to 21.9% in the other within 2 to 3 days. These concentrations subsequently declined. Meanwhile the concentration of Hb A gradually increased and ultimately reached 100% (Table VIII and IX in Appendix and Figure 8 and 9). Figure 10 and 11 are the transformations of Figure 8 and 9 to

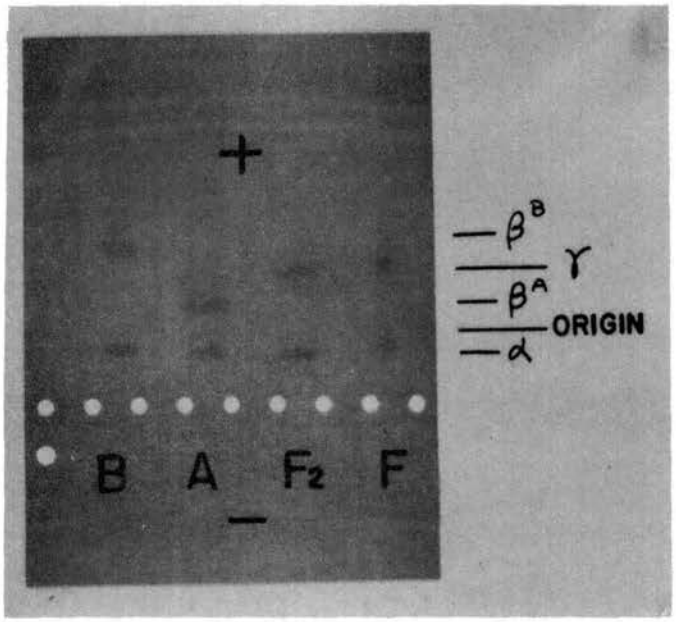
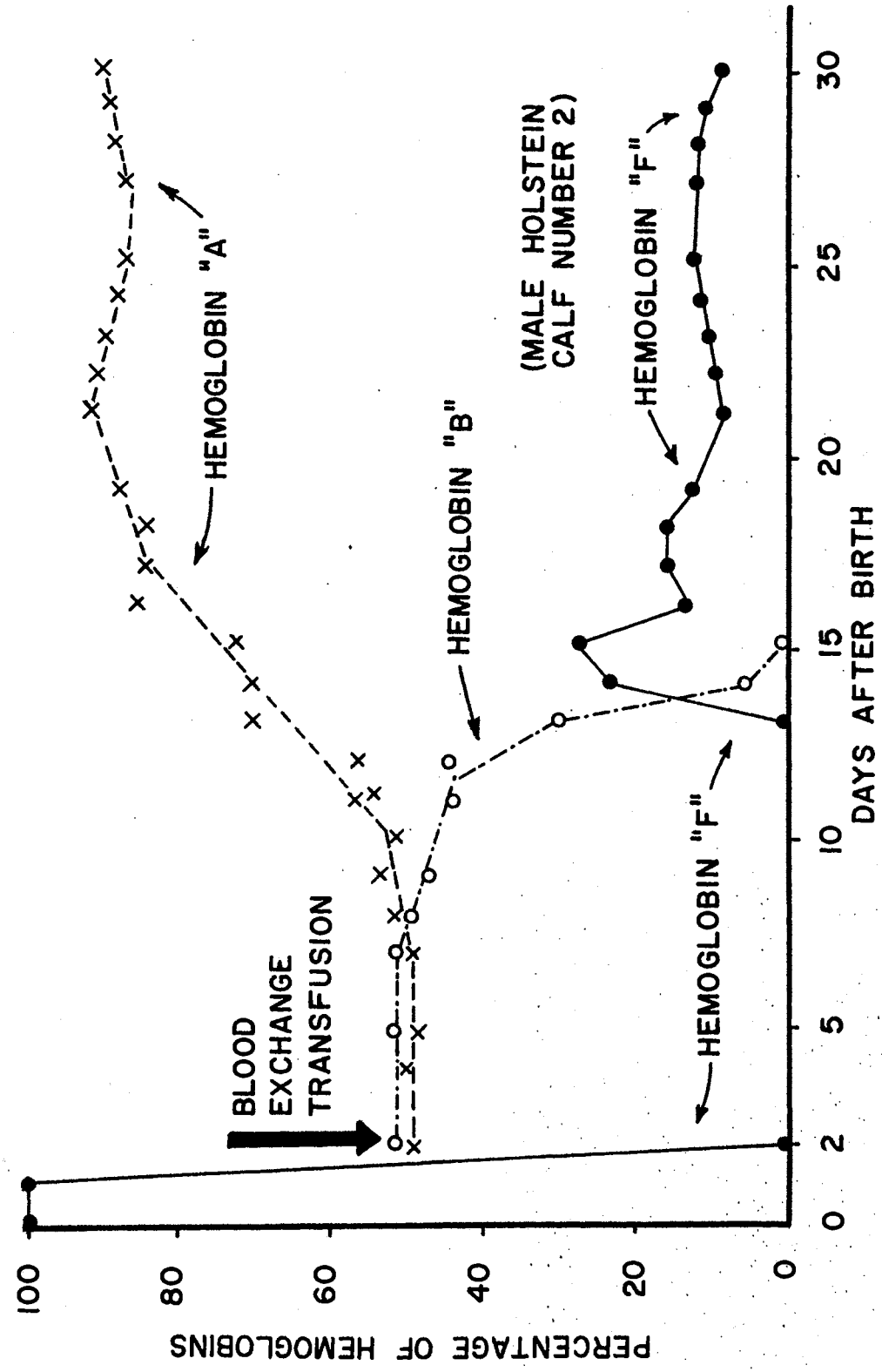


Figure 7. Electrophoretic Pattern of Bovine Hemoglobin Subunits in TEB-6M urea,2-mercapto-ethanol pH 8.6 Buffer.

Electrophoresis was conducted in Beckman Microzone Model R-101 cell. 500 volt. was applied for 20 minutes.

Figure 8. Variation of Hemoglobins in Exchange Transfused Calf (1).

Animal: Holstein male calf, No. 2. The blood of calf was exchanged with adult blood (Hb AB type). Each hemoglobin percent was determined as given in Figure 1 and 2. Data is listed in Table VIII (Appendix).


PERCENTAGE OF HEMOGLOBINS
100
80
60
40
20
0
0
2
5
10
15
20
25
30
DAYS AFTER BIRTH
(MALE HOLSTEIN
CALF NUMBER 2)
HEMOGLOBIN "A"
HEMOGLOBIN "B"
HEMOGLOBIN "F"
HEMOGLOBIN "F"
BLOOD
EXCHANGE
TRANSFUSION

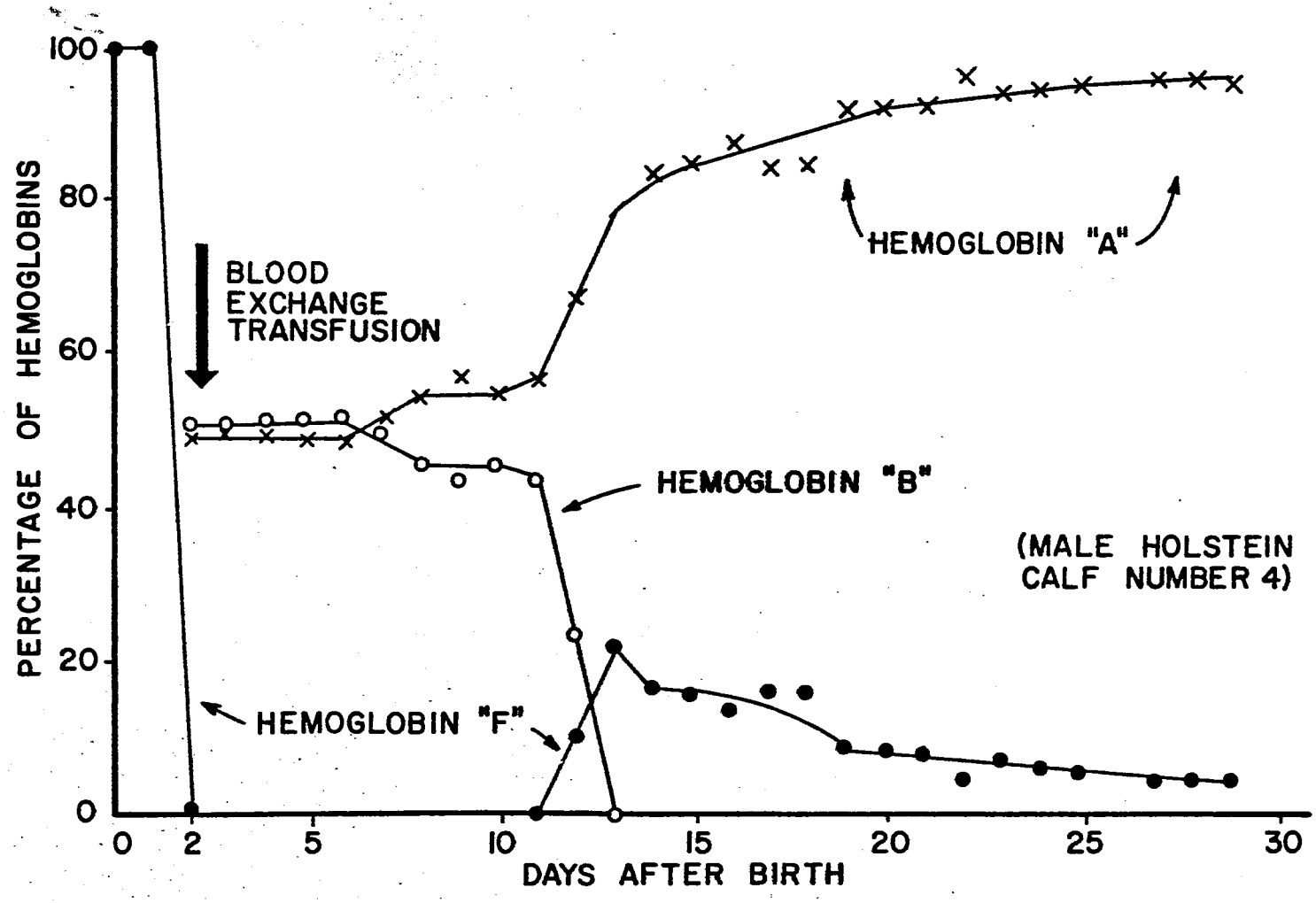


Figure 9. Variation of Hemoglobins in Exchange Transfused Calf (2). Conditions Are the Same as Figure 8.

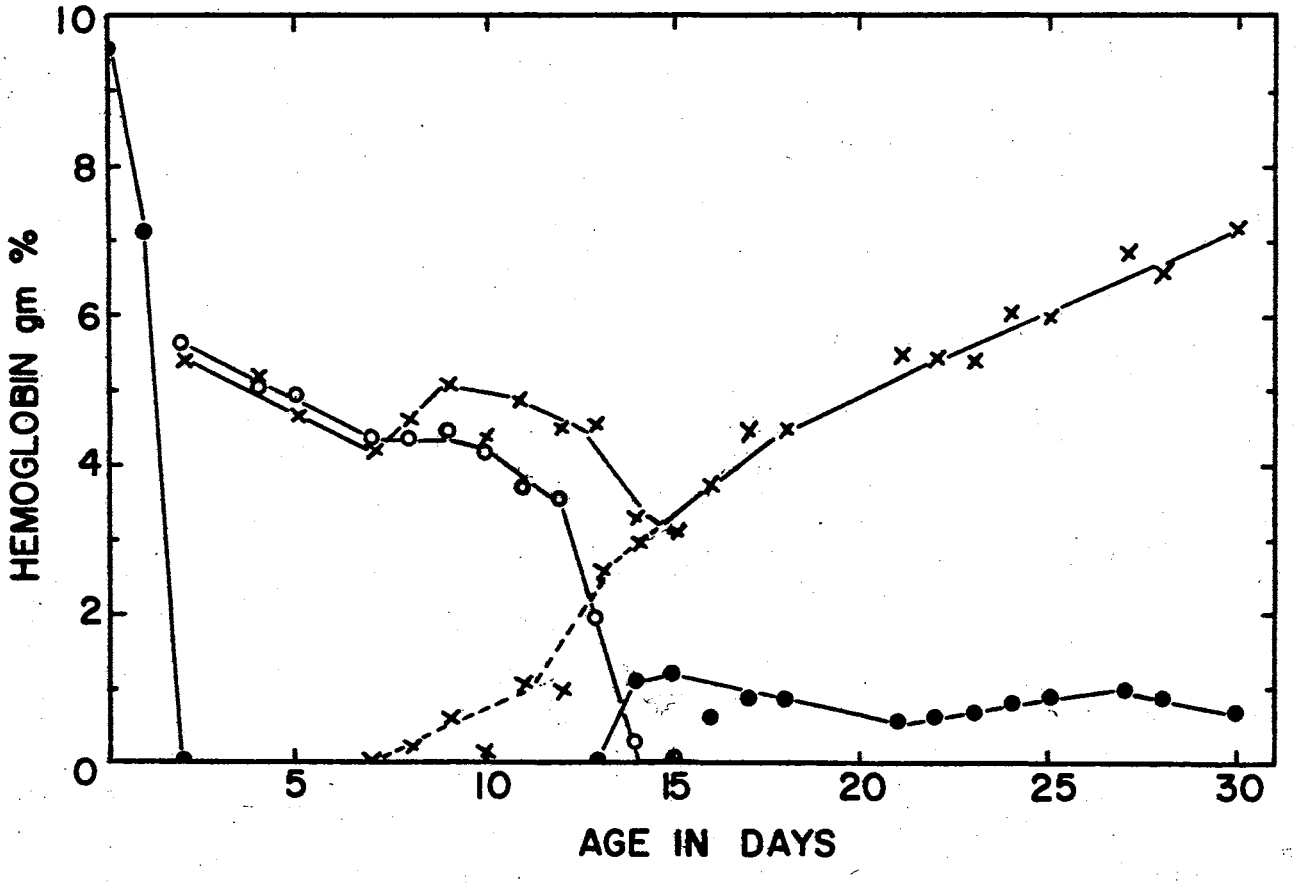


Figure 10. Exchange Transfusion Data (Figure 8) Expressed in Hemoglobin gm %.

Animal: Holstein male calf No. 2. (●——●): Hb F, (o——o): Hb B, (x——x): Hb A, (x---x): Hb A - Hb B.

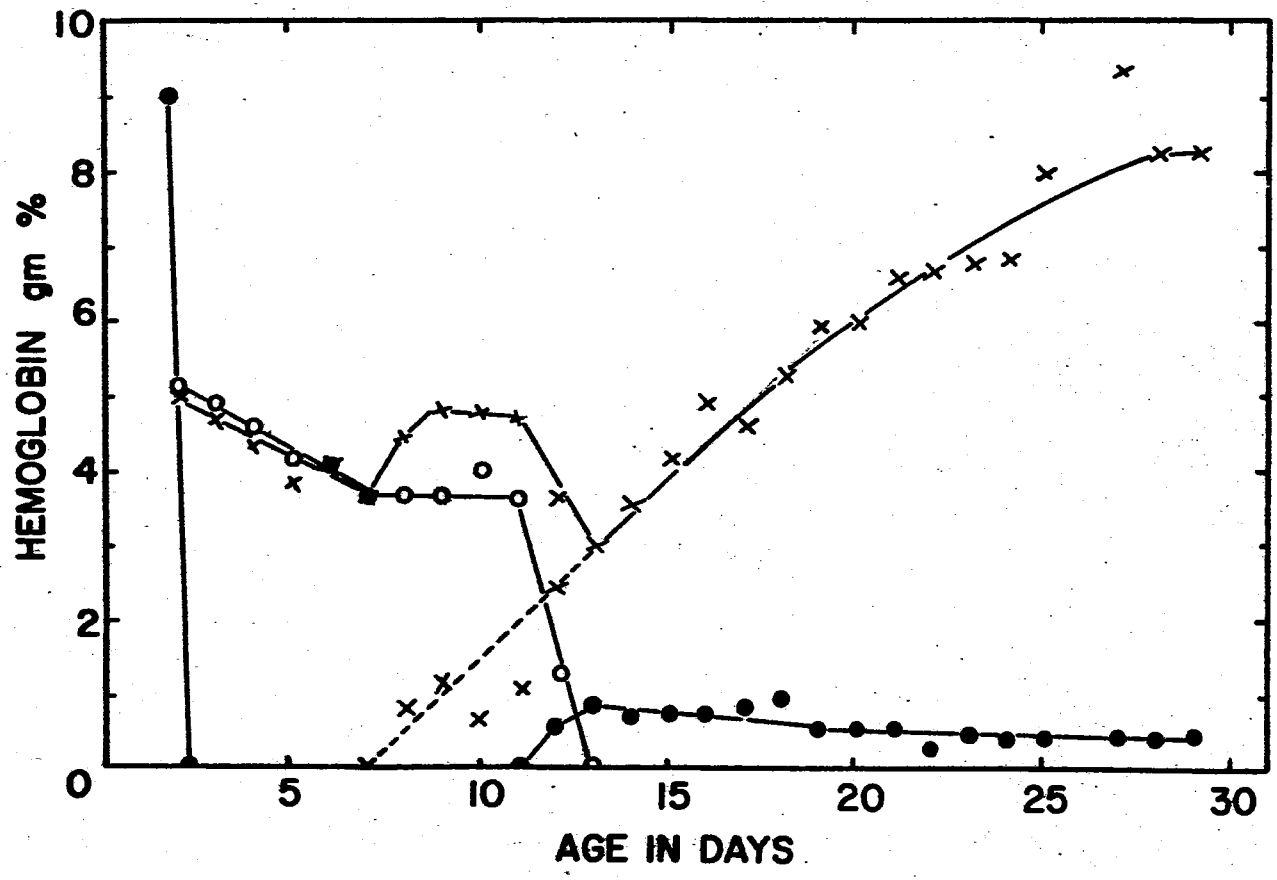


Figure 11. Exchange Transfusion Data (Figure 9) Expressed in Hemoglobin-gm %.

Animal: Holstein male calf No. 4. (●——●): Hb F, (o——o): Hb B, (x——x): Hb A, (x---x): Hb A - Hb B.

hemoglobin gm percent.

Exchange transfusion of a newborn Holstein calf with Hb A type blood was not complete due to jugular vein clotting and some Hb F was demonstrated in blood samples of post-transfusion. No rejections of the transfused cells could be detected during a 25 day study as indicated by no decrease in RBC, PCV and hemoglobin % (Figure 12).

Hemorrhage and Hemoglobin Change

On hemorrhage of 2 six-months old Hereford cows (Hb A type) to levels of 5.9 and 6.3 gm % of hemoglobin respectively, there was no evidence of change in hemoglobin type during the recovery from anemia. On hemorrhage of a 2 day old Angus calf to 40 gm % hemoglobin, the proportion of Hb F decreased and that of the Hb A increased in a manner similar to that of untreated young calves. Only slight increases of Hb F were observed after 25 to 31 days of age as seen in Figure 13, 14, Table X and XI (Appendix).

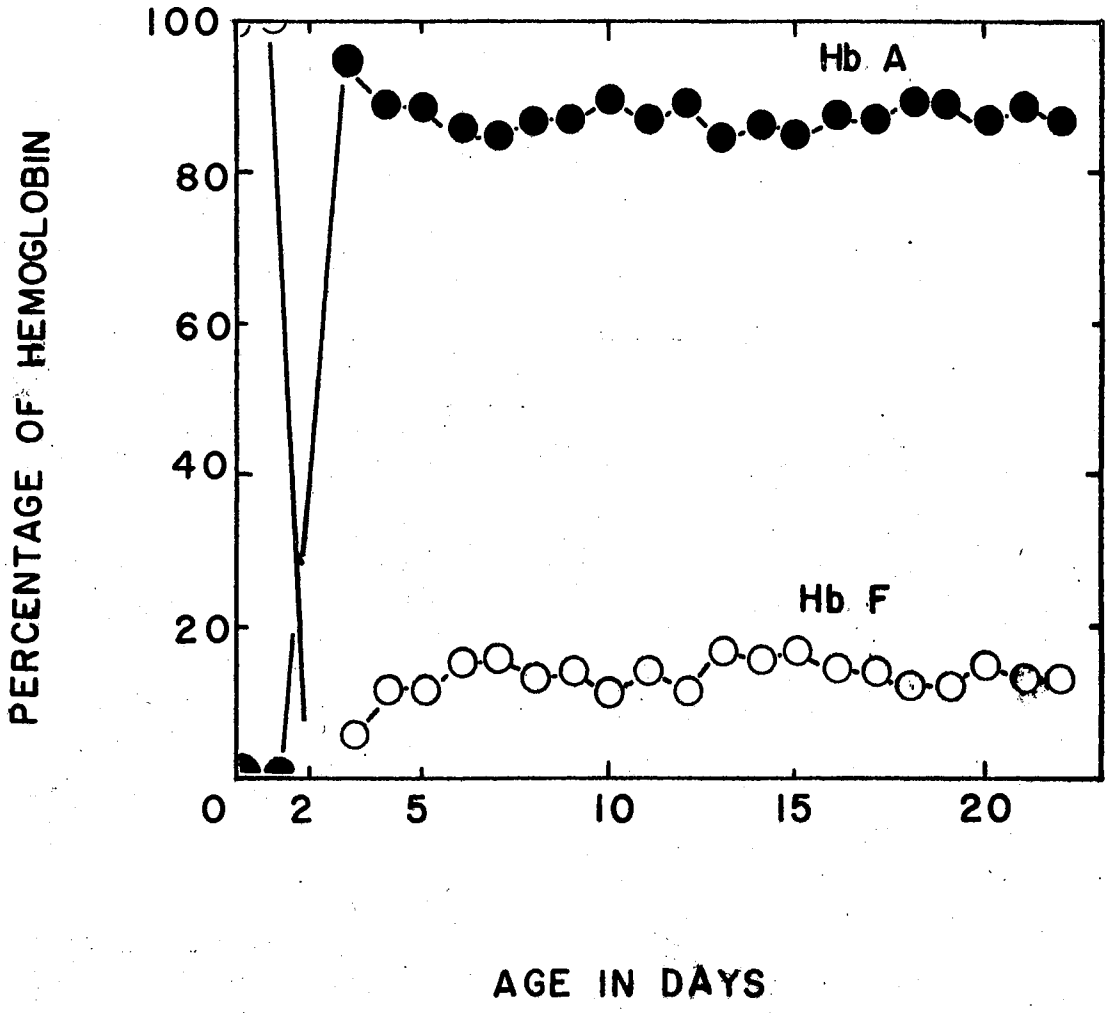


Figure 12. Variation of Hemoglobins in Exchange Transfused Calf (3).

Animal: Holstein male calf No. 5. The blood of calf was exchanged with adult blood (Hb A type). Each hemoglobin percent was determined as given in Figure 1 and 2.

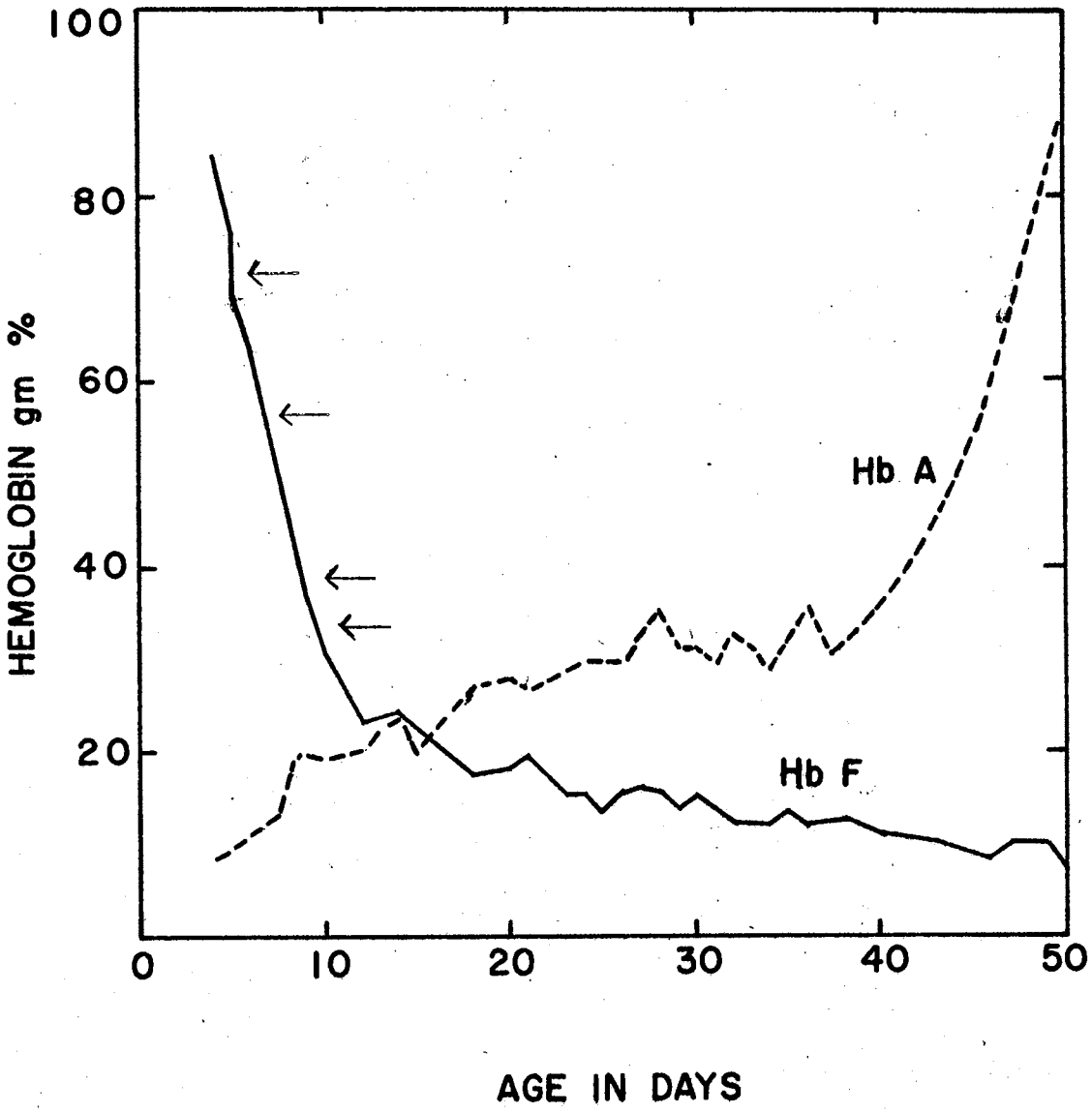
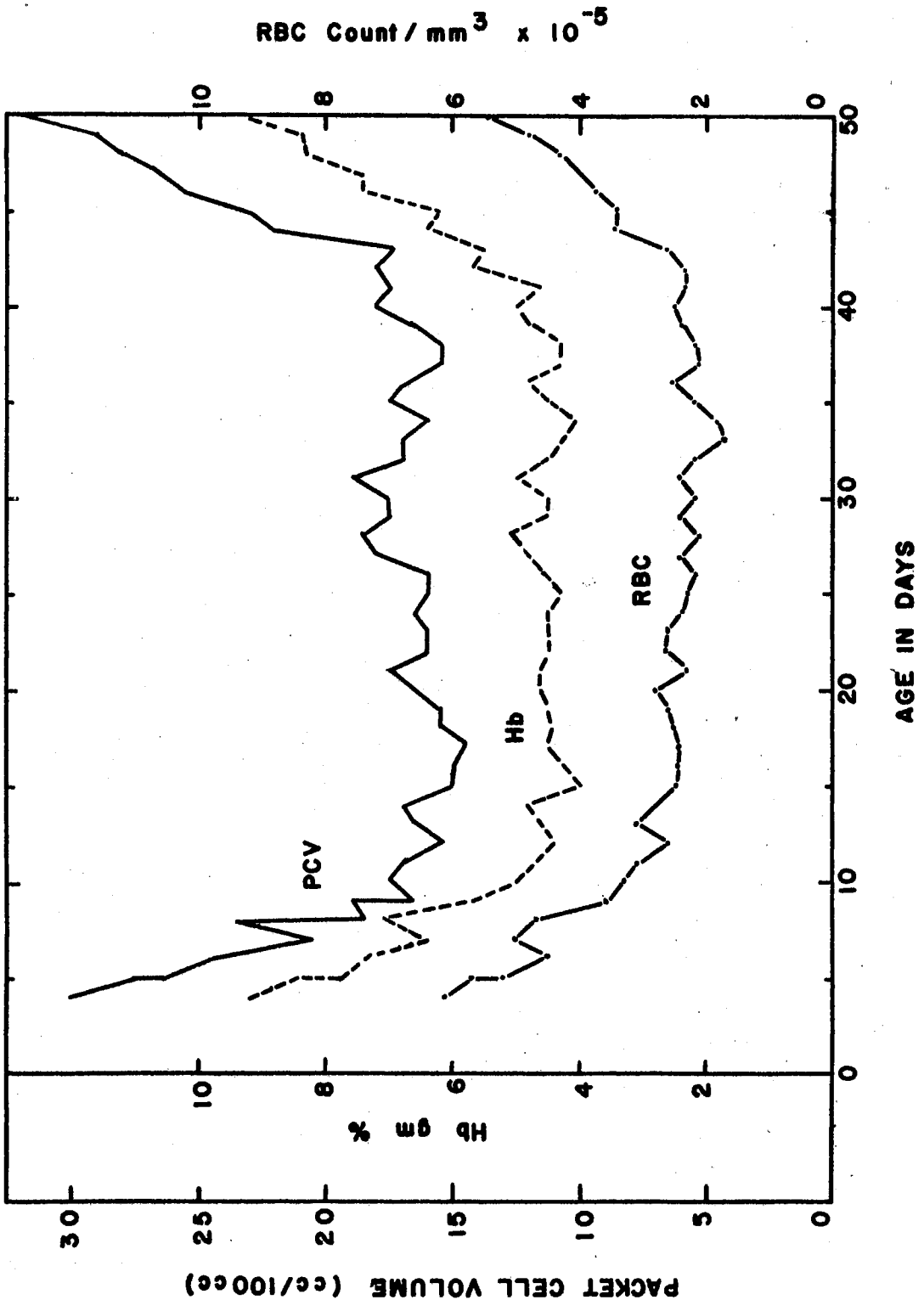


Figure 13. Hemoglobin A and F Changes in an Anemic Calf by Blood Loss.

Animal: Angus female calf No. 205 (⟵): Point of hemorrhage. 300 ml. of blood was withdrawn every point of hemorrhage. Hemoglobins were separated and determined as given in Figure 1 and 2. See Table X (Appendix) for data.

Figure 14. Hematologic Observations in an Anemic Calf by Blood Loss.

Packed cell volume (PCV), hemoglobin % and RBC count were determined daily during the anemic period. This data was obtained from the Veterinary Research, Oklahoma State University. See Table XI (Appendix) for data.


RBC Count / mm3 x 10-5
0
2
4
6
8
10
50
40
30
20
10
0
AGE IN DAYS
RBC
Hb
PCV
Hb gm %
PACKET CELL VOLUME (cc/100cc)
0
5
10
15
20
25
30

CHAPTER IV

ABSENCE OF FETAL HEMOGLOBIN IN THE NEWBORN DOG (BEAGLE)

It has been established that newborn infants are resistant to malaria (4) and newborn calves to anaplasmosis (8,9) and babesiosis (91). The fetal hemoglobin in newborn infants has been suggested as the cause of the resistance to malaria (4). It has been suggested that fetal hemoglobin in newborn calves may play a role in the resistance to anaplasmosis (12).

In contrast to newborn calves, newborn puppies are highly susceptible to babesiosis (16). The verification of presence of absence of a fetal hemoglobin in newborn puppies could give a valuable information which may aid in explanation of fetal hemoglobin resistance to this type parasitemia.

Experimental Procedure

Chemicals and Equipments

Ampholytes and model 8101 Isoelectric focusing column were purchased from LKB Instruments; trypsin from Calbiochem; pyridine from J. T. Baker Chemical Co.; n-butanol from Fisher Scientific Company. For high voltage electrophoresis of peptides, a varsol-cooled Savant high voltage electrophoresis tank and flat type electrophoresis cell were used.

Animals and Blood Samples

Adult and newborn dog blood samples (Beagle) were supplied in part by Veterinary Research, Oklahoma State University. Other adult and newborn dog blood samples were supplied from the Tri-Co Research Project, Inc., Kalamazoo, Michigan. The blood samples were refrigerated during the transportation, received within 24 to 48 hours after collection and treated immediately. Puppy blood samples were obtained by heart puncture of the newborn.

Methods

Alkali Denaturation of Hemoglobin

The alkali denaturation study of adult and newborn dog hemoglobins mostly followed the method described by Jonxis et al. (222). The concentration of sodium hydroxide solution used for the denaturation was 0.01 N instead of 0.06 N. The higher concentration of sodium hydroxide gave a too rapid denaturation of dog hemoglobin. Since equivalent concentrations of hemoglobin was desired in aliquots of same samples for the denaturation study, hemoglobin solutions were prepared and used rather than using whole blood. Hemoglobin solutions (0.01 ml) were diluted to 10 ml with water, 2 drops of 10% NH_4OH were added and the extinction was measured at 576 nanometers (E_B) as a control. Hemoglobin solutions were diluted with 10 ml 0.01 N NaOH, 2 drops NH_4OH were added and the extinction was measured at 576 nanometers each minute for 15 minutes (E_T). Then, the solution was placed in a water bath at 39° C for 15 minutes, cooled to room temperature and the extinction was measured at 576 nanometers (Ee). The quotient E_B/Ee should be constant.

The concentration of undenatured hemoglobin at a certain moment (U) was calculated by the following equation:

$$U(\%) = \frac{E_T - Ee}{E_B - Ee} \times 100$$

Isoelectric Focusing

An LKB isoelectric focusing column 8181 of 110 ml capacity was used. The top electrode was used as the anode and the bottom electrode as cathode. The cathode solution was prepared by dilution of 0.4 ml ethylenediamine or ethanolamine with 11 ml deionized water containing 12 g of sucrose which was slowly drained into the column. A dense solution was prepared by dilution of 7.5 ml of 8% carrier ampholytes to 42 ml of deionized water and 28 g of sucrose. A less dense solution was prepared by dilution of 2.5 ml of 8% carrier ampholytes to 60 ml with deionized water. Dense and less dense solutions were mixed using 2 buretts to yield a density gradient according to LKB 8100 Electrofocusing Equipment Instruction manual receiving each fraction (4.6 ml) in a test tube. A protein solution which had been dialyzed against deionized water to remove any salt and small ions present was added in a density gradient fraction replacing a corresponding volume of less dense solution. The amount of protein was 5 to 20 mg per zone. The gradient solution fractions were introduced into the column by slow drain starting from the most dense fraction. The anode solution was prepared by dilution of 0.2 ml H_3PO_4 or H_2SO_4 with 20 ml H_2O and introduced into the column. All of the steps in filling the column were done in a cold room with cooling water flow in operation for the column. Temperature of cooling water was 6° C throughout the operation. A carrier ampholyte solution of pH

range 6-8 was used. Initially 300 volt. were applied and increased to 500 V stepwise in a period of 2 hours. Maximum power at the beginning was not to exceed 3 watts. Five hundred volts was maintained about 24 hours and then decreased to 300 volts. Electrofocusing was continued about 12 more hours until the current through the column became constant. After the top electrode reservoir was closed and the electrode solution was removed using a syringe and fine tubing, the column contents were drained while collecting each 1 ml fraction in a fraction collector. A flow rate of 1-2 ml/min. was maintained. Absorbance of each fraction was measured by Beckman-DU Spectrophotometer and the pH was measured by Beckman or Fisher model 320 expanded scale research pH meter to determine isoelectric point of the protein.

Tryptic Digest of Globin and Peptide Mapping

Hemoglobin solutions were obtained by a modification of Drabkin's method and the protein concentration was determined. In order to precipitate the globin, each volume of hemoglobin solution containing 60 mg of the protein was poured slowly with stirring into 24 ml of 1.5% HCl in acetone previously cooled to -20^{o} C. The heme group remained in solution. Precipitated globin was washed 3 to 4 times with acetone to remove heme and then dried in a vacuumed dessicator (223). Performic acid was prepared as given by Nathans (224). For each 10 gm of protein, 0.5 ml of performic acid was added to oxydize free sulfhydryl groups. The solution was allowed to stand in room temperature for 20 minutes. It became a yellowish clear solution. An excess of water was added and the solution was lyophilized. The tryptic digest and peptide mapping were done with some modification of the method given by Beale (225).

The performic acid treated globin (60 mg) was dissolved in 6 ml of water and 2 drops of 10% NH_4OH were added to give a pH of 8.47. Several drops of 0.5 M NH_4HCO_3 were added and gave a pH of 8.2. Trypsin (0.6 ml, 2 mg/ml in water) was added and digestion carried out at 37° C for 3 hours with continuous stirring. The final pH was 8.25 to 8.29. The pH was then lowered to 6.4 with N acetic acid and the insoluble core peptides were precipitated and coagulated by heating the digest in a water bath at 90° C for 5 minutes. The precipitate was spun down at 36,500 x g for 30 minutes. The supernatant containing the soluble tryptic peptides were lyophilized to yield 34 mg of white material. Ten mg of the peptides were dissolved in 0.1 ml of water and 4.5 to 5.0 mg of peptides was applied to Whatmann 3 MM paper (47.2 x 54.5 cm) using a Spinco paper electrophoresis applicator. Four samples were applied on the same paper with even spacing (9.0 cm). The line of application was 12 cm from the short edge. The paper was wetted carefully with the electrophoresis buffer (pyridine-water-acetic acid, 25:225:1, by volume), blotted and hung in a Savant varsol cooled electrophoresis tank or flat type electrophoresis cell with the lines of application nearest to the anode. Electrophoresis was carried out with a potential gradient of 33 V/cm for 2 hours. The electropherograms were air dried and cut into 4 pieces leaving one sample on each paper strip. A new Whatman 3MM paper (42.0 x 54.5 cm) was sewn to each paper strip overlapping 1.5 cm with a serate edge design. (The strip was sewn two times, once for each paper edge and along the long edge nearest from the separated peptides.) Descending chromatography was then carried out for 10 hours with a solvent of butanol-acetic acid-pyridine-H_2O (15:3:10:12, by volume). All 4 chromatograms were developed in the same descending chromatography tank.

After the chromatograms were air dried, they were dipped into 0.02% (W/V) ninhydrin in acetone and dried in an oven at 80° C for 10 to 15 minutes to reveal the peptide spots.

Results

Electrophoretic Pattern of Canine Hemoglobin

Hemoglobins obtained by the modified Drabkin's method from adult and newborn dog (Beagle) blood samples were subjected to electrophoresis on cellulose acetate membrane using Tris-EDTA-borate buffer, pH 8.6 at 4° C. The number of animals screen tested were 27 mature and 17 newborn Beagles. Both mature and newborn hemoglobin solutions gave only one protein band with identical electrophoretic mobility. Mixture of both hemoglobins also gave one protein band. Figure 15 shows the electrophoretic mobility of newborn canine hemoglobin, adult canine hemoglobin and mixture of both hemoglobins in 6% polyacrylamide gel at pH 8.1. There was no difference on electrophoretic mobilities between newborn and adult canine hemoglobins.

Isoelectric Focusing of Canine Hemoglobin

The canine hemoglobins from adult and newborn gave one protein on isoelectric focusing with the same isoelectric point of 7.12 and sometimes 7.08. The electrofocusing of a mixture of adult and newborn canine hemoglobin gave one protein band and is shown on Figure 16. The elution pattern of the electrofusing column after canine hemoglobin stopped at its isoelectric point and pH profile are shown on Figure 17.

Hemoglobins from three adult and five newborn dogs (Beagle) blood samples were tested by isoelectrofusing.

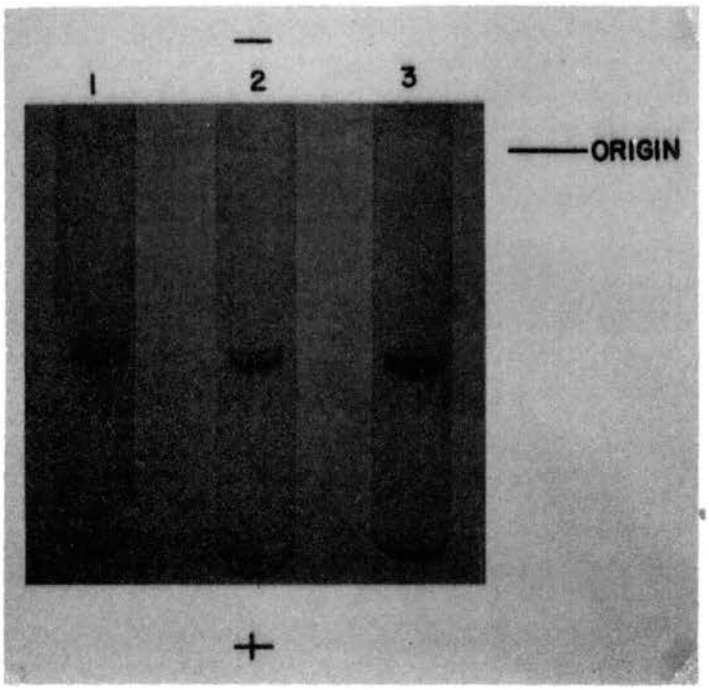


Figure 15. Electrophoretic Pattern of Adult and Newborn Canine Hemoglobins in Acrylamide Gel.

Electrophoresis was conducted in Hoefer electrophoresis cell. Electrophoretic conditions were 6% gel, Tris-glycine buffer pH 8.1, 5 ma per gel and 30 minutes at room temperature. 1: newborn canine hemoglobin, 2: adult canine hemoglobin, 3: mixture of 1 and 2.

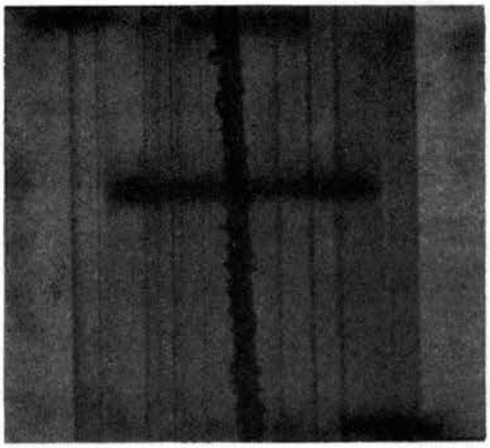


Figure 16. Isoelectric Focusing of an Adult and Newborn Canine Hemoglobin Mixture.

An equal amount of newborn and adult canine hemoglobins were mixed and 15 mg. of the protein mixture was introduced into the column. Instruments: LKB 8101 isoelectric focusing column and Savant H.V. 1000A/ power supply. Anode solution: 0.2 ml. H_3PO_4 in 20 ml. H_2O. Cathode solution: 0.4 ml. ethanolamine in 11 ml. H_2O. Temperature: 6^{o} C. Voltage: 300 V was applied initially, increased to 500 V stepwise in a period of 2 hours and maintained for 24 hours. Decreased to 300 V and electrofocusing was continued for 12 more hours.

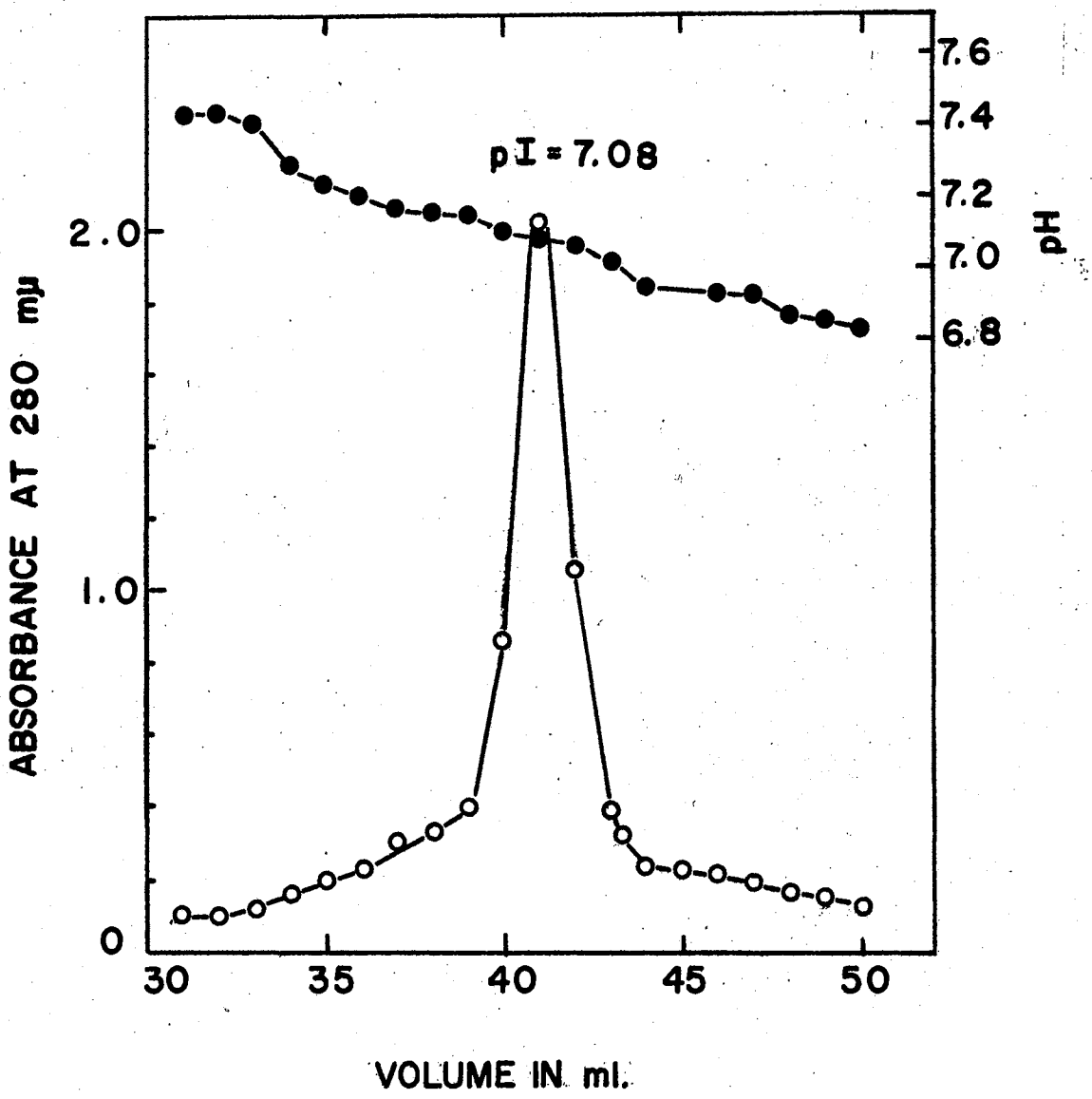


Figure 17. Isoelectric Point Determination for Canine Hemoglobin.

Sample: 15 mg. of hemoglobin from newborn Beagle dog. Electrofocused column (pH 6-8) was drained receiving 1 ml fractions by fraction collector. Absorbance (o——o) and pH (●——●) of each fraction were measured to find isoelectric point.

Alkali Denaturation Study

Five adult and 15 newborn dogs were studied. The results obtained with hemoglobins from 2 dams and 6 puppies (3 for each dam) are shown on Figure 18 and Table XII (Appendix). Mean values were plotted. The whole blood of adult and newborn dogs was also used for this study and gave results almost identical with those obtained for hemoglobin solutions.

Essentially, there was no difference between adult and newborn canine hemoglobins on alkali denaturation rate. The slight difference between two samples may represent the errors in experiment.

Tryptic Digest and Peptide Mapping

The identical electrophoretic mobility, isoelectric point and similar alkali denaturation rate do not necessarily mean that the two hemoglobins are the same. Peptide mapping of both proteins was attempted under identical conditions. The experiment was repeated several times until consistent results were obtained. Globins obtained from 7 adult and 6 newborn dogs were tested by this method. The two proteins gave identical peptide maps with 19 peptide spots as shown on Figure 19.

The above results verify that adult and newborn canine hemoglobins are the same.

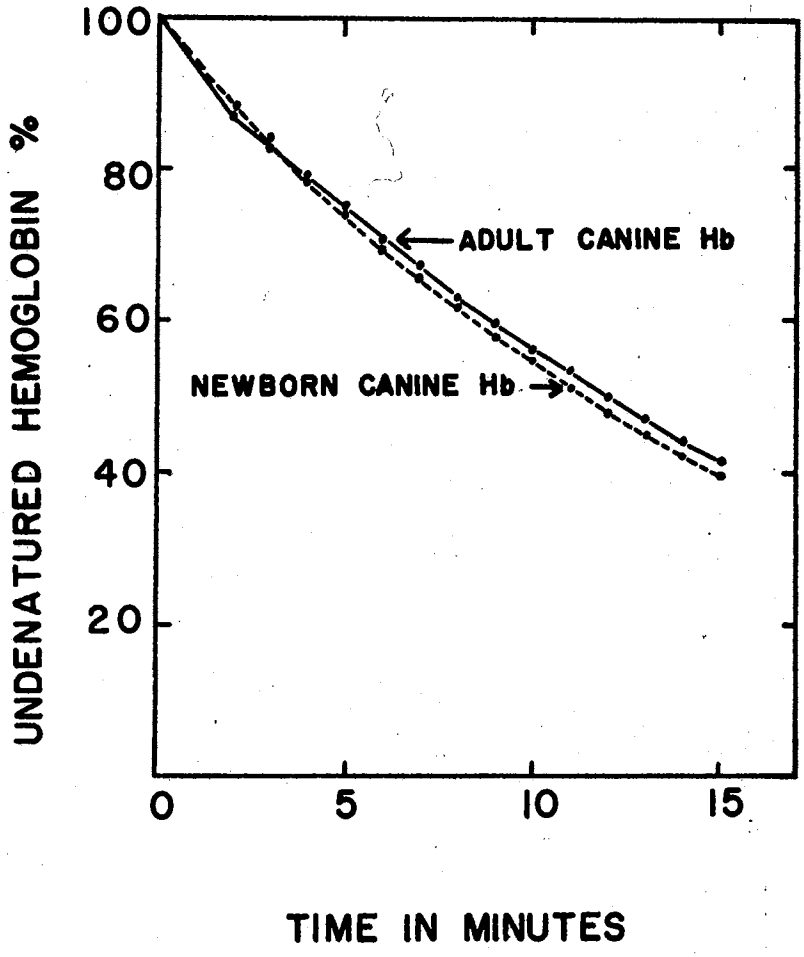


Figure 18. Alkali Denaturation Pattern of Canine Hemoglobins.

The results are mean values of hemoglobin solutions obtained from 2 dams and 6 newborn puppies (3 for each dam). Breed: Beagle. The data is listed in Table XII (Appendix).

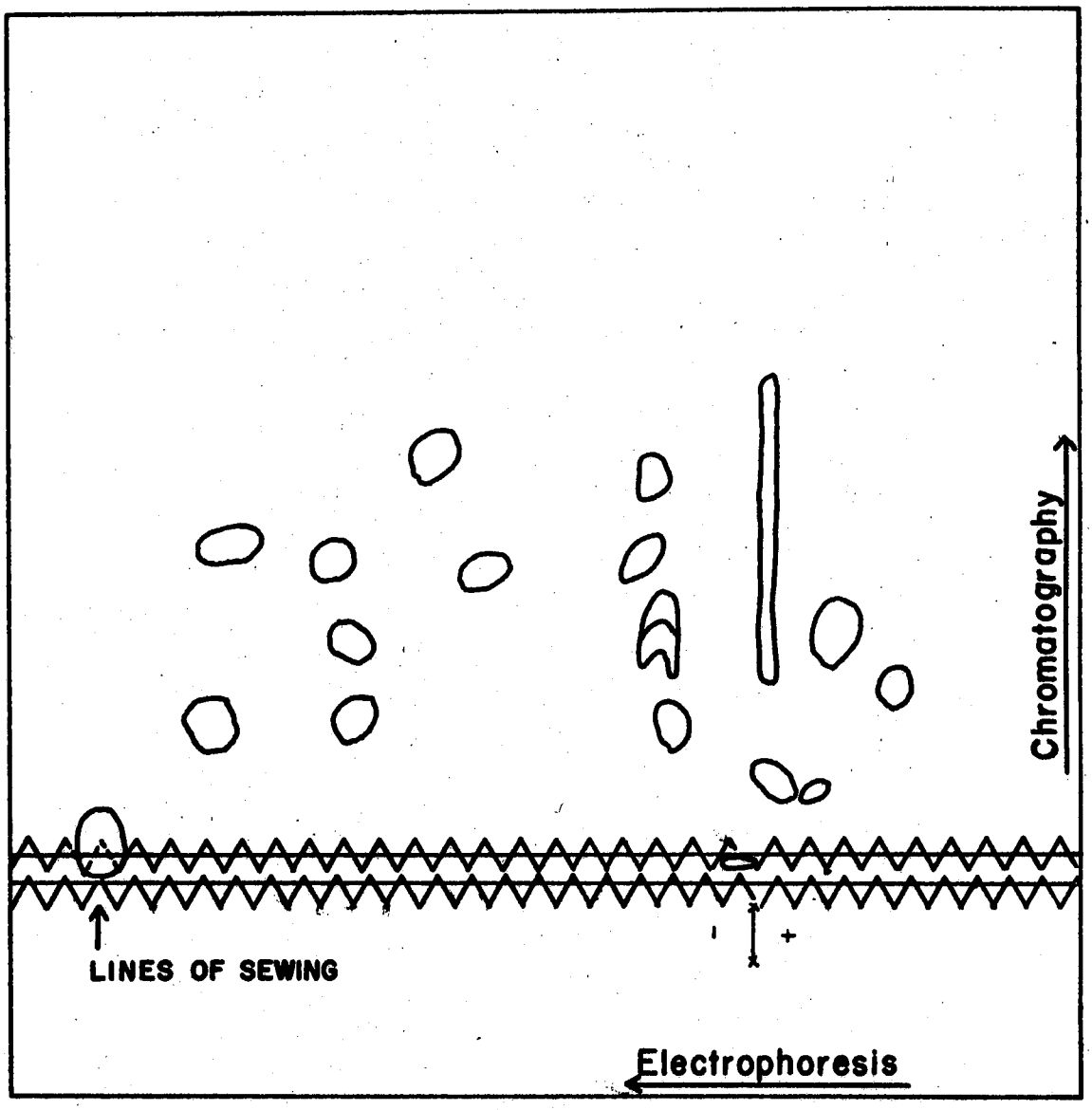


Figure 19. Peptide Map of Canine Globin After Tryptic Digest.

Performic acid treated canine globins were incubated with trypsin at 37° C and pH 8.2 for 3 hours. Electrophoresis of the resultant peptides was conducted in pyridine-water-acetic acid (25:225:1, by volume) on Whatman No. 3 paper applying 33V/cm for 2 hours. The electrophorogram was air dried, cut into 4 pieces leaving one sample on each strip. A new Whatman No. 3 paper was attached to each strip and second dimensional descending chromatography was carried out for 10 hours with a solvent of butanol-acetic acid-pyridine-H_2O (15:3:10:12, by volume).

CHAPTER V

STUDIES ON ERYTHROCYTE MEMBRANE PROTEINS AND TOCOPHEROLS

The erythrocyte membrane is the barrier of the red blood cell to the outside environment and is composed of proteins, lipids and carbohydrates. All of the blood parasites and drugs must penetrate the cellular membrane to grow and function inside of cell. The cellular membrane may allow selective penetration of some parasites and drugs, but may resist penetrations of other parasites and drugs. Some forms of innate resistance could be due to impermeability of cellular membranes to blood parasites. Therefore, the composition and construction of cellular membrane is of interest and importance. There have been no reports on membrane protein differences which may affect penetration of an A. marginale initial body between cow and newborn calf erythrocytes.

Tocopherols are known to have antioxidant activity. The composition of the membrane with respect to tocopherols may differ in calf and cow erythrocytes. It has not been established at this time if the fatty acid and tocopherol composition of erythrocyte membranes affect penetration of blood parasites.

Erythrocyte membrane proteins and tocopherols of the cow, A. marginale infected cow and the newborn calf were studied and are described in this chapter.

Experimental Procedure

Chemicals

Sodium dodecyl sulfate (SDS) was purchased from Sigma Chemical Co., St. Louis, Missouri and recrystallized from aqueous ethanol; Di and monobasic sodium phosphate from Fisher Scientific Co.; coomassie brilliant blue R-250 from Colab Lab, Inc., Chicago Heights, Illinois; activated charcoal, 6-14 mesh from Hoefer Scientific Instruments, San Francisco, California; α-tocopherol, N.F. from Cabiochem; β-tocopherol from Pierce Chemical Co., Rockford, Illinois; γ-tocopherol N.F. from Distillation Products Industries Division, Eastman Organic Chemicals, Rochester, N. Y.; Gas Chrom Q. and trimethylchlorosilane from Applied Science Laboratories, State College, Pa.; and squalene from K and K Laboratories, Plainview, N. Y.

Methods

Calf, cow and A. marginale infected cow (33 and 38% infected) blood samples were supplied by Veterinary Research, Oklahoma State University. Blood samples were withdrawn from animals by jugular venipuncture and received in tubes containing heparin or EDTA solution as anticoagulants.

Erythrocyte membranes from the above blood samples were prepared by the method suggested by Hanahan and his collaborators (206). Anticoagulant treated blood samples were centrifuged, plasma and the buffy leucocyte layer were removed by aspiration, and the erythrocytes were washed three times with 0.15 M (isotonic) NaCl solution. The cells were routinely centrifuged for 20 minutes at 1000 x g. All manipulations

were conducted at 0-4° C. The cells were resuspended in isotonic saline to a hematocrit of 60-80% and hemolyzed within 1 day for erythrocyte ghost preparation. Hemolysis was accomplished by mixing one volume of washed red cell suspension into 14 volumes of 20 ideal milliosmolar (isOsm) phosphate buffer, pH 7.4. The contents were mixed by gentle swirling and centrifuged at 35,000 x g in a Servall RC-2 centrifuge for 20-30 minutes. Four to six additional washes with 20 imOsm, pH 7.4, phosphate buffer followed to remove hemoglobins in the red cell stroma. The final product was a white stroma without any pink or yellow color. As before, all the manipulations were carried out at 0-4° C. The prepared stroma, which was essentially free from hemoglobins, was lyophilized with a minimum amount of the above phosphate buffer for a membrane protein study. For the tocopherol analysis, the prepared stroma was washed two additional times with deionized water and lyophilized.

Erythrocyte ghosts from a normal and an A. marginale infected cow were stained with Wright's stain following the procedure described by Schalm (226). Anaplasma marginale infected cow erythrocytes were stained similarly. Four to five drops of 0.07% Wright's stain in absolute methanol were added to erythrocyte or stroma on a slide and allowed to act 3 minutes. An equal amount of 0.133 M phosphate buffer, pH 6.6 was added and mixed thoroughly. Then the dilute stain was permitted to act for 5 minutes. The slide was washed with distilled water, dried under a lamp and mounted for microscopic examination.

For each 1 mg of lyophilized ghost, 80 microliters of 3% SDS - 0.1 M phosphate buffer pH 7.8, 20 microliters of glycerol and 1.1 microliter of a 2-mercaptoethanol were added. This mixture was incubated at room temperature for 18 hours to solubilize membrane proteins. The membrane

protein solutions obtained were subjected to electrophoresis in 0.1 M phosphate - 0.1% SDS buffer pH 7.2 in the Hoefer electrophoresis cell. The polyacrylamide separating gel (6%) was prepared in a 11.3 x 0.5 cm column using the electrophoretic buffer as solvent and pre-electrophoresed for 45 minutes applying 8 milliampere per column to remove excess persulfate. One to two hundred microliters of the membrane protein solution in glycerol was applied to the column. Electrophoresis was for $2\frac{1}{2}$ hours applying 8 m.a. per column, stained with 0.025% coomassie brilliant blue in 7% acetic acid for 8 hours and then destained with 7% acetic acid with continuous stirring in Hoeffer diffusion destainer (209). Reference proteins with known subunit molecular weights were electrophoresed in SDS according to the procedure given by Weber and Osborn (227). Relative mobilities were determined and plotted in semi-log scale. The molecular weights of membrane proteins which appeared in the gels were determined by comparing the relative mobilities with those of reference proteins.

The destained gels were scanned at 590 nanometers with a Gelman gel scanner, Gelman 2410 Linear transport, attached to Beckman D.U. spectrophotometer and Gelman recorder.

For detection of carbohydrates, a method described by Zacharius et al. (210) was employed. Membrane proteins electrophoresed in an SDS-acrylamide gel (11.3 x 0.5 cm) as previously described, were immersed in 12.5% trichloroacetic acid for 30 minutes, rinsed lightly with distilled water and immersed in 1% periodic acid (made in 3% acetic acid) for 50 minutes to oxidize the carbohydrates. The gel was washed overnight in 200 ml distilled water with stirring and a few changes of distilled water. The gel was then immersed in fuchsin-sulfite in the dark for 50

minutes and washed with freshly prepared 0.5% sodium metabisulfite 3 times for 10 minutes each. The gel was then transferred to water. Immediate note was taken of the position of the pink carbohydrate bands before the pink or reddish background color developed as suggested by Dulaney and Touster (228).

In the tocopherol analysis of erythrocyte membranes, tocopherols were extracted by the method given by Slover et al. (215) with a slight modification as suggested by Rudolph (229). A gas chromatographic method of analysis was employed. Ten milligrams of lyophilized stroma were placed in a 2-neck flask and 25 ml of ethyl alcohol and 0.5 ml of 10% alcoholic pyrogallol were added. This mixture was heated in a nitrogen atmosphere for 5 minutes at 55-60° C. One ml of saturated KOH was added to saponify lipids, boiled and refluxed gently 8-10 minutes. The mixture was then cooled in an ice bath and 25 ml of petroleum ether (Skelly-F) and deionized water were added. The mixture was shaken in a separatory funnel, and the aqueous layer was separated. The extraction step was repeated with 25 ml petroleum ether, and the two petroleum ether fractions were combined. The extract was further washed 4 times with 25 ml of deionized water and 2 ml of 10% alcoholic pyrogallol. This extract was placed in a rotary boiling flask and evaporated to dryness in hot water and under vacuum by aspiration. The residue was washed with benzene 2-3 times, and the benzene was evaporated in a N_2 atmosphere.

Trimethylsilyl (TMS) ethers of the tocopherols were prepared by adding 0.1 ml of a mixture of hexamethyldisilazane-trimethylchlorosilane-anhydrous pyridine (9:6:10, by volume). After standing at least 15 minutes, 3 μl of the TMS ether solution was injected onto a 4% OV-17

(methyl phenyl silicon) or 3% OV-1 (methyl silicon) gas liquid chromatography column. The GLC OV-1 column was stabilized at an isothermal temperature (225° C) for several hours prior to use. An internal standard, squalene in benzene, was added to the tocopherol extract prior to the evaporation of benzene step in the procedure. Eack peak on a gas chromatogram was identified by comparing retention time with standard α-, β- and γ-tocopherol derivatives and comparison with the data of Rudolph (229).

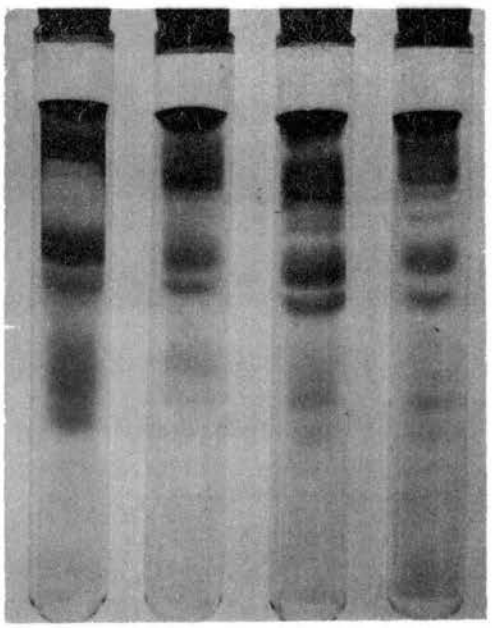
Results

Microscopic Examination

Anaplasma marginale infected erythrocytes and red cell stromata prepared from A. marginale infected erythrocytes showed a number of A. marginale bodies. These findings are comparable to the results reported by Wallace et al. (169). These results suggest that A. marginale bodies are membrane bound in erythrocytes.

Electrophoretic Studies of Erythrocyte Membrane Proteins

The membrane proteins of uninfected calf and cow and A. marginale infected cow erythrocytes were electrophoresed in SDS-acrylamide gel columns with the results shown in Figure 20. Four major and 3 to 8 minor proteins were observed from the above samples. Anaplasma marginale infected erythrocyte membrane gave four more protein bands than uninfected cow erythrocyte membranes with molecular weights of 320,000, 71,500, 51,500 and 29,500 respectively. The molecular weight of each protein was estimated by use of a standard molecular weight curve (Figure 21) prepared according to the procedure given by Weber and



1 2 3 4

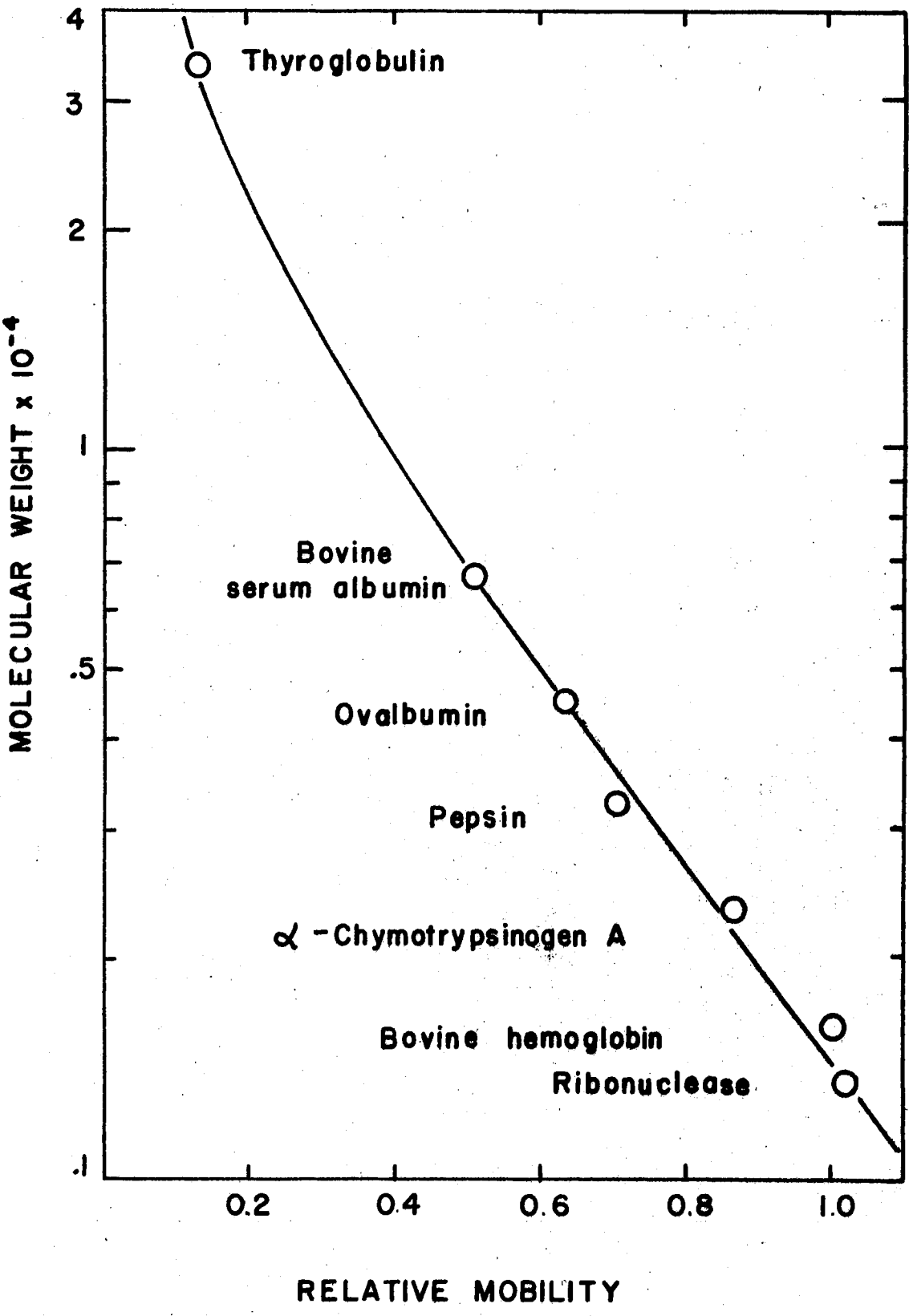
Figure 20. Electrophoretic Pattern of Bovine Erythrocyte Membrane Proteins in SDS-polyacrylamide Gel (6%).

1, calf; 2 and 3, cows; 4, A. marginale infected cow. Electrophoresis was conducted in 0.1 M phosphate buffer with 0.1% SDS, pH 7.2, applying 8 ma per gel for $2\frac{1}{2}$ hours at room temperature.

Figure 21. Electrophoretic Mobilities of Various Protein Subunits in SDS-polyacrylamide Gel.

Proteins were incubated at 37° C for 2 hours in 0.01 M phosphate buffer, pH 7.0, 1% in SDS, and 1% in 2-mercaptoethanol. Electrophoresis was conducted in 0.1 M phosphate buffer pH 7.1 with 0.1% SDS. Relative mobility to tracking dye, Bromphenol blue, was calculated as follows:

$$\text{Relative mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before destaining}}{\text{distance of dye migration}}$$


Thyroglobulin
Bovine serum albumin
Ovalbumin
Pepsin
α -Chymotrypsinogen A
Bovine hemoglobin
Ribonuclease
MOLECULAR WEIGHT x 10⁻⁴
4
3
2
1
.5
.1
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

Osborn (227). The reference proteins incubated in both 1% and 3% SDS gave identical electrophoretic mobilities. The percentage of each protein in the coomassie brilliant blue stained gel was estimated by densitometric scanning (Figure 22). There was some variation of composition of membrane protein for different cows. Molecular weight and per cent of erythrocyte membrane proteins for the gels shown in Figure 20 are tabulated in Table II. There was no difference in membrane proteins between newborn calf and cow erythrocyte in qualitative sense. Two carbohydrate bands have been detected (Figure 23) in the uninfected calf, uninfected cow, and A. marginale infected cow erythrocytes stromata as reported in human erythrocytes (198). The slower moving band of the two would denote glycoprotein and corresponded to the 120,000 molecular weight band. The faster moving and broader band would be glycolipid as in human erythrocytes (198).

Studies on Tocopherols

The gas-liquid chromatographic analysis of TMS ethers of α-, β-, and γ-tocopherols along with the internal standard, squalene, on a 3% OV-1 column gave a clear separation of the above compounds as shown in Figure 24. Calf, cow and A. marginale infected cow erythrocyte membranes (2 samples each) demonstrated the presence of α-tocopherol by GLC analysis. Calf erythrocyte ghosts have a higher amount of α-tocopherol than cow erythrocyte ghosts. A trace amount of β-tocopherol was detected in calf, cow and A. marginale infected erythrocyte membranes (Table III).

The GLC tracings of TMS ethers of tocopherols in the above samples and standard tocopherols were reproducible with about $\pm$ 5% error.

TABLE II

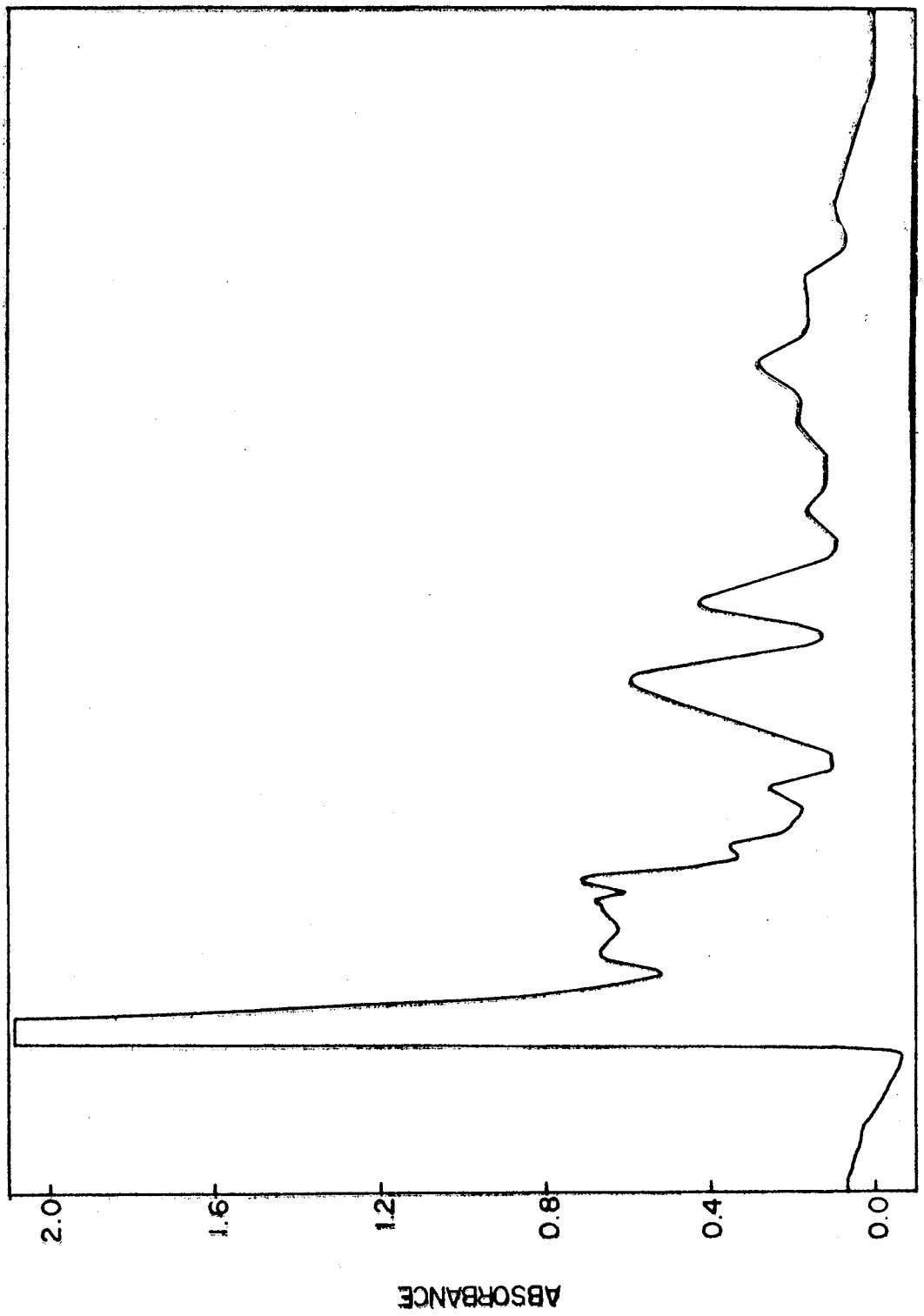
ESTIMATION OF MOLECULAR WEIGHT AND PERCENTAGE OF ERYTHROCYTE MEMBRANE PROTEINS BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Molecular weight	Percentages from Gel Scan			
	Calf	Cow (1)	Cow (2)	Infected cow
-		6.2		
320,000				12.9
279,000	17.1[1]	25.2	26.2	11.2
247,000	6.3[1]	6.7	9.5	9.3
231,000	1.6[1]	4.5	3.1	5.7
196,500			3.0	3.6
185,000			3.8	
120,000	28.3	30.7	24.5	20.2
95,000	10.5	12.6	11.9	10.7
71,500				4.3
51,500				4.1
44,700	12.2	11.3	13.0	7.4
37,300	9.2	2.8	5.1	6.3
29,500				4.1

[1]These proteins may have higher molecular weights as exact correspondence was not attained.

Figure 22. Densitometric Scan of Erythrocyte Membrane Proteins Separated in SDS-polyacrylamide Gel.

Sample: A. marginale infected erythrocyte membrane proteins.
Instrument: Gelman 2410 linear transport attached to Beckman D.U. spectrophotometer and Gelman Recorder.
Wave length: 590 nanometers. Scan rate: 1 cm/minute.


ABSORBANCE
2.0
1.6
1.2
0.8
0.4
0.0
PROTEINS SEPARATED

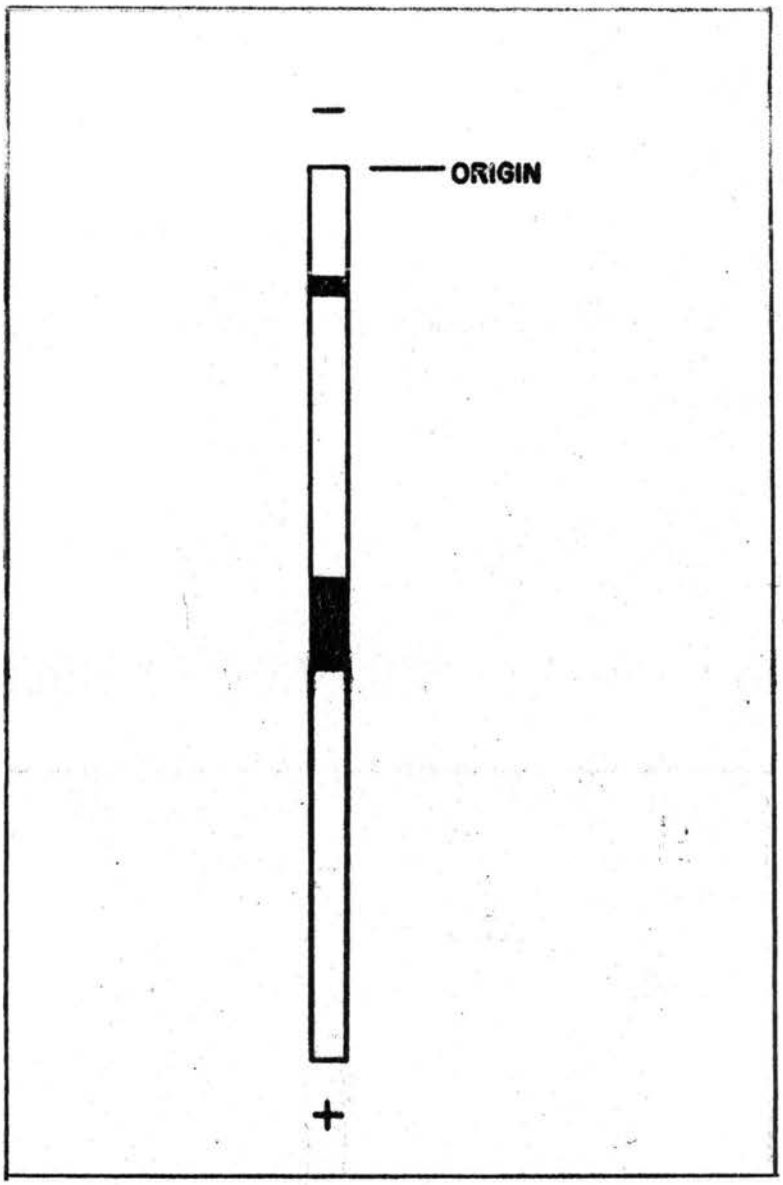


Figure 23. Carbohydrates in Bovine Erythrocyte Membrane.

Electrophoresis was conducted in 0.1 M phosphate buffer with 0.1% SDS, pH 7.2, applying 8 ma per gel for $2\frac{1}{2}$ hours. Carbohydrates were detected by periodate-fuchsin sulfite reaction.

Figure 24. GLC Tracing of α-, β-, and γ-tocopherol TMS Ethers and the Squalene Internal Standard.

Column - 6' x 1/4" glass, silanized.

Column packing - 3% OV-1 on Gas Chrom Q.

Column temperature - 225° C.

Injection port temperature - 265° C.

Detector temperature - 202° C.

Detector - H_2 flame.

Carrier gas - N_2.

Flow rate - 75 ml./min.

Hydrogen press. - 20 lbs.

Air press. - 40 lbs.

N_2 press. - 75 lbs.

Instrument - Perkin Elmer 801.

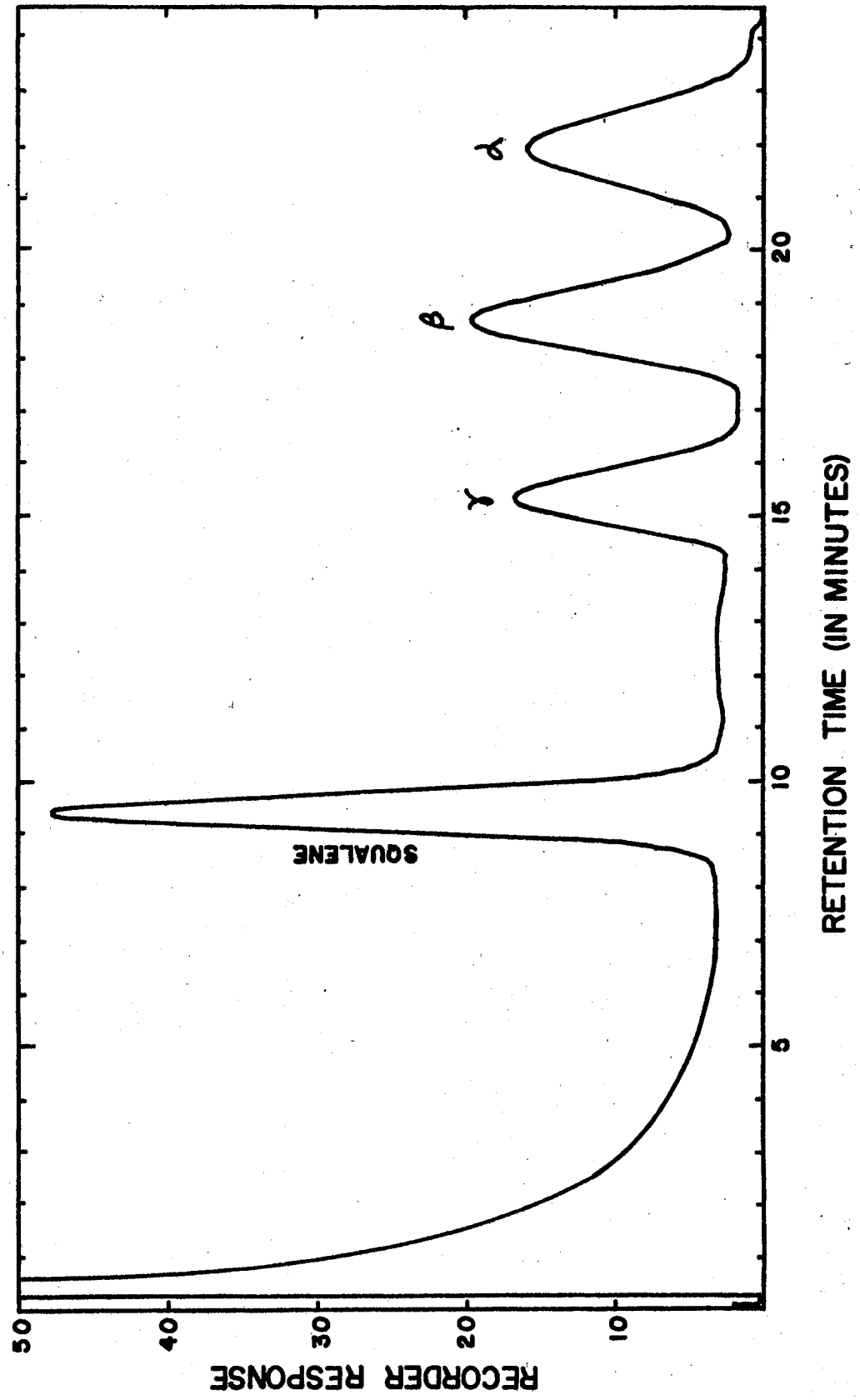

SQUALENE
γ
β
δ
RECORDER RESPONSE
50
40
30
20
10
5
10
15
20
RETENTION TIME (IN MINUTES)

TABLE III

TOCOPHEROL CONTENT OF BOVINE ERYTHROCYTE MEMBRANES

Sample	α-tocopherol	β-tocopherol
	μg/mg membrane	
Calf	1.78 $\pm$ 0.22	trace
Cow	0.94 $\pm$ 0.39	trace
Infected cow	1.16 $\pm$ 0.36	trace

CHAPTER VI

ANAPLASMA MARGINALE INFECTION IN NEWBORN BOVINE[1]

Quantitative variation of fetal hemoglobin in the newborn bovine has been established. There was no significant increase or decrease of bovine Hb A and F % in circulating blood of a hemorrhaged newborn calf (Chapter III). Erythrocyte membrane protein studies revealed that there is no marked difference between calf and cow RBC membrane proteins. Studies on antioxidant protection by tocopherols of fatty acids did not give any clue that tocopherol composition in calf RBC membrane plays a role in resistance to anaplasma infection (Chapter V).

It has been suggested that certain hemoglobin types of animals may provide resistance to hemotropic parasitism, such as bovine Hb F to anaplasmosis (12) and bovine Hb AB to trypanosomiasis (14). The newborn canine is highly susceptible to Babesia canis infection (18). The absence of a fetal hemoglobin in newborn canis has been established (Chapter IV). In this view, the variation of bovine Hb F and A in A. marginale infected calves have been studied to attempt to find a role of Hb F in anaplasmosis.

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[1]This study is collaborated work with Dr. E. W. Jones' group at Veterinary Research, Oklahoma State University and statistical evaluation was done by Drs. Ian Anderson, Veterinary Research, and Robert Morrison, Mathematics and Statistics Department, Oklahoma State University, Stillwater, Oklahoma.

Experimental Procedure

Animals and Methods

Five intact male Holstein calves were infected with A. marginale within 48 hours of birth by injecting 0.5 cc of infected blood showing 31% parasitized erythrocytes. Hematologic observations, recorded 3 times weekly during the prepatent period and daily during patent disease, included PCV, RBC, hemoglobin gm %, reticulocyte counts and percentage of parasitized erythrocytes. Hemoglobins were analyzed by cellulose acetate electrophoresis.

Result

Variations of hemoglobin A and F in the 5 male Holstein calves infected with A. marginale are shown in Figure 25 to 29 along with the percentage of the infected erythrocytes.

The quantities of fetal hemoglobin diminished and adult hemoglobin increased at expected normal rates during the prepatent period. The Anaplasma marginale began to appear in blood specimens 26 to 34 days after the inoculation. At this point, mean fetal hemoglobin concentration was 37.53% (range: 7.48 - 46.45%) in the calves. Onset of patent parasitemia was characterized by decreased hematocrit, accelerated net Hb F removal and marked check in net Hb A rate of increase. At the maximum point of infection, mean fetal hemoglobin concentration was 25.23% (range: 5.63 - 38.10%). The decreases of Hb F in normal and A. marginale infected calves are compared in Figure 30.

Hemoglobin A, which should increase continuously to approach 100%, decreased during the patent parasitemic period. The slope change of Hb

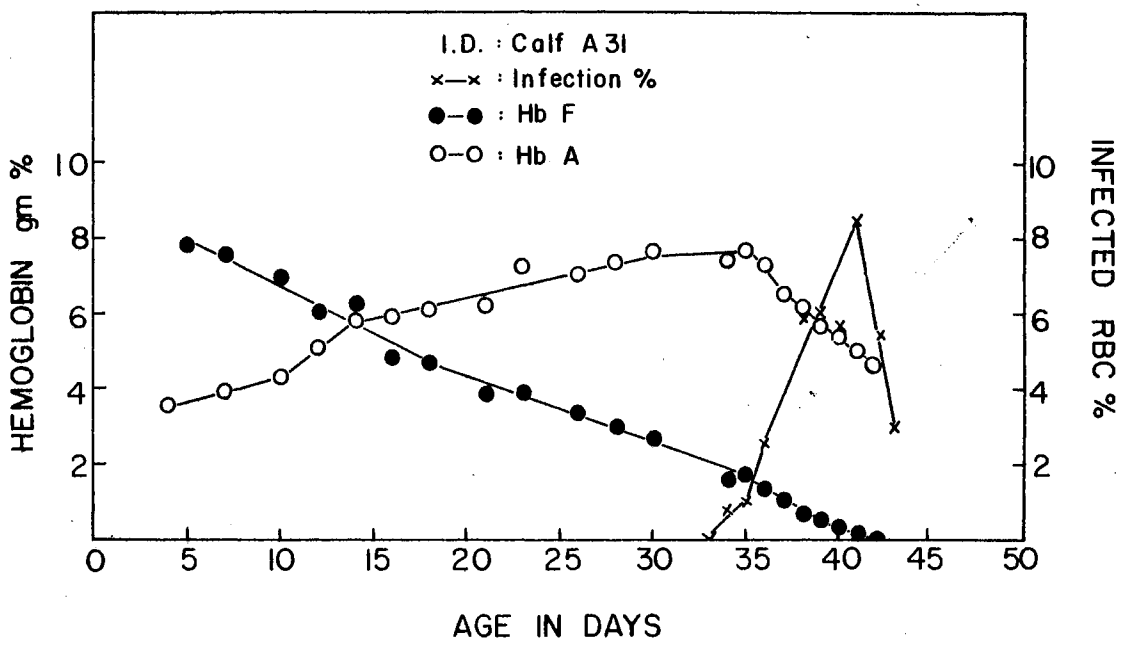

Figure 25. Variation of Hemoglobins in A. marginale Infected Calf (1). See Figure 1 and 2 for Separation and Determination of Bovine Hemoglobin A and F.

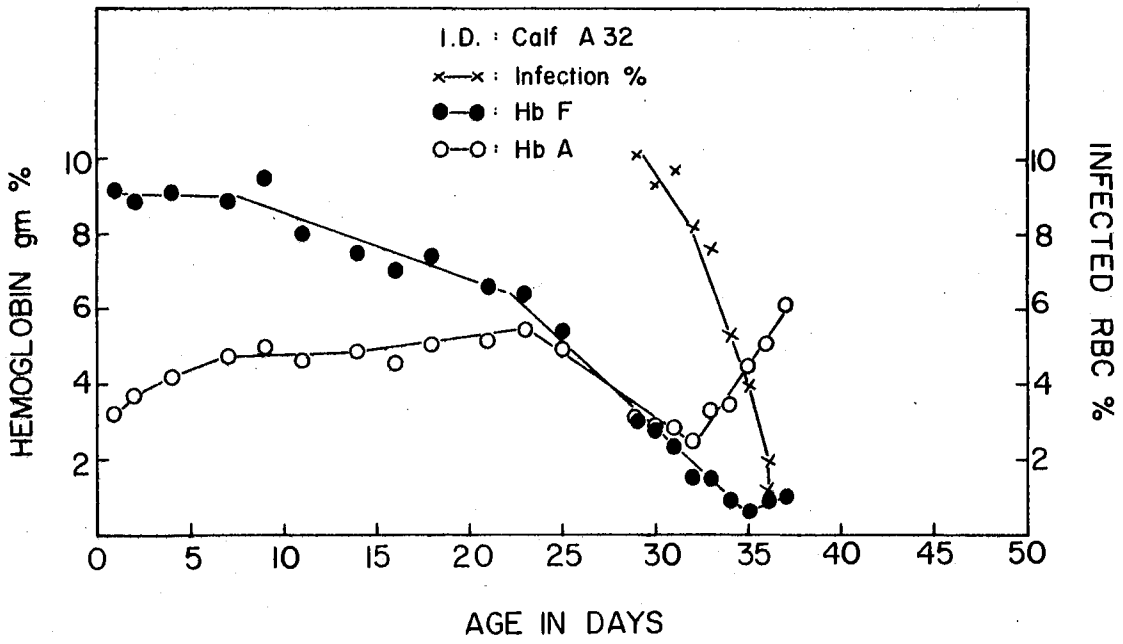

Figure 26. Variation of Hemoglobins in A. marginale Infected Calf (2). See Figure 1 and 2 for Separation and Determination of Bovine Hemoglobin A and F.

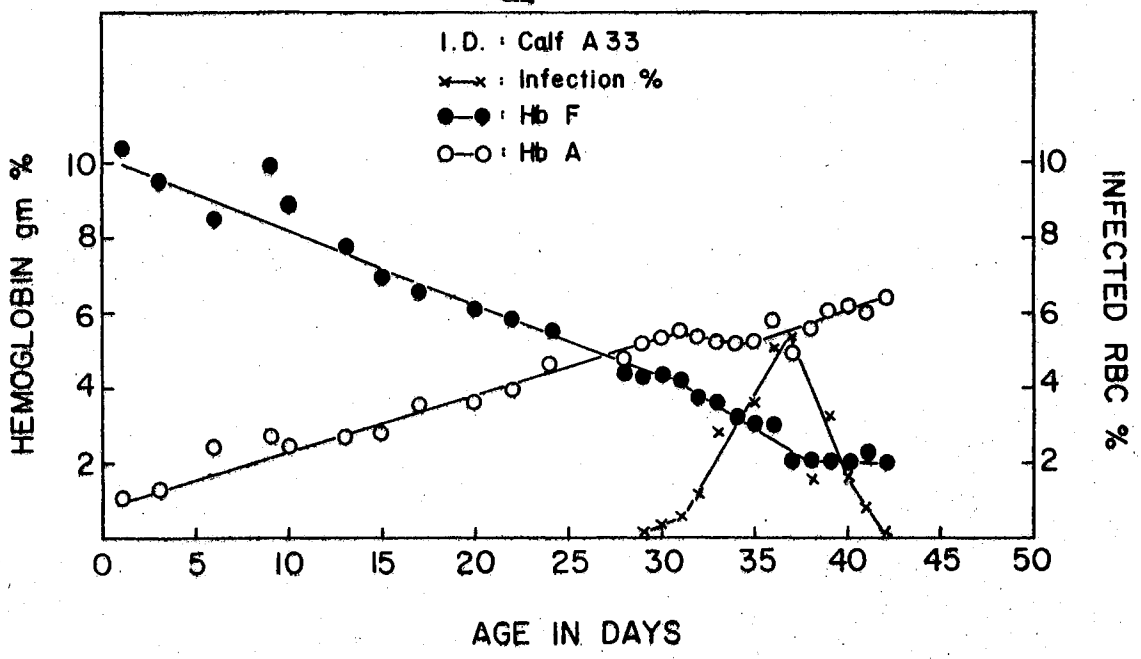

Figure 27. Variation of Hemoglobins in A. marginale Infected Calf (3). See Figure 1 and 2 for Separation and Determination of Bovine Hemoglobin A and F.

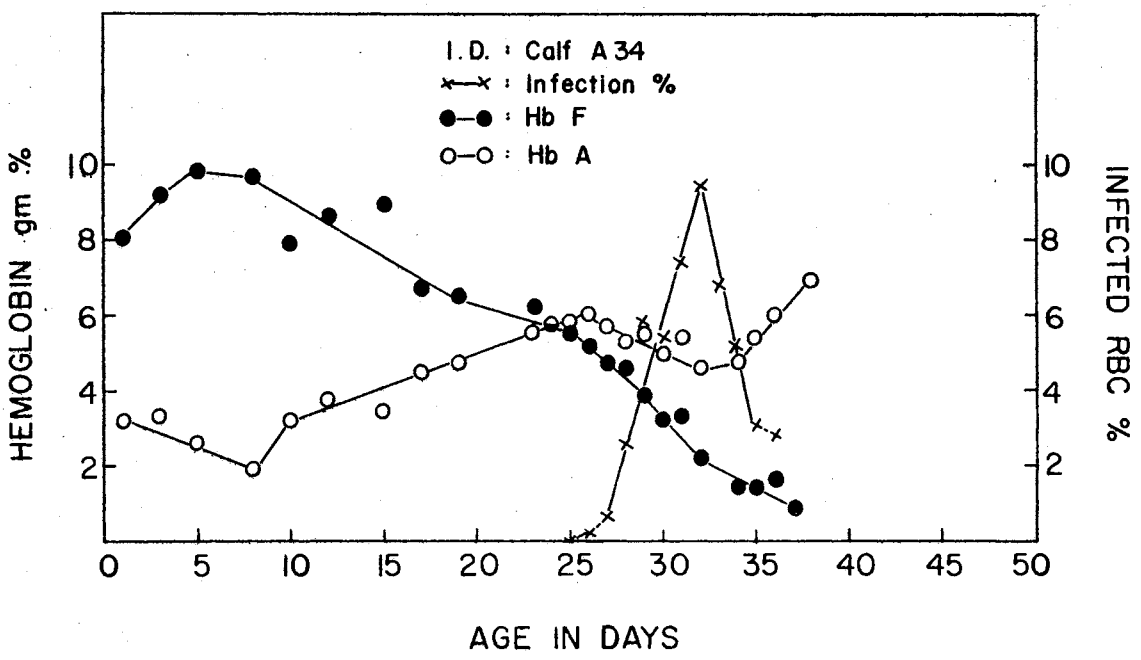

Figure 28. Variation of Hemoglobins in A. marginale Infected Calf (4). See Figure 1 and 2 for Separation and Determination of Bovine Hemoglobin A and F.

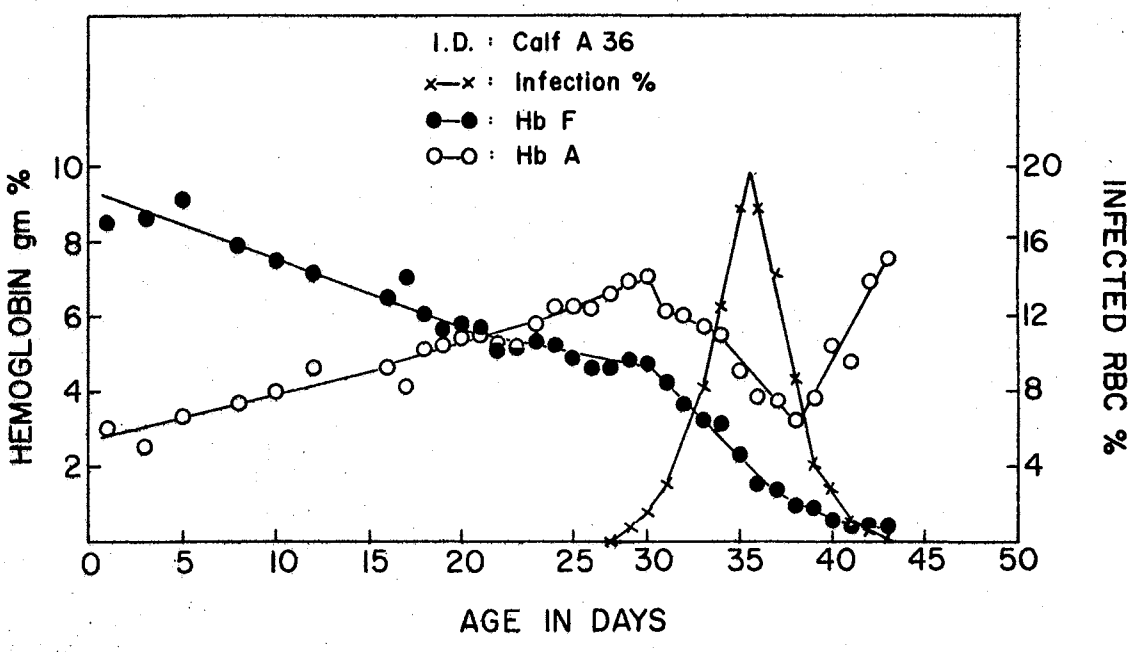

Figure 29. Variation of Hemoglobins in A. marginale Infected Calf (5). See Figure 1 and 2 for Separation and Determination of Bovine Hemoglobin A and F.

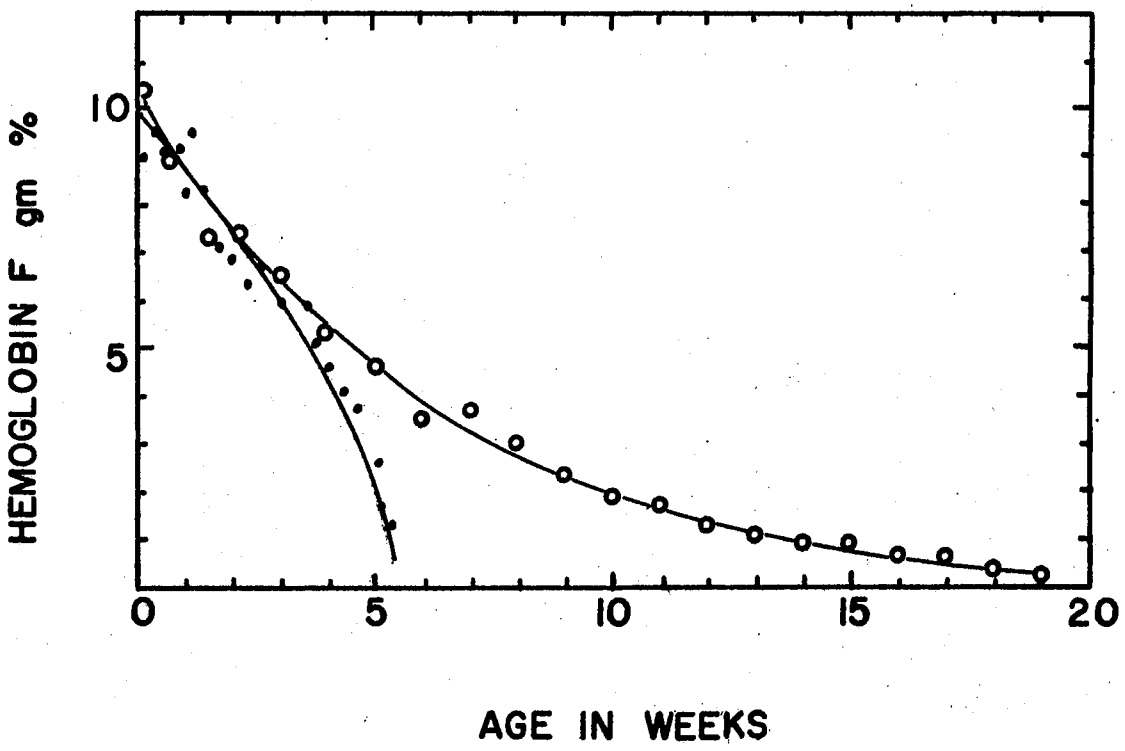

Figure 30. Hemoglobin F Decrease in Intact (o——o) and A. marginale Infected (•——•) Calves.

Each point in the figure represents mean value. Figures 4 and 25-29 were used to obtain the mean values.

A and Hb F concentration patterns (cf. Figure 4 and 5) due to A. marginale infection need to be examined to establish the effect of each hemoglobin. For analysis the preinfection period included the prepatent period and ended when hematocrit began to fall, denoting the commencement of the postinfection period. This period ended with onset of reticulocyte response. Seven consecutive daily data points were used in each period, missing observations being obtained by linear interpolation. These data were subjected to computer analysis of variance for a factorial experiment. The factors and levels were calves (5), periods (2), hemoglobin (2), and days (7). Figure 31 shows regression slopes of hemoglobins A and F pre and post infection. The angles θ and $\emptyset$ represent the change in slopes due to the infectious anemia. Table IV shows an abbreviated analysis of variance for the data. Tests for nonlinearity indicate practically no curvature is present in any of the slopes. The test of interaction $D_L \times P \times H$ represents a test for equality of the two angles θ and $\emptyset$. The value for $F = 7.02$ assuming 1 and 96 degrees of freedom is significant ($P < 0.01$), indicating the angles θ and $\emptyset$ are different. Because of nonindependence of errors the correct denominator degrees of freedom may not be quite as high as 96 although significance at the 0.05 level is demonstrated with as few as 5 denominator degrees of freedom. This significant difference in change of slopes Hb A vs Hb F is interpreted as an indication of selective Hb A removal during infection.

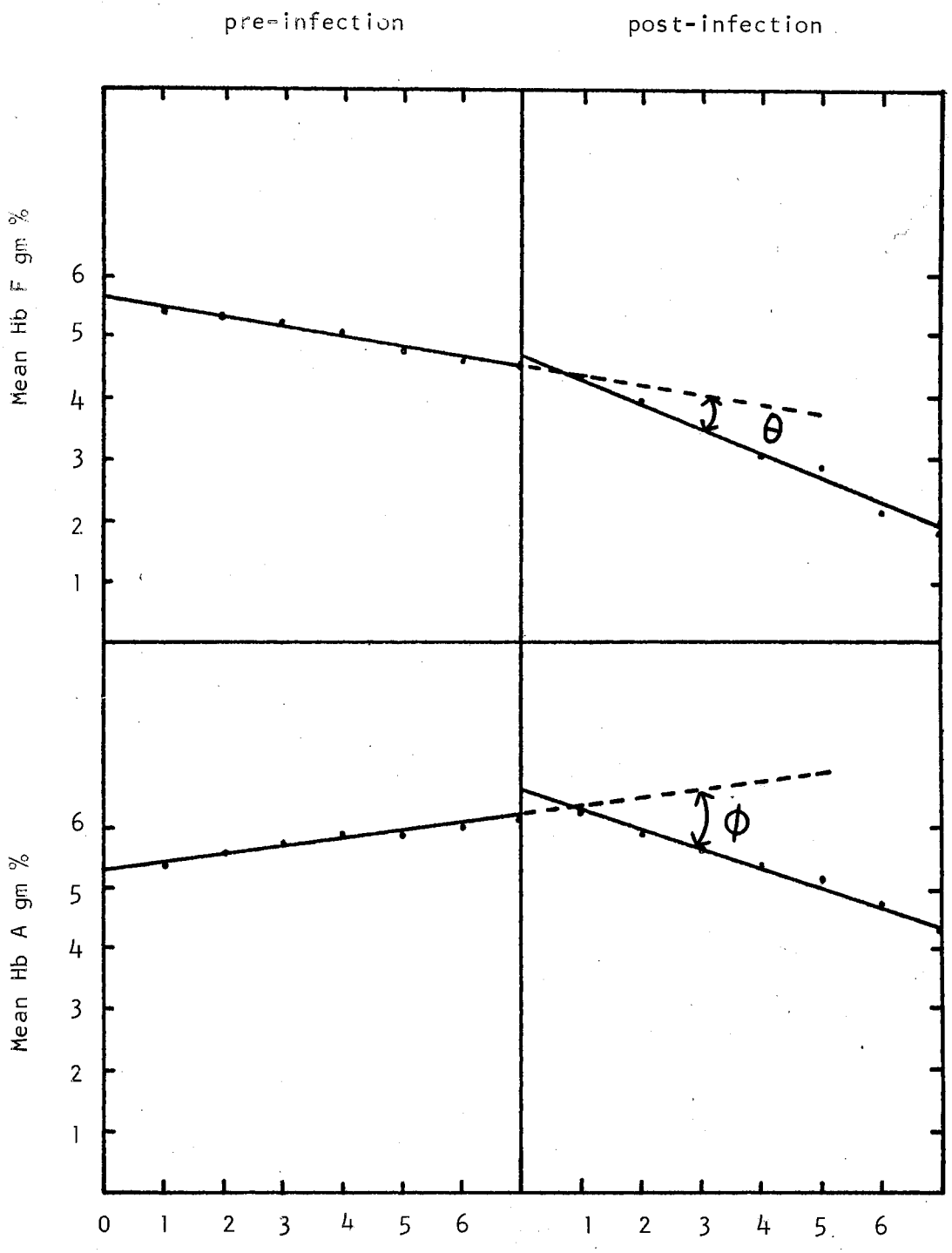

Figure 31. Regression of Mean Hemoglobins F and A gm % on Days Pre and Post A. marginale Infection. See Table XIII in Appendix Also.

TABLE IV

ANALYSIS OF VARIANCE OF HEMOGLOBINS A AND F
PRE AND POST A. MARGINALE INFECTION

Source	df	MS	F	Level of Significance
Total	139			
Calves (C)	4	4.8191	0.34	
Periods (P)	1	50.2081	3.56	
Hemoglobin (H)	1	82.7905	5.87	$P < 0.05$
P x H	1	19.3588	1.37	
Error (a) CP+CH+CPH	12	14.0954		
Days (D)	6			
Linear	(1)	22.2603	185.04	$P < 0.01$
Quadratic	(1)	0.1184	0.98	
Residual	(4)	0.0188		
D x P	6			
D_L x P	(1)	17.1464	142.53	$P < 0.01$
D_Q x P	(1)	0.0090	0.07	
Residual	(4)	0.0634		
D x H	6			
D_L x H	(1)	6.2477	51.93	$P < 0.01$
D_Q x H	(1)	0.0008	0.01	
Residual	(4)	0.0227		
D x P x H	6			
D_L x P x H	(1)	0.8447	7.02	$P < 0.01$
D_Q x P x H	(1)	0.0015	0.01	
Residual	(4)	0.0199		
Error (b) CD+CPD+CHD+CPHD	96	0.1203		

CHAPTER VII

DISCUSSION

Since hemoglobin S is known to cause sickle cell anemia and resistance to malaria in the persons who carry the trait, hemoglobin types of man and animals have attracted attention. It has been reported that aggregated Hb S prevent phagotrophy of P. falciparium resulting in no growth of this blood parasite even though the organism can penetrate into the erythrocytes through the membrane (4,101). It has been found that newborn infants, who have Hb F, are resistant to malaria and that Hb AB type cattle are resistant to trypanosomiasis (4,14). However, no definite conclusions have been drawn from this research for these types of resistance. Bovine hemoglobin F differs from bovine hemoglobin A by amino acid composition (55,56,57), alkali denaturation rate (75), solubility (83), and O_2 dissociation (80). All of these differences result from primary structure difference. Solubility, viscosity, immunologic behavior, O_2 dissociation, primary and tertiary structure of human fetal hemoglobin would be the most likely properties which provide a resistance to malaria.

Hemoglobin types observed from Ayrshire, Holstein, Angus, Hereford, Jersey, Brahman and Santa Gertrudis agree with the previous published results. Hemoglobin type (Hb A) in the Scottish Highland breed is a new finding by the author. It was suggested that Hb A and B are controlled by co-dominant alleles. If true, the mating between Hb AB type

cattle should produce Hb B homozygous cattle by Medelian genetic law. However, no Hb B homozygote of Santa Gertrudis cows have been observed either by author or others (45). As Santa Gertrudis cattle are hybrids of Shorthorn (5/8) and Brahman (3/8) breeds, the absence of Hb B homozygous cattle in the Santa Gertrudis breed may be because the homozygous condition of Hb B for Santa Gertrudis is lethal or is a consequence of natural selection.

The decrease of fetal hemoglobin in newborn infants has been very well established (66,107). Hemoglobin F in the adult is less than 1% of total hemoglobin. This suggests that the gene for the γ-chain is not fully inactivated during the adult life. There must be a rate controlling step in hemoglobin synthesis and it should be at somewhere the translation stage of protein synthesis.

Grimes et al. (42) reported Hb F decrease in dairy cattle (Ayrshire, Holstein and Jersey). Their research was based on a paper electrophoretic technique, in which about 10% error for the data (117) would be present. They also reported that Hb F was replaced by Hb A in 14 bull and 4 heifer calves at the average ages of 65 and 97 days respectively. If so, there could be a hormonal effect on the decrease of Hb F. The author observed that in calves Hb F decreased exponentially without an abrupt decline to zero and a logarithmic plot of Hb F decrease produced a straight line. There was no differences in the decrease of Hb F of heifer and bull calves. Grimes et al. could not separate Hb F from Hb B at pH 9.0 and stated that Hb F failed to disappear in the blood of 3 calves (Hb FB type). As shown in Figure 1, a clear separation of Hb B from Hb F was achieved on cellulose acetate membranes. Hemoglobin B migrated faster than Hb F toward the anode at pH 8.6.

The decreasing concentration pattern of Hb FB and Hb FAB type newborn calves was not studied, but it is most probable that the Hb F decrease in these calves would be similar or identical with that in Hb FA type newborn calves.

The equation derived for the estimation of Hb F in calves has been used and found that the estimated values by the equation give values close to the observed. The logarithm plot of mean Hb A percent against age did not give a linear relationship. But the estimation of Hb A in calves can be done by substracting the percent of Hb F from 100%.

$$\text{Hb A \%} = 100 - \text{Hb F \%}$$

The RBC life span of 3 months old calves has been reported to be 54 days by mean of radioactive iron (26). Carbon^{14} studies have revealed the RBC life span of the mature cow to be 160 days (27). If the life span of the RBC of the neonatal bovine is indeed shorter than that of the adult then the decline of Hb F concentration to less than 1% in 21 to 23 weeks would seem to indicate a continuing production of Hb F for up to 100 days after birth.

Muta (109) reported a second fetal hemoglobin in newborn Holstein calves. This has been identified as a denatured Hb F according to the polypeptide chain separation data (Chapter III).

Chain separation of hemoglobins by 2M acetate buffer, pH 4.7 was incomplete. Urea (6M) and 2-mercaptoethanol (1%) in TEB buffer, pH 8.6, at 4^{o} C yielded a complete separation of hemoglobin molecule with 24 hours of incubation. The slow moving extra protein band observed in chain separation study with 2M acetate buffer, pH 4.7, would be undissociated fetal hemoglobin.

Eliot (16) found that newborn Beagle puppies are very susceptible to B. canis and the resulting disease is usually fatal. In contrast to babesiosis in puppies, Neitz (181) found that newborn calves were relatively resistant to babesiosis. Babesia is found in animals whose erythrocytes have relatively high concentrations of sodium ion and glucose-6-phosphate dehydrogenase activity and these factors may be related to the disease (15). It should be stated that there is still a possibility that the hemoglobin types of animals may affect the growth and development of Babesia parasites. In the present study, the absence of a fetal hemoglobin in newborn Beagle puppies was verified by electrophoresis, alkali denaturation, isoelectric focusing and peptide mapping of isolated canine hemoglobins. LeCrone (45) reported the absence of fetal hemoglobin in Beagle dogs based on the results of agar-gel electrophoresis, alkali denaturation, blood smears, and column chromatography. In his paper, there was no clear statement that alkali denaturation was identical for hemoglobins of newborn and adult Beagle dogs. The same electrophoretic mobility or elution pattern from a chromatography column does not necessarily mean that two proteins are identical in structure. There could be a number of proteins which show the same electrophoretic mobility and chromatographic behavior. Human hemoglobin H and I; J and LiB_1; K, LiB_2 and Dunham; F and G, L and Galveston; S and D have identical electrophoretic mobilities on paper at pH 8.6 (235). Tyuma et al. (231) observed that rabbit fetal and adult hemoglobins have identical electrophoretic mobilities and the existence of the fetal hemoglobin was proven by an alkali denaturation study. A similar amino acid composition with the same net charge of proteins would result in similar electrophoretic mobilities and column chromatographic behavior. It is

known that a considerable zone spreading occurs in the agar gel electrophoresis of hemoglobins and in any column chromatography zone spreading is even greater. The author could observe no differences in electrophoretic mobilities, alkali denaturation rates and isoelectric points between the hemoglobins obtained from newborn and adult Beagle dogs. The author also examined the 2 proteins by peptide mapping to obtain further evidence. The results proved they were identical.

In peptide mapping, trypsin digested peptides were applied on Whatman No. 3 paper by the sample applicator of Spinco paper electrophoresis system. This application method gave a series of well separated peptide bands on first dimensional electrophoresis. In contrast to this, peptides applied by syringe gave peptide spots with much more zone spreading on first dimensional electrophoresis. A Savant varsol cooled high voltage electrophoresis tank gave better results on peptide mapping with less zone spreading than a Savant flat type high voltage electrophoresis cell. Electrophoresis and descending chromatography of the peptides from tryptic digest of the newborn and adult canine hemoglobins were done using the same conditions for both peptides.

Although the alkali denaturation rate of canine hemoglobin was not a straight line, but it does not mean that there are two hemoglobins present. Isoelectric focusing of the canine hemoglobin showed isoelectric point of 7.08 to 7.12. Reproducibility for this system was 0.04 pH unit according to author's experiments.

The absence of fetal hemoglobin and high susceptibility to B. canis infection is indirect evidence that fetal hemoglobins provide resistance. Further research is needed to establish how fetal hemoglobins, enzyme levels (such as glucose-6-phosphate dehydrogenase) or cationic

compositions provide resistance to infectious parasitemia.

Bell and Huisman (147) failed to observe any new hemoglobin in anemic Hb AB type Guernsey cow, which was bled down to 5.4 gm % of hemoglobin. These observations coincide with those of this author. Two Hereford cows were hemorrhaged severely for a period of one month. They did not have any detectable change of hemoglobin type. There have been no reports of reactivation of Hb F synthesis in cattle.

In the hemorrhaged Angus newborn calf, there was no significant increase of Hb F in the circulation (Figure 11). However, it appeared as though there was some increase of Hb F at 25 to 31 days of age. This small increase coincides with increase of PCV, Hb and RBC count (Figure 12). As hemoglobin F was present in the subject animal throughout the experiment, minor quantitative variation of hemoglobin F due to hemorrhage could not be clearly established.

In exchange transfused calves, hemoglobin F synthesis was demonstrated with an anemic condition (anemia due to loss of hemoglobin B containing erythrocytes). In these calves, Hb F could not be detected for about 10 days post-transfusion by cellulose acetate electrophoresis. This method can detect approximately 0.3 microgram of protein or about 1% of Hb F in blood sample. In view of this, it is surprising that Hb F increased by 1.06 and 0.55 gms per 100 ml blood during a 24 hours period in calves 2 and 4 respectively. The fetal hemoglobin appeared at the time of a hemolytic crisis and subsequently declined slowly. Since it requires about 4 days for a pronormoblast to become a mature erythrocyte and fetal hemoglobin could not be observed for 10 days, it would appear fetal hemoglobin synthesis in this case originates in bone marrow. The author recognizes the possibility that spleen can hold a

considerable amount of Hb F type red cells and release these during an anemic period. Immature erythrocytes in bone marrow or in circulation may synthesize fetal hemoglobin rapidly in the condition of hypoxia. Allen and Jandl (232) reported that conditions of hypoxia favored the formation of fetal hemoglobin compared to the rate of synthesis of adult hemoglobin. This research is based on reticulocyte incubation with C^{14}-leucine or Fe^{59}.

In present study, hemoglobin F decreased slowly in blood of the exchange transfused calves as hemoglobin A synthesis increased to reach normal hemoglobin concentration. Other workers have observed that in hemorrhaged sheep (42) Hb C appeared 2 weeks post hemorrhage, and the amount increased as the amount of hemorrhage increased. Sheep Hb C decreased as the normal Hb A synthesis increased (48). The author's observation on the decrease of Hb F in exchange transfused calves and the reports of decrease of Hb C in anemic sheep as synthesis of normal adult hemoglobins increase cannot be fully explained at this time.

The exchange transfused calves had a hemolytic crisis due to the exclusion of Hb B containing foreign cells. The exchange transfused calves must be synthesizing hemoglobins and erythrocytes during and after the exchange transfusion. The amount of hemoglobin F synthesized in these calves was easily and clearly detected by electrophoresis as shown on Figure 8 and 9. However, the amount of hemoglobin A synthesized could not be determined from Figure 8 and 9.

As discussed earlier, hemoglobin A and B are codominant in heterozygotes for this trait (40). Huisman (147) reported mean value of 41.5% for Hb A and 58.5% for Hb B in Guernsey cattle heterozygous for hemoglobins according to the study with DEAE-cellulose chromatography.

The hemoglobin A and B should be 50% each in heterozygous cattle on the basis of codominance. The exchange transfused calves showed essentially the same amount of hemoglobin A and B immediately after the exchange transfusion. If hemoglobin A and B are in same cell based on codominance, hemoglobin A should decrease at the same rate of hemoglobin B in exchange transfused calves. In fact, hemoglobin A decreased with hemoglobin B at the same rate for 4 to 5 days post exchange transfusion (Figure 10 and 11). Then, an increase of hemoglobin in Figure 13 and 14 should be due to the appearance of hemoglobin A type erythrocytes in the blood. The differences between hemoglobin A and B in gms per 100 ml blood are plotted (Figure 10 and 11). The increasing hemoglobin A pattern gave reasonably well defined curves comparable to hemoglobin A synthesis in normal newborn calves (Figure 5). The exchange transfusion and the anemic crisis due to exclusion of the foreign erythrocytes in the calves could have stimulated bone marrow tissue to accelerate erythropoiesis. The exchange transfused erythrocytes (Hb AB type) in the calves were excluded from the animal completely probably by antibodies produced by the calves. In this case, a low concentration of hemoglobin in the blood stream and a hypoxic condition in the calves must be stimulatory factors for Hb F synthesis if Hb F originates from bone marrow.

The primary purpose of the exchange transfusion with hemoglobin AB type blood was to attempt to study RBC factors on traits involved in A. marginale infection. As the transfused calves have received mature cattle erythrocytes, it was predicted that one could study the RBC factor in the resistance of young calves to anaplasmosis. As appearance of Hb F in the circulation occurred sooner than the A. marginale incubation period, the infection study was not performed. The third exchange

transfused Holstein calf with Hb A type adult blood did not show any exclusion of transfused erythrocytes. This suggests that this calf did not develop antibodies to the transfused cells and probably Hb A type adult erythrocytes are proper for physiology of Holstein calves, which will have the same type of erythrocytes at maturity. If bovine Hb F in erythrocytes of newborn calves cause the resistance to anaplasmosis as suggested by Williams and Jones (12), anaplasma organisms would not grow in Hb F type erythrocytes but would grow in Hb A type erythrocytes. The computer analysis of hemoglobin F and A variation in A. marginale infected calves (Figure 31 and Table IV) verified that there was a selective removal of Hb A type erythrocytes during the infection. Calves approximately 30 days old were bled daily (300-400 cc) until PCV values were approximately 50% pretreatment values and hematologic observations and Hb A and F concentrations were determined as in the study of A. marginale infected calves. No significant difference was found between the decreasing rates of Hb A and F in the hemorrhaged calves (233).

The above observations support the suggestions of Williams and Jones (12) and Allison (15) that Hb F may play a role in neonatal humans and animals against hemotropic parasitisms.

As stated before under the results, microscopic examination revealed that A. marginale bodies are membrane bound in the erythrocyte. Four additional protein bands observed in A. marginale infected erythrocyte membranes (Table III) may originate from A. marginale. Calf, cow and A. marginale infected cow erythrocyte stromata demonstrated the presence of 4 common major proteins (Figure 20) which is comparable with those in human erythrocytes (198). Probably, there are no differences between erythrocyte membrane proteins of newborn calf and cow (Table II).

Phospholipids in erythrocyte membrane can change the protein structure. It is not known if differences between calf and cow erythrocyte membrane could result in the differences observed for in A. marginale penetration.

The carbohydrate study revealed that there is no difference between calf and cow erythrocyte membrane glycoprotein and glycolipid in qualitative sense.

The tocopherol study showed that α-tocopherol is present in both newborn calf and cow erythrocyte membranes. A higher content of α-tocopherol was found in erythrocyte membranes of calf than that of cow, and the amounts were around 1 μg per mg of erythrocyte ghosts. A trace of β-tocopherol was found in erythrocyte membranes of newborn calves and the cows.

It is not known whether different fatty acid composition and tocopherol concentration of erythrocyte membranes can make a difference in the A. marginale penetration rate. Therefore, it is not possible at this moment to correlate amount of tocopherols present in erythrocyte membrane to A. marginale penetration.

Bradley et al. (68) reported that Hb F in proportions of 25 percent uniformly distributed in all erythrocytes protects against hemolysis in patients heterozygous for both Hb S and hereditary persistance of Hb F. As bovine fetal hemoglobin decreases while adult hemoglobin increases in neonatal calves, it is probable that some portion of erythrocytes in neonatal calf contain both hemoglobin A and F. Figure 30 shows that hemoglobin F in A. marginale infected calves decreased more rapidly than hemoglobin F decrease in normal calves. This faster decrease of Hb F in A. marginale infected calves reflects that Hb F type erythrocytes

were also influenced by the parasites as well as Hb A type erythrocytes. Since it is probable that some erythrocytes contain both Hb F and A, they could be invaded by the parasites and removed from the circulation.

The benefit which can be obtained by A. marginale organism during residency in erythrocytes should be carefully studied. When this is fully known, one can prevent Anaplasma marginale growth in host cell by blocking or disturbing the metabolic pathway. One should consider the nutritional source for the A. marginale in the host cell. The plasma has a large amount of protein and other nutritional materials, but the parasites may not use these as the parasites are inside of the erythrocyte membrane. One might imagine the proteins of the erythrocyte are the chief source of parasite protein. About 90 percent of the red blood cell protein is hemoglobin. There are, also, enzymes and membrane proteins which could be utilized by A. marginale for the protein source. Lipids and carbohydrates should be considered for the nutritional source for the organism. There is evidence that the concentration of RBC phospholipid decreases in bovine anaplasmosis (170).

There is a significant decrease of glycolytic and hexose monophosphate pathway enzymes activities as the calf matures (149). It has been reported that erythrocytes with high potassium ion levels are readily infected with plasmodium and erythrocytes with low levels of potassium ion are not infectible (234). Babesia are found in species with relatively high erythrocyte sodium ion levels.

The author feels this study has proven that Hb F type erythrocytes provide the resistance to anaplasmosis in the newborn bovine. Hemoglobin type, lipid composition, some enzyme levels and cationic composition are other factors which may provide resistance of Hb F type ery-

throcytes to the anaplasmosis. The biochemical mechanism of this resistance is not known. Probably, the biochemical mechanism could be clarified after the success of *in vitro* cultures of *A. marginale*.

There is evidence that the malarial parasites actively metabolize hemoglobin (235). A much more satisfying result on the gross aspects of hemoglobin digestion has been given by electron microscopic studies of Rudzinska and Trager (99,100) on *P. lophurae* and *P. berghei*. Their thin-section electron micrographs showed that the parasites feed by phagotrophy; that is, they engulf portions of the erythrocyte cytoplasm by invaginating their limiting membranes. They also indicated the site of hemoglobin digestion.

If the fetal hemoglobin provides the resistance to anaplasmosis in newborn calves, the resistance could be due to the higher solubility of Hb F, higher intracellular viscosity by Hb F or a resistance of Hb F to the protease(s) of *A. marginale*.

SUMMARY

Hemoglobin types of cattle depend on breed and age. Two electrophoretically distinct hemoglobins have been detected in adult and newborn equine. No additional hemoglobin which may be regarded as a fetal form has been detected in the newborn equine. Adult and newborn canines demonstrated the presence of only one hemoglobin by electrophoresis. The absence of a fetal hemoglobin in newborn canine, which is quite susceptible to babesiosis, has been proved by electrophoresis, an alkali denaturation study, isoelectric focusing and peptide mapping. The hemoglobin composition of newborn calves is Hb F, 89% and Hb A, 11%. The bovine Hb F decreased to less than one percent after 21 to 23 weeks of birth and Hb A approached 100 percent. No second type of bovine fetal hemoglobin has been found. The synthesis of Hb F in young calves was studied by exchange transfusion with adult cow blood of Hb AB type. It appears that the exchange transfused erythrocytes have been completely excluded from the calves and production of Hb A containing erythrocytes was observed. Hemoglobin F containing erythrocytes appeared in the exchange transfused calves when the animals became anemic by exclusion of the transfused cells. In a Holstein calf exchange transfused with Hb A type adult cow blood, no exclusion of the exchange transfused erythrocytes was observed. Two young cows and one newborn calf were hemorrhaged and no hemoglobin type changes were observed.

There is probably no difference between calf and cow erythrocyte membrane proteins. The Anaplasma marginale organisms appeared to be

attached to membranes in erythrocytes. Analysis of the Anaplasma marginale organisms infected erythrocyte membranes demonstrated the presence of four additional proteins when compared to the erythrocyte membranes of a normal cow. These additional proteins may be a part of A. marginale organism. Calf erythrocyte membranes have a higher α-tocopherol content than cow erythrocyte membranes. A trace amount of β-tocopherol has been detected in calf and cow erythrocyte membranes.

Newborn calves infected with A. marginale showed a selective removal of Hb A containing erythrocytes. It is proposed that Hb F containing bovine erythrocytes provide the resistance to A. marginale organisms.

A SELECTED BIBLIOGRAPHY

(1) Rhinesmith, H. S., Schroeder, W. A., and Martin, N. J., J. Amer. Chem. Soc., 80, 3358 (1958).

(2) Neel, J. V., Science, 110, 64 (1949).

(3) Ingram, V. M., Biochem. Biophys. Acta, 28, 539 (1958).

(4) Allison, A. C., Exptl. Parasitol., 6, 418 (1957).

(5) Garnham, P. C. C., Ann. Trop. Med. Parasitol., 43, 47 (1949).

(6) Macfarlane, A. S., Brit. Med. Bull., 8, 213 (1952).

(7) Weinman, D., and Ristic, M., Infectious Blood Diseases of Man and Animals, II, Academic Press, New York and London, 1968, p. 474.

(8) Jones, E. W., and Brock, W. E., J. Am. Vet. Med. Assoc., 149, 1624 (1966).

(9) Jones, E. W., Kliewer, I. O., Norman, B. B., and Brock, W. E., Am. J. Vet. Res., 29, 535 (1968).

(10) Ingram, D. G., and Smith, A. N., Can. Vet. J., 6, 194 (1965).

(11) Smith, A. N., and Ingram, D. G., Can. Vet. J., 6, 226 (1965).

(12) Williams, E. I., and Jones, E. W., Am. J. Vet. Res., 29, 703 (1968).

(13) Jones, E. W., and Norman, B. B., Proc. 4th Natl. Anaplasmosis Conf., 3 (1962).

(14) Bangham, A. D., and Blumberg, B. S., Nature, 181, 1551 (1958).

(15) Allison, A. C., Immunity to Protozoa, (Garnham, P. C. C., Pierce, A. C., and Roitt, I., ed.) Blackwell Sci. Publ., Oxford, England, 1963, p. 109.

(16) Eliot, F. D., M. S. Thesis, Oklahoma State University (1970).

(17) Schalm, O. W., Veterinary Hematology, Lea and Febiger, Philadelphia, 1965, p. 225.

(18) Wintrobe, M. M., Clinical Hematology, ed. 5, Lea and Febiger, Philadelphia, 1961, p. 124.

(19) Murphy, J. R., J. Lab. and Clin. Med., 55, 286 (1960).

(20) Murphy, J. R., J. Lab. and Clin. Med., 61, 567 (1963).

(21) Leavell, B. S., and Thorup, O. A., Fundamentals of Clinical Hematology, W. B. Saunders Co., Philadelphia and London, 1966, p. 28.

(22) Miescher, F., Cov. Bl. F. schweiz. Aertz., 23, 830 (1893).

(23) Ferguson, J. H., Blood and Body Functions, F. A. Davis, Philadelphia, 1965, p. 199.

(24) Schalm, O. W., Veterinary Hematology, Lea and Febiger, Philadelphia, 1965, p. 142.

(25) Harris, J. W., The Red Cell, Harvard University Press, 1963, p. 235.

(26) Baker, N. F., Douglas, J. R., Am. J. Vet. Res., 18, 295 (1957).

(27) Kaneko, J. J., Ann. N. Y. Acad. Sci., 104, 689 (1963).

(28) Brown, I. W., Jr., and Eadie, G. S., J. Gen. Physiol., 36, 327 (1953).

(29) Solomon, J. B., Biochem. Animal Development, I, Academic Press, New York and London, 1965, p. 145.

(30) Perutz, M. F., Muirhead, H., Cox, J. M., and Gouman, L. C. G., Nature, 219, 131 (1968).

(31) Koshland, D. E., Jr., and Neet, K. E., Ann. Rev. Biochem., 37, 359 (1968).

(32) Lehmann, H., and Huntsman, R. G., Man's Haemoglobins, J. B. Lippincott Co., Philadelphia and Montreal, 1966, p. 47.

(33) Rosemeyer, M. A., and Huehns, E. R., J. Mol. Biol., 25, 253 (1967).

(34) Wilson, S., and Smith, D., Canad. J. Biochem. Physiol., 37, 405 (1959).

(35) Chernoff, A. I., and Nelson, M. P., Blood, 24, 750 (1964).

(36) Perutz, M. F., and Lehmann, H., Nature, 219, 902 (1968).

(37) Lehmann, H., and Huntsman, R. G., Man's Haemoglobins, J. B. Lippincott Co., Philadelphia and Montreal, 1966, p. 8.

(38) Wintrobe, M. M., Clinical Hematology, ed. 5, Lea and Febiger, Philadelphia, 1961, p. 154.

(39) Lehmann, H., and Carrell, R. W., Brit. Med. Bull., 25, 14 (1969).

(40) Bangham, A. D., Nature, 179, 467 (1957).

(41) Efremov, G., and Braend, M., Acta Vet. Scand., 6, 109 (1965).

(42) Grimes, R. M., Duncan, C. W., and Lassiter, C. A., J. Dairy Sci., 4, 1527 (1958).

(43) Ranjekar, P. K., and Barnabas, J., Comp. Biochem. Physiol., 28, 1395 (1969).

(44) Schmid, D. O., and Thein, P., Zentraldl. Veterinarmed. Beine A., 14, 32 (1967).

(45) Crockett, J. R., Koger, M., and Chapman, H. L., Jr., J. Animal Sci., 22, 173 (1963).

(46) Harris, H., and Warren, F. L., Biochem. J., 60, p. xxix (1955).

(47) Van der Helm, H. J., van Vliet, G., and Huisman, T. H. J., Arch. Biochem. Biophys., 72, 331 (1957).

(48) van Vliet, G., and Huisman, T. H. J., Biochem. J., 93, 401 (1964).

(49) Huisman, T. H. J., Dozy, A. M., Wilson, J. B. Efremov, G. D., and Vaskov, B., Biochem. Biophys. Acta, 160, 467 (1968).

(50) Gratzer, W. B., and Allison, A. C., Biol. Rev. Cambridge Phil. Soc., 35, 459 (1960).

(51) LeCrone, C. N., Blood, 35, 451 (1970).

(52) Cabannes, R., and Serain, C., C. R. Soc. Biol., Paris, 149, 1193 (1955).

(53) Bangham, A. D., and Lehmann, H., Nature, 181, 267 (1958).

(54) Kilmartin, J. V., and Clegg, J. B., Nature, 217, 269 (1967).

(55) Schroeder, W. A., Shelton, J. R., Shelton, J. B., Robberson, B., and Babin, D. R., Arch. Biochem. Biophys., 120, 1 (1967).

(56) Schroeder, W. A., Shelton, J. R., Shelton, J. B., Robberson, B., and Babin, D. R., Arch. Biochem. Biophys., 120, 124 (1967).

(57) Babin, D. R., Schroeder, W. A., Shelton, J. R., Shelton, J. B., and Robberson, B., Biochem. J., 85, 1297 (1966).

(58) Epstein, C. J., and Motulsky, A. G., Progr. Med. Genet., 4, 97 (1965).

(59) Tavill, A. S., Grayzel, A. I., London, I. M., Williams, M. K., and Vanderhoff, G. A., J. Biol. Chem., 243, 4987 (1968).

(60) Bruns, G. P., and London, I. M., Biochem. Biophys. Res. Commun., 18, 236 (1965).

(61) Cantarow, A., and Schepartz, B., Biochemistry, ed. 4, W. B. Saunders, 1967, p. 609.

(62) Karpatkin, S., J. Lab. and Clin. Med., 62, 121 (1963).

(63) Weatherall, D. J., The Thalassaemia Syndromes, F. A. Davis, Philadelphia, 1965.

(64) Smith, E. W., and Torbert, J. V., Bull. Johns Hopkins Hosp., 102, 38 (1958).

(65) Pearson, H. A., and Moore, M. M., Am. J. Human Genet., 17, 125 (1965).

(66) Zuckerkandl, E., Sci. Amer., 212, 110 (1965).

(67) Beaven, G. H., Ellis, M. J., White, J. C., Brit. J. Haemat., 6, 201 (1960).

(68) Bradley, T. B., Jr., Brawner, J. N., III, and Conley, C. L., Bull. Johns Hopkins Hosp., 108, 242 (1961).

(69) Jacob, F., and Monod, J., J. Mol. Biol., 3, 318 (1961).

(70) Britten, R. J., and Davidson, E. H., Science, 165, 349 (1969).

(71) Tomkins, G. M., Geleluter, T. D., Granner, D., Martin, D., Jr., Samueles, H. HI, and Thomson, E. D., Science, 166, 1474 (1969).

(72) Baglioni, C., and Compana, T., European J. Biochem., 2, 480 (1967).

(73) Adams, H. R., Boyd, E. M., Wilson, J. B., Miller, A., and Huisman, T. H. J., Arch. Biochem. Biophys., 127, 721 (1968).

(74) Dayhoff, M. O., Atlas of Protein Sequence and Structure, Natl. Biomed. Res. Found., Silver Spring, 1969, D34.

(75) Brinkman, R., and Jonxis, J. H. P., J. Physiol., 88, 162 (1936).

(76) White, J. C., and Beaven, G. H., Brit. Med. Bull., 15, 33 (1959).

(77) Beaven, G. H., Hoch, H., and Holiday, E. R., Biochem. J., 49, 374 (1951).

(78) Itano, H. A., Science, 117, 89 (1953).

(79) Schroeder, W. A., Shelton, J. R., Shelton, J. B., and Cormick, J., Proc. Natl. Acad. Sci., 48, 284 (1962).

(80) Roos, J., and Romjin, C., J. Physiol., 92, 249 (1938).

(81) McCarthy, E. F., J. Physiol., 80, 260 (1933).

(82) McCarthy, E. F., Cold Spr. Harb. Symp. Quant. Biol., 19, 133 (1954).

(83) Wyman, J., Jr., Rafferty, J. A., and Ingalls, E., J. Biol. Chem., 153, 275 (1944).

(84) Itano, H. A., Arch. Biochem. Biophys., 47, 148 (1953).

(85) Roughton, F. J. W., and Kendrew, J. C., Haemoglobin, Interscience, New York, 1949, p. 261.

(86) Haurowitz, F., Hoppe-Seyl. Z., 232, 125 (1935).

(87) Zinsser, H. H., and Tang, Y. C., Arch. Biochem. Biophys., 34, 81 (1951).

(88) Bragg, W. L., and Perutz, M. F., Acta Cryst. Camb., 5, 277 (1952).

(89) Bragg, W. L., and Perutz, M. F., Acta Cryst. Camb., 5, 323 (1952).

(90) Aksoy, M., Acta Hemat., 13, 226 (1955).

(91) Goodman, M., and Campbell, D. H., Blood, 8, 422 (1953).

(92) Brinkman, R., and Jonxis, J. H. P., J. Physiol., 88, 162 (1936).

(93) Lecks, H. L., Amer. J. Dis. Child., 79, 117 (1950).

(94) Betke, D., Naturwissen schaften, 40, 60 (1953).

(95) Darling, R. C., Smith, C. A., Asmussen, E., and Cohen, F. M., J. Clin. Invest., 20, 739 (1941).

(96) Allen, D. W., Wyman, J., Jr., and Smith, C. A., J. Biol. Chem., 203, 81 (1953).

(97) Gitlin, D., Sasaki, T., and Vuopio, P., Blood, 32, 796 (1968).

(98) Fulton, J. D., and Flewett, T. H., Trans. Roy. Soc. Trop. Med. Hyg., 50, 150 (1956).

(99) Rudzinska, M. A., and Trager, W., J. Protozool., 4, 190 (1957).

(100) Rudzinska, M. A., and Trager, W., J. Biophys. Biochem. Cytol., 6, 103 (1959).

(101) Honigberg, B. M., In Chemical Zoology, I, (Florkin, M., and Scheer, B. T., eds.,) Academic Press, New York, 1967, p. 695.

(102) Rudzinska, M. A., Trager, W., and Bray, R. S., J. Protozool., 12, 563 (1965).

(103) Moulder, J. W., Biochem. Intracellular Parasitism, Univ. Chicago Press, Chicago, 1962, p. 26.

(104) Allison, A. C., Brit. Med. J., I, 290 (1954).

(105) Gilles, H. M., Ann. Trop. Med. Parasitol., 50, 103 (1957).

(106) Lehmann, H., Man, 59, 66 (1959).

(107) Chernoff, A. I., and Singer, K., Pediatrics, 9, 409 (1952).

(108) Lehmann, H., and Huntsman, R. G., Man's Haemoglobins, J. B. Lippincott Co., Philadelphia and Montreal, 1966, p. 48.

(109) Muta, I., Acta Med. Nagasaki., 9, 172 (1964).

(110) Huisman, T. H. J., Brande, J. V. D., and Meyering, C. A., Clin. Chem. Acta, 5, 375 (1960).

(111) Muller, C. J., "Molecular Evolution. A Comparative Study on the Structure of Mammalian and Avian Hemoglobins." The Netherlands, Van Gorcum, Assen. (1961).

(112) Mikle, S., and Merckur'eva, E. K., Nauchn. Dokl. Vysshei. Shkoly, Biol. Nauki, 4, 178 (1963).

(113) Pauling, L., Itano, H. A., Singer, S. J., and Wells, I. C., Science, 110, 543 (1949).

(114) Kunkel, H. G., and Tiselius, A., J. Gen. Physiol., 35, 89 (1952).

(115) Smithies, O., Biochem. J., 61, 629 (1955).

(116) Ornstein, L., Ann. New York Acad. Sci., 121, 321 (1964).

(117) Robinson, A. R., Robson, M., Harrison, A. P., and Zuehzer, W. W., J. Lab. and Clin. Med., 50, 745 (1957).

(118) Zak, B., Eggers, E. M., Jarkowski, T. L., and Williams, L. A., J. Lab. and Clin. Med., 54, 288 (1959).

(119) Yakulis, V. J., Heller, P., Josephson, A. M., and Singer, L., Am. J. Clin. Path., 34, 28 (1960).

(120) Kohn, J., Tr. Roy. Soc. Trop. Med. and Hyg., 52, 304 (1958).

(121) Graham, J. L., and Grumbaum, B. W., Am. J. Clin. Path., 39, 567 (1963).

(122) Smithies, O., Vox Sanguinis, 10, 359 (1965).

(123) Boardman, N. K., and Partridge, S. M., Biochem. J., 59, 543 (1955).

(124) Prins, H. K., and Husiman, T. H. J., Nature, 175, 903 (1955).

(125) Prins, H. K., and Huisman, T. H. J., Nature, 177, 840 (1956).

(126) Gutter, F. J., Peterson, E. A., and Sober, H. A., Arch. Biochem. Biophys., 80, 353 (1959).

(127) Peterson, E. A., and Sober, H. A., J. Am. Chem. Soc., 78, 751 (1956).

(128) Huisman, T. H. J., and Dozy, A. M., J. Chromatog., 7, 180 (1962).

(129) Huisman, T. H. J., and Dozy, A. M., J. Chromatog., 19, 160 (1965).

(130) Svensson, H., Acta Chem. Scand., 15, 325 (1961).

(131) Kolin, A., J. Chem. Phys., 22, 1628 (1954).

(132) Veterberg, O., and Svensson, H., Acta Chem. Scand., 20, 820 (1966).

(133) Haglund, H., Science Tools, 14, 17 (1967).

(134) Lemberg, R., and Legge, J. W., Hematin Compds. and Bile Pigments, Interscience Publishers, New York, 1949, p. 255.

(135) Itano, H. A., Bergren, W. R., and Sturgeon, P., Medicine, 35, 121 (1956).

(136) Haurowitz, F., Hardin, R., and Dicks, M., J. Am. Chem. Soc., 58, 103 (1953).

(137) Hirs, C. H. W., J. Biol. Chem., 219, 611 (1956).

(138) Clegg, J. B., Naughton, M. A., and Weatherall, D. J., J. Mol. Biol., 19, 91 (1966).

(139) Huehns, E. R., and Shooter, E. M., J. Mol. Biol., 3, 257 (1961).

(140) Jones, R. T., Schroeder, W. A., and Vinograd, J. R., J. Amer. Chem. Soc., 81, 4749 (1959).

(141) Huisman, T. H. J., van Vliet, G., and Sebens, T., Nature, 182, 171 (1958).

(142) Blunt, M. H., and Evans, J. V., Nature, 200, 1215 (1963).

(143) Tucker, E. M., Res. Vet. Sci., 7, 368 (1966).

(144) Beale, D., Lehmann, H., Drury, A., and Tucker, E. M., Nature, 209, 1099 (1966).

(145) Blunt, M. H., Am. J. Physiol., 209, 986 (1965).

(146) Huisman, T. H. J., Adams, H. R., Dimmock, M. O., Edwards, W. E., and Wilson, J. B., J. Biol. Chem., 242, 2534 (1967).

(147) Bell, J. T., and Huisman, T. H. J., Am. J. Vet. Res., 29, 479 (1968).

(148) Blunt, M. H., Blalock, E., Kitchens, J., Huisman, T. H. J., Proc. Soc. Exp. Biol. Med., 127, 38 (1968).

(149) Smith, J. E., and Jones, E. W., Personal Communication.

(150) Christensen, J. F., Diseases of Cattle, Amer. Vet. Pub., Evanston, Ill., 1956, p. 657.

(151) Carricaburn, J. B., Proc. 5th Natl. Anaplasmosis Conf., p. 19 (1959).

(152) Ristic, M., Advan. Vet. Sci., 6, 111 (1960).

(153) Kreier, J. P., Ristic, M., and Schroeder, W. F., Am. J. Vet. Res., 25, 343 (1964).

(154) Summers, W. A., Proc. 4th Natl. Anaplasmosis Conf., p. 70 (1962).

(155) Brock, W. E., Ph.D. Dissertation, University of Oklahoma, Norman, Oklahoma, 1958.

(156) Anthony, D. W., and Roby, T. O., Am. J. Vet. Res., 27, 191 (1966).

(157) Weinman, D., and Ristic, M., Infectious Blood Diseases of Man and Animals, II, Academic Press, New York and London, 1968, p. 493.

(158) Ristic, M., and Kreier, J. P., Am. J. Vet. Res., 24, 985 (1963).

(159) Ristic, M., and Watrach, A., Am. J. Vet. Res., 22, 109 (1961).

(160) Scott, W. L., Greer, J. C., and Foote, L. E., Am. J. Vet. Res., 22, 877 (1961).

(161) Ristic M., Am. J. Vet. Res., 28, 63 (1967).

(162) Bedell, D. M., and Dimopollous, G. T., Am. J. Vet. Res., 24, 278 (1963).

(163) Foote, L. E., Greer, J. C., and Stich, Y. E., Science, 128, 147 (1958).

(164) Christensen, J. F., Osebold, J. W., and Rosen, M. N., J. Am. Vet. Med. Assoc., 132, 289 (1958).

(165) Howell, D. E., Proc. 5th Natl. Anaplasmosis Conf., p. 164 (1968).

(166) Ristic, M., and Watrach, A., Am. J. Vet. Res., 24, 267 (1963).

(167) Rogers, T. E., and Dimopoullos, G. T., Proc. Soc. Exptl. Biol. Med., 120, 685 (1965).

(168) Rogers, T. E., Hidalgo, R. J., and Dimopoullos, G. T., J. Bact., 88, 81 (1964).

(169) Wallace, W. R., Dommert, A. R., and Dimopoullos, G. T., Am. J. Vet. Res., 28, 443 (1967).

(170) Dimopoullos, G. T., and Bedell, D. M., Proc. Soc. Exptl. Biol. Med., 105, 463 (1960) and Am. J. Vet. Res., 23, 813 (1962).

(171) Dimopoullos, G. T., and Bedell, D. M., Am. J. Vet. Res., 25, 394 (1964).

(172) Schrader, G. T., and Dimopoullos, G. T., Bacteriol. Proc., 62, 43 (1962) and Am. J. Vet. Res., 24, 283 (1963).

(173) Mann, D. K., Ph.D. Thesis, University of Illinois, Urbana, Ill., 1967.

(174) Mason, R. A., and Ristic, M., J. Infect. Diseases, 116, 335 (1966).

(175) Dimopoullos, G. T., Proc. 5th Natl. Anaplasmosis Conf., p. 33 (1968).

(176) Pilcher, K. S., Wu, W. G., and Muth, O. H., Am. J. Vet. Res., 22, 298 (1961).

(177) Rao, P. J., Ph.D. Thesis, University of Illinois, Urbana, Ill.

(178) Jones, E. W., Proc. 5th Natl. Anaplasmosis Conf., p. 75 (1968).

(179) Roby, T. O., and Gates, D. W., Am. J. Vet. Res., 22, 982 (1961).

(180) Fernandez, L., and Lova, C. A., Proc. 5th Pan Am. Congr. Vet. Med. Zootech., Caracas, Venezuela, p. 1 (1966).

(181) Neitz, W. O., Ann. N. Y. Acad. Sci., 64, 56 (1956).

(182) Malherbe, W. D., Ann. N. Y. Acad. Sci., 64, 128 (1956).

(183) Nuttall, G. H. F., and Graham-Smith, G. S., J. Hyg., 6, 586 (1906).

(184) Nuttall, G. H. F., and Graham-Smith, G. S., J. Hyg., 7, 232 (1907).

(185) Krylov, M. V., Acta Protozool (Warsaw), 2, 97 (1964).

(186) Rudzinska, M. A., and Trager, W. J., Protozool., 9, 279 (1962).

(187) Wilson, S. G., Tijdschr. Diergeneesk, 89, 1783 (1964).

(188) Riek, R. F., In Infectious Blood Disease of Man and Animals, (Weinman, D., and Ristic, M., ed.) II, Academic Press, New York and London, 1968, p. 219.

(189) Hoyte, H. M. D., J. Protozool., 12, 83 (1965).

(190) Enigk, K., and Friedhoff, K., Z. Tropenmed. Parasitol., 14, 502 (1963).

(191) Mahoney, D. F., Ann. Trop. Med. Parasitol., 63, 1 (1969).

(192) Hirato, K. N., Uwano, Y., and Kutii, T., Japan J. Vet. Sci., 7, 197 (1945).

(193) Schindler, R., and Dennig, H. K., Z. Tropenmed. Parasitol., 13, 480 (1962).

(194) Hall, W. T. K., Australian Vet. J., 39, 386 (1963).

(195) Mahoney, D. F., Australian Vet. J., 38, 48 (1962).

(196) Bakermann, S., and Wasemiller, G., Biochemistry, 6, 1100 (1967).

(197) Maddy, A. H., Biochem. Biophys. Acta, 117, 193 (1966).

(198) Lenard, J., Biochemistry, 9, 1129 (1970).

(199) Behrendt, H., Chemistry of Erythrocytes, C. C. Thomas, Springfield, Ill., 1969, p. 227.

(200) Reed, C. R., Swisher, S. N., Marinetti, G. V., and Eden, E. G., J. Lab. and Clin. Med., 56, 281 (1960).

(201) Hoffman, J. F., Circulation, 26, 1201 (1962).

(202) Korn, E. D., Ann. Rev. Biochem., 38, 263 (1969).

(203) Bishop, C., and Surgenor, D. M., The Red Blood Cell, Academic Press, New York, 1964, p. 278.

(204) McManus, T. J., Fed. Proc., 26, 1821 (1967).

(205) Van Deenen, L. L. M., Prog. Chem. Fats and Other Lipids, 5, 1 (1965).

(206) Burger, S. P., Fujii, T., and Hanahan, D. J., Biochemistry, 7, 3682 (1968).

(207) Korn, E. D., In Theoretical and Experimental Biophysics (Cole, A., Ed.) Marcel Dekker, New York, 1969.

(208) Lenard, J., Biochemistry, 9, 1129 (1970).

(209) Kobylka, D., and Carraway, K., Abstract, Oklahoma ACS Tetrasectional Meeting, 1970.

(210) Zacharius, R. M., Zell, T. E., Morrison, J. H., and Woodlack, J. J., Anal. Biochem., 30, 148 (1969).

(211) Anon, Biochem. Biophys. Acta, 107, 1 (1965).

(212) Richards, J. H., and Hendrickson, J. B., The Biosynthesis of Steroids, Terpenes, and Acetogenins, W. A. Benjamin, New York, 1964, p. 70.

(213) Lea, C. H., and Ward, R. J., J. Sci. Agric., 10, 537 (1959).

(214) Bunyan, J., McHale, D., Green, J., and Marcinkiewiez, S., Brit. J. Nutr., 15, 253 (1961).

(215) Slover, H. T., Lehmann, J., and Valis, R. J., J. Amer. Oil Chem. Soc., 46, 417 (1969).

(216) Walker, B. L., and Dummerow, I. A., J. Nutr., 81, 75 (1963).

(217) Hepler, O. E., Manual of Clinical Laboratory Methods, 4th ed., C. C. Thomas, Springfield, Ill., 1963.

(218) Drabkin, D. L., J. Biol. Chem., 164, 704 (1946).

(219) News and Views, Blood, 29, 282 (1967).

(220) Huisman, T. H. J., Martis, E. A., and Dozy, A., J. Lab. and Clin. Med., 52, 312 (1958).

(221) Brodbeck, U., Denton, W. L., Tanahashi, N., and Ebner, K. E., J. Biol. Chem., 242, 1391 (1967).

(222) Jonxis, J. H. P., and Visser, H. K. A., J. Disease Child., 92, 588 (1956).

(223) Ingram, V. M., and Stretton, A. O. W., Biochim. Biophys. Acta, 62, 456 (1962).

(224) Nathans, D., J. Mol. Biol., 13, 521 (1965).

(225) Beale, D., Biochem. J., 103, 129 (1967).

(226) Schalm, D. W., Veterinary Hematology, Lea and Febiger, Philadelphia, 1965, p. 62.

(227) Weber, K., and Osborn, M., J. Biol. Chem., 244, 4406 (1969).

(228) Dulaney, J. T., and Touster, O., Biochim. Biophys. Acta, 196, 20 (1970).

(229) Rudolph, C. J., Jr., Ph.D. Dissertation, Oklahoma State University, 1971.

(230) Ingram, V. M., Hemoglobin and Its Abnormalities, C. C. Thomas, Springfield, Ill., 1965, p. 61.

(231) Tyuma, I., Enoki, Y., and Morikawa, S., Japan. J. Physiol., 14, 573 (1964).

(232) Allen, D. W., and Jandl, J. H., J. Clin. Invest., 39, 1107 (1960).

(233) Anderson, I. L., and Jones, E. W., Personal Communication.

(234) McGhee, R. B., J. Infec. Dis., 92, 14 (1953).

(235) Deegan, T., and Maegraith, B. G., Ann. Trop. Med. Parasitol., 50, 194 (1956).

APPENDIX

TABLE V

COMPILATION OF DATA ON BOVINE HEMOGLOBIN F %
FROM ZERO TO 23 WEEKS OF AGE

Age		Mean	S.D.	Range	N
Day	0-1	89.20	9.76	68.65 - 97.08	7
	2-8	79.34	9.73	56.10 - 92.86	16
	9-13	68.11	12.10	52.12 - 85.82	9
Week	2	68.43	10.49	50.56 - 82.78	10
	3	59.61	11.04	42.23 - 76.13	15
	4	50.30	10.90	34.24 - 62.45	12
	5	40.71	8.06	24.92 - 56.93	14
	6	33.74	7.71	19.71 - 41.72	7
	7	30.42	8.82	13.06 - 44.15	12
	8	22.20	7.75	13.09 - 33.92	9
	9	19.93	8.35	5.33 - 31.56	10
	10	15.37	4.07	10.93 - 22.00	11
	11	15.67	4.92	9.24 - 22.79	11
	12	10.86	3.46	7.74 - 18.05	12
	13	9.56	2.58	7.23 - 15.05	10
	14	7.43	2.46	3.98 - 14.29	12
	15	8.05	3.19	4.26 - 14.09	10
	16	5.60	1.81	3.79 - 9.92	9
	17	5.18	1.43	3.11 - 7.60	9
	18	3.71	0.91	2.10 - 5.44	11
	19-20	1.86	0.55	1.01 - 3.04	15
	21-23	0.92	0.39	0.44 - 1.55	14

S.D.: sample standard deviation
N: number of samples

TABLE VI

COMPILATION OF DATA ON BOVINE HEMOGLOBIN A %
FROM ZERO TO 23 WEEKS OF AGE

	Age	Mean	S.D.	Range	N
Day	0-1	10.80	9.77	3.74 - 31.35	7
	2-8	20.66	9.73	7.14 - 33.34	16
	9-13	31.89	12.00	14.18 - 47.79	9
Week	2	31.57	10.52	17.22 - 49.44	10
	3	39.46	12.37	17.22 - 57.77	15
	4	49.70	10.02	37.23 - 63.18	12
	5	59.29	8.06	43.07 - 75.08	14
	6	66.26	7.71	58.28 - 80.29	7
	7	69.58	8.78	55.85 - 86.94	12
	8	77.80	7.75	66.08 - 86.91	9
	9	80.05	8.38	68.44 - 94.67	10
	10	84.63	4.08	78.00 - 89.75	11
	11	84.33	4.93	77.21 - 90.76	11
	12	89.14	3.49	81.95 - 92.26	12
	13	90.44	2.58	84.95 - 93.97	10
	14	92.57	2.48	85.71 - 96.02	12
	15	91.95	3.20	85.91 - 95.74	10
	16	94.78	1.81	90.08 - 96.21	9
	17	94.82	1.43	92.40 - 96.89	9
	18	96.29	0.91	94.56 - 97.90	11
	19-20	98.14	0.55	96.96 - 98.99	15
	21-23	99.08	0.39	98.45 - 99.56	14

S.D.: sample standard deviation
N: number of samples

TABLE VII

COMPUTATION OF REGRESSION EQUATION FOR Hb F DECREASE IN CALVES

Age in weeks, X	log Hb F %, Y	Age in weeks, X	log Hb F %, Y
0.071	1.9504	10	1.1867
0.714	1.8995	11	1.1951
1.571	1.8332	12	1.0359
2	1.8353	13	0.9805
3	1.7753	14	0.8710
4	1.7016	15	0.9058
5	1.6097	16	0.7482
6	1.5281	17	0.7143
7	1.4382	18	0.5694
8	1.3464	19.5	0.2695
9	1.2996		

$\Sigma X = 191.8560$ $\Sigma Y = 26.7387$ $n = 21$

$\bar{X} = 9.1360$ $\bar{Y} = 1.2733$

$\Sigma X^2 = 2491.232$ $\Sigma Y^2 = 38.7331$ $\Sigma XY = 185.8349$

$(\Sigma X)^2/n = 1752.7964$ $(\Sigma Y)^2/n = 34.0456$ $(\Sigma X)(\Sigma Y)/n = 244.2848$

$\Sigma x^2 = 738.4366$ $\Sigma y^2 = 4.6875$ $\Sigma xy = 58.4499$

slope, $b = \Sigma xy/\Sigma x^2 = -0.0792$

log Hb F %, $Y = \bar{Y} - b(X - \bar{X}) = 1.2733 - 0.0792\ (X - 9.136)$

$= 1.9969 - 0.0792X$

$\Sigma d_{y \cdot x}^2 = \Sigma y^2 - (\Sigma xy)^2/\Sigma x^2 = 0.0610$

$s_{y \cdot x}^2 = \Sigma d_{y \cdot x}^2/(n - 2) = 0.0032$

$s_{y \cdot x} = 0.0566$

TABLE VIII

PACKED CELL VOLUME (PCV) AND HEMOGLOBIN (Hb) VALUES IN EXCHANGE TRANSFUSED CALF (1), ANIMAL: HOLSTEIN CALF NO. 2

Calf Age in Days	Hb gm/100cc Blood[1]	PCV cc/100cc Blood[1]	% Hb			Hb gm/100cc Blood		
			F	A	B	F	A	B
0	9.6	35.5	100.00	00.00	00.00	9.60	0.00	0.00
1	7.1	27.5	100.00	00.00	00.00	7.10	0.00	0.00
2	Blood was exchanged with Hb AB type adult blood							
2	11.0	33.5	0.00	49.04	50.96	0.00	5.39	5.61
3	9.2	31.0	0.00	---	---	0.00	--	--
4	10.3	32.0	0.00	50.00	50.00	0.00	5.15	5.15
5	9.6	30.5	0.00	48.56	51.44	0.00	4.66	4.95
6	--	--	0.00	---	---	0.00	--	--
7	8.5	27.5	0.00	49.26	50.74	0.00	4.19	4.31
8	8.9	28.0	0.00	51.12	48.88	0.00	4.60	4.35
9	9.6	31.0	0.00	53.24	46.76	0.00	5.11	4.49
10	8.5	27.0	0.00	50.99	49.01	0.00	4.33	4.17
11	8.5	28.0	0.00	56.64	43.36	0.00	4.81	3.69
12	8.0	26.0	0.00	56.05	43.95	0.00	4.48	3.52
13	6.5	18.0	0.00	70.21	29.79	0.00	4.56	1.94
14	4.6	16.0	23.14	71.07	5.79	1.06	3.27	0.27
15	4.3	15.0	27.27	72.73	0.00	1.17	3.13	0.00

TABLE VIII (Continued)

Calf Age in Days	Hb gm/100cc Blood[1]	PCV cc/100cc Blood[1]	% Hb			Hb gm/100cc Blood		
			F	A	B	F	A	B
16	4.3	17.0	13.13	86.25	0.00	0.57	3.71	0.00
17	5.3	19.0	16.07	83.93	0.00	0.85	4.45	0.00
18	5.3	20.0	16.05	83.95	0.00	0.85	4.45	0.00
19	--	21.0	12.40	87.60	0.00	--	--	0.00
20	--	22.5	---	---	0.00	--	--	0.00
21	6.0	23.0	8.49	91.51	0.00	0.51	5.49	0.00
22	6.0	22.5	9.56	90.44	0.00	0.57	5.43	0.00
23	6.0	23.5	10.40	89.60	0.00	0.62	5.38	0.00
24	6.8	24.5	11.48	88.52	0.00	0.78	6.02	0.00
25	6.8	24.0	12.73	87.27	0.00	0.87	5.93	0.00
26	--	--	---	---	0.00	--	--	0.00
27	7.8	27.0	12.14	87.86	0.00	0.95	6.85	0.00
28	7.4	25.0	11.74	88.26	0.00	0.87	6.53	0.00
29	--	--	14.55	85.45	0.00	--	--	0.00
30	7.8	26.5	8.72	91.28	0.00	0.68	7.12	0.00

[1]These values were determined at the Veterinary Research, Oklahoma State University.

TABLE IX

PACKED CELL VOLUME (PCV) AND HEMOGLOBIN (Hb) VALUES IN EXCHANGE TRANSFUSED CALF (2), ANIMAL: HOLSTEIN CALF NO. 2

Calf Age in Days	Hb gm/100cc Blood[1]	PCV cc/100cc Blood[1]	% Hb			Hb gm/100cc Blood		
			F	A	B	F	A	B
0	--	--	100.00			--		
1	--	--	100.00			--		
2	9.1	32.5	100.00			9.10		
			Blood was exchanged with Hb AB type adult bloog					
2	10.1	31.5	0.00	49.98	50.52	0.00	5.00	5.10
3	9.6	28.0	0.00	48.98	51.02	0.00	4.70	4.90
4	9.0	26.5	0.00	48.68	51.32	0.00	4.38	4.62
5	8.0	24.5	0.00	48.15	51.85	0.00	3.85	4.15
6	8.1	25.5	0.00	50.33	49.67	0.00	4.08	4.02
7	7.3	24.5	0.00	49.55	50.45	0.00	3.62	3.68
8	8.1	24.5	0.00	54.88	45.12	0.00	4.45	3.65
9	8.5	25.0	0.00	56.86	43.14	0.00	4.83	3.67
10	8.8	27.5	0.00	54.43	45.57	0.00	4.79	4.01
11	8.3	26.0	0.00	56.58	43.42	0.00	4.70	3.60
12	5.5	17.5	9.92	66.94	23.14	0.55	3.68	1.27
13	3.8	12.3	21.79	78.21	0.00	0.83	2.97	0.00
14	4.2	14.5	16.47	83.53	0.00	0.69	3.51	0.00

TABLE IX (Continued)

Calf Age in Days	Hb gm/100cc Blood[1]	PCV cc/100cc Blood[1]	% Hb			Hb gm/100cc Blood		
			F	A	B	F	A	B
15	4.9	16.5	15.38	84.62	0.00	0.75	4.15	0.00
16	5.7	19.0	13.19	86.81	0.00	0.75	4.95	0.00
17	5.5	20.5	16.07	83.93	0.00	0.88	4.62	0.00
18	6.3	21.0	15.70	84.30	0.00	0.99	5.31	0.00
19	6.5	21.5	8.51	91.49	0.00	0.55	5.95	0.00
20	6.6	22.0	8.11	91.89	0.00	0.54	6.06	0.00
21	7.2	23.5	7.92	94.08	0.00	0.57	6.63	0.00
22	7.0	24.0	4.24	95.76	0.00	0.30	6.70	0.00
23	7.3	24.5	6.72	93.28	0.00	0.49	6.81	0.00
24	7.3	24.5	5.88	94.12	0.00	0.43	6.87	0.00
25	8.5	29.0	5.30	94.70	0.00	0.45	8.05	0.00
26	--	--	---	---	0.00	--	--	0.00
27	9.8	31.0	4.67	95.33	0.00	0.46	9.34	0.00
28	8.7	28.0	4.73	95.27	0.00	0.41	8.29	0.00
29	8.7	27.0	5.59	94.41	0.00	0.49	8.21	0.00
30	--	--	---	---	0.00	--	--	0.00

[1]These values were determined at the Veterinary Research, Oklahoma State University.

TABLE X

HEMOGLOBIN A AND F CHANGES IN AN ANEMIC CALF BY BLOOD LOSS

Age in days	Hb A gm/100 ml blood	Hb F gm/100 ml blood	Blood volume withdrawn (ml)
4	0.84	8.36	
5	0.91	7.59	
			300
6	1.03	6.27	
			300
7	1.24	5.16	
8	1.88	5.19	
			300
9	1.99	3.71	
			300
10	1.89	3.11	
11	2.74	2.06	
12	2.06	2.34	
13	2.22	2.38	
14	2.38	2.42	
15	2.00	2.00	
16	2.25	2.05	
17	--	--	
18	2.66	1.74	
19	2.73	1.77	
20	2.80	1.80	
21	2.67	1.93	
22	2.75	1.75	
23	2.91	1.59	
24	2.97	1.53	
25	2.98	1.32	
26	2.94	1.56	
27	3.20	1.60	
28	3.51	1.59	
29	3.10	1.40	
30	3.10	1.53	
32	3.27	1.23	
33	3.12	1.18	
34	2.91	1.19	
35	3.17	1.33	
36	3.60	1.20	
37	3.15	1.23	
38	3.04	1.26	
39	--	--	
40	3.80	1.10	
41	--	--	
42	--	--	
43	4.47	1.03	
44	--	--	
45	5.32	0.88	

TABLE X (Continued)

Age in days	Hb A gm/100 ml blood	Hb F gm/100 ml blood	Blood volume withdrawn (ml)
46	6.35	0.85	
47	6.40	1.00	
48	7.35	0.95	
49	7.39	1.01	
50	8.86	0.75	

Animal: Angus female calf No. 205

TABLE XI

HEMATOLOGIC OBSERVATION IN AN ANEMIC CALF BY BLOOD LOSS[1]

Age in days	Hb gm %	PCV cc/100cc	RBC count/ cmm x 10^{-6}
4	9.2	30.0	6.10
5	8.5	27.5	5.40
5	7.8	26.5	5.20
6	7.3	24.5	4.50
7	6.4	20.5	5.05
8	7.1	23.5	4.79
8	-	18.5	--
9	5.7	19.0	3.62
10	5.0	17.5	3.32
11	4.8	16.5	3.14
12	4.4	15.5	2.64
13	4.6	16.0	3.14
14	4.8	17.0	2.82
15	4.0	15.0	2.52
16	4.3	15.0	2.44
17	4.5	14.5	2.42
18	4.4	15.5	2.50
19	4.5	15.5	2.66
20	4.6	16.5	2.78
21	4.6	17.5	2.34
22	4.5	16.0	2.64
23	4.5	16.0	2.60
24	4.5	16.5	2.38
25	4.3	16.0	2.32
26	4.5	16.0	2.20
27	4.8	18.0	2.42
28	5.1	18.5	2.16
29	4.5	17.5	2.10
30	4.5	17.5	2.18
31	5.0	19.0	2.42
32	4.5	17.0	2.20
33	4.3	17.0	1.74
34	4.1	16.0	1.84
35	4.5	17.5	2.22
36	4.8	17.0	2.58
37	4.3	15.5	2.12
38	4.3	15.5	2.16
39	4.8	16.5	2.38
40	5.0	18.0	2.48
41	4.6	17.5	2.36
42	5.7	18.0	2.34
43	5.5	17.5	2.62
44	6.4	22.0	3.44
45	6.2	23.0	3.44

TABLE XI (Continued)

Age in days	Hb gm %	PCV cc/100cc	RBC count/ cmm x 10^{-6}
46	7.4	25.5	3.74
47	7.4	26.5	--
48	8.3	28.0	4.32
49	8.4	29.0	4.78
50	9.6	32.0	5.48

[1]The data were obtained from the Veterinary Research Laboratories, Oklahoma State University.

Animal: Angus female calf No. 205

TABLE XII

ALKALI DENATURATION RATE OF NEWBORN AND MATERNAL CANINE HEMOGLOBINS

Time in minutes	% Undenaturation	
	Newborn canine Hb[1]	Maternal canine Hb[2]
0	---	---
1	88.32	87.15
2	83.85	82.97
3	78.90	78.78
4	73.94	74.99
5	69.67	70.80
6	65.43	67.80
7	61.53	67.42
8	57.74	62.79
9	54.49	59.44
10	51.01	56.10
11	47.89	53.56
12	44.98	50.22
13	42.10	47.28
14	39.99	44.30
15	37.32	41.77

[1]Mean values of hemoglobins from 6 newborn Beagle dogs.

[2]Mean values of hemoglobins from 2 maternal Beagle dogs of above puppies.

TABLE XIII

REGRESSION OF MEAN HEMOGLOBINS F AND A GM % OF NEWBORN CALVES ON DAYS PRE AND POST *A. marginale* INFECTION

	Hemoglobin A		Hemoglobin F	
	Pre-infection	Post-infection	Pre-infection	Post-infection
Slope	0.120	-0.308	-0.169	-0.441
Intercept	5.310	6.571	5.674	4.821
$s_{\bar{y}\cdot x}$	0.058	0.103	0.066	0.112

VITA

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