ENGLISH, Darrel Starr, 1936-
THE DEVELOPMENTAL GENETICS OF
HEMOGLOBIN SYNTHESIS IN CHIRONOMUS.

Iowa State University, Ph.D., 1968
Biology-Genetics

THE DEVELOPMENTAL GENETICS OF HEMOGLOBIN SYNTHESIS IN *CHIRONOMUS*

by

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A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Genetics

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

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Signature was redacted for privacy.

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Iowa State University
Of Science and Technology
Ames, Iowa

1968

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INTRODUCTION

Normal and Abnormal Hemoglobins

The red substance of blood has excited the interest of man since his earliest history. This pigment, hemoglobin, has now been under intensive investigation by chemists, physical chemists and biochemists for a considerable number of years. It was one of the first proteins to be crystallized and was described as a tetrahedral crystal by K. E. Reichert in 1849. The hemoglobins and a closely related muscle protein myoglobin were shown to be conjugated proteins containing the prosthetic group heme and a protein portion called globin. It was demonstrated that hemin crystals from different species were always the same and the differences were due to the globin portion of the molecule. It was later shown that the differences between hemoglobins resided in amino acid substitutions throughout the polypeptide chain of the globin. These substitutions were probably, in turn, the result of single base changes in the DNA strands of the hemoglobin genes.

Complex proteins such as hemoglobin and myoglobin exhibit several levels of organization. Their primary structure implies the number and type of amino acids which make up the polypeptide chain in the globin portion of the molecule. By their secondary structure, the regular arrangement in space of the polypeptide chain is implied, as for example the alpha helix. Their tertiary structure refers to the arrangement of such helices in space, giving the super folding of the molecule in three dimensions. Finally, the quarternary structure of a protein refers to the assembly of two or more subunits to form the final molecule.

The hemoglobins found in most vertebrates are composed of four such subunits (Schroeder 1959). The globin portion of each tetramer typically contains two pairs of identical monomers. Adult hemoglobin A, for example, contains two alpha and two beta polypeptide chains, each of which is combined with a heme group containing an iron atom at the center. It is the ferrous iron atom at the center of each heme group which is functionally the most important part of the molecule, since it is at this site that oxygen attaches reversably during oxygenation and deoxygenation of hemoglobin in its normal physiological role. Perutz and co-workers (1960) determined the complete three dimensional structure of horse hemoglobin using X-ray diffraction analysis.

One notable exception to the tetrameric form of hemoglobin is found in the lamprey, a primitive chordate containing a hemoglobin molecule that resembles myoglobin, because it possesses a monomeric heme-globin configuration. The molecular weight of this molecule was calculated to be approximately 17,000. Hagfish hemoglobin was also determined to be very similar or possibly a dimer of 34,000 molecular weight. The tetrameric hemoglobins have been dissociated into dimers and then monomeric units in solutions containing urea or guanidine or low pH (Braunitzer et al. 1964). These monomeric subunits likewise have a molecular weight of roughly 17,000, thus making the total molecular weight of the tetrameric form 68,000. The monomeric form of the molecule was regarded to be more primitive than the tetramer, and the monomer existing in the lamprey was presumed to be the archetypal molecular form. During the course of evolution a series of fortunate mutational events modified the primitive molecule, enabling it to associate into tetrameric groups and thereby increased its effectiveness

in an oxygen transport mechanism.

In contrast to the normal human hemoglobin A type, the human fetus has a different type of hemoglobin which is distinguished chemically, but has the same overall complexity. Human fetal hemoglobin is composed of two alpha polypeptide chains identical to the adult type and two gamma polypeptide chains chemically different from the beta chains. Also found in humans is a minor hemoglobin designated as hemoglobin A_2 which is electrophoretically different from the others. It also consists of four subunits, two alphas which are the same as before and two delta chains which are distinguishable from beta and gamma chains. Thus, there are four types of polypeptide chains in the normal human, which make up three types of hemoglobins. There is good evidence that these polypeptide chains have their primary structure controlled by four different genes.

Over thirty abnormal hemoglobin forms have been characterized in humans. Each known variant has resulted in a change of at least one amino acid residue in one of the two polypeptide chain types. Each of these single amino acid mutations, in turn, corresponds to a single base change in the genetic code. One of the first mutant hemoglobins to be discovered was hemoglobin S, the abnormal hemoglobin that occurred in sickle cell anemia (Pauling et al. 1949). Ingram (1957) showed that this disease is due to a substitution of a glutamic acid residue for a valine in position six of the beta polypeptide. This seemingly minor change has a profound effect on the afflicted individual. The red blood cells are deformed and the oxygen carrying capacity is impaired. Individuals who are heterozygous are apparently healthy, but carry some sickle shaped erythrocytes. Homozygous individuals have severe hemolytic anemia. It was rather

surprising to find that individuals heterozygous for hemoglobin S are more resistant than normal individuals to malaria (Allison 1954, 1964).

This single change in the amino acid sequence of the beta polypeptide chain is ascribed in turn to a change of a single AT base pair to TA in the human genome (Ingram 1958). Some abnormal hemoglobins involve an unusual combination of chain types. Two examples are hemoglobin H, which contained four beta peptide chains, and hemoglobin Barts, which contained four gamma chains. Both have been detected because they result in inherited hemolytic anemia. It appears that they involve a relative overproduction of beta and gamma chains within a cell, leading to tetramer formation. These examples of abnormal situations, often referred to as "molecular diseases", have had a profound influence in reinforcing modern concepts of molecular genetics.

Hemoglobin Synthesis

An understanding of the mechanism of hemoglobin synthesis was greatly aided by the fact that immature red blood cells produce only a single protein, hemoglobin. Dintzis (1962) showed that the polypeptide grows in a linear fashion from one end to the other in rabbit reticulocytes. Furthermore, he was able to show by pulse labeling that the hottest amino acid (number 16) in the alpha chain is at the C-terminus. This indicated that the C-terminal peptide is the last one laid down in the synthesis of the alpha chain and that the growth of the chain begins at the N-terminus and proceeds toward the C-terminus.

Knopf (1962), utilizing the fact that rabbit ribosomes are free from the endoplasmic reticulum, was able to prepare a cell-free system. His results confirmed the experiments of Dintzis, but differed from the

reticulocyte system in that the cell-free system was unable to start a new round of synthesis. He found that the ribosomes are only able to complete the synthesis of the chain already started before their isolation.

Von Ehrenstein and Lipmann (1961) were able to show the universality of the protein synthesizing mechanism. They reconstituted a cell-free hemoglobin synthesizing system consisting of rabbit ribosomes and Escherichia coli sRNA with C^{14}-leucine. Upon suitable incubation leucine went directly into hemoglobin. Therefore, the leucine specific sRNA from E. coli possessed the same recognition mechanism for the ribosomal template as did the rabbit reticulocyte sRNA.

Von Ehrenstein, Weisblum and Benzer (1963) were able to demonstrate that once the amino acid was attached to the sRNA, in the case of E. coli, the placement of the amino acid on the template was solely a function of the sRNA. He was able to remove the sulfur of a cysteinyl-sRNA complex thus transforming the cysteine to alanine. The rabbit hemoglobin synthesized had alanine where normally cysteine was found. These findings concerning protein synthesis correlated nicely with the current central dogma of protein coding.

Another illuminating article by Winterhalter and Huehns (1964) described the steps involved in tetramer formation. Their data suggested that during protein synthesis the alpha chain is not released from the ribosome until it has combined with a beta, gamma or delta chain. These chains are released from the ribosome before dimer formation. The dimers are then combined with identical dimers and four heme groups to complete the hemoglobin molecule. The 25:1 ratio of Hb A to Hb A_2 and replacement of Hb F by Hb A normally found in adult humans would then depend on the

relative rates of synthesis of the beta, gamma and delta chains. The number of polysomes carrying mRNA for these chains regulates the alpha chain production at the polysome level since their release in dimer formation depends on the presence of one of the other chains. This also accounts for the absence of tetramers of alpha chains and accounts for the presence of tetrameric beta and delta chains, if the polysomes producing alpha chains were absent. Ingram (1963), Baglioni (1963) and Zuckerkandl (1964) have proposed a regulatory mechanism for the production of different polypeptide chains and the rate of hemoglobin production, based on the operon model in Escherichia coli as proposed by Jacob and Monod (1961).

Developmental Aspects

A differentiating cell was characterized by the variety of proteins it produced (Markert 1963). The diverse hemoglobin types produced in man were very illuminating with regard to the ontogenetic sequence of protein production and gene action. Early in fetal life the production of Hb F precedes that of the normal Hb A molecule. The difference in structure between the two hemoglobins was shown to involve the production of alpha and gamma chains as opposed to alpha and beta chains. The production of fetal hemoglobin predominates until the time of birth, at which time approximately 70% of the blood contains Hb F and the remaining 30% is Hb A. By the end of the first year, all fetal hemoglobin is replaced by adult hemoglobin. It appeared that the gene controlling the gamma chain is suppressed during infancy and the activity of the beta gene is switched on, while the alpha chain remains functional at all times. Why the fetus needs a different hemoglobin remains unclear. Although the oxygen affinity of hemoglobin F in the fetal circulation was shown to be higher than the

oxygen affinity of adult hemoglobin, Allen and Jandl (1960) were unable to find evidence that this had any conclusive physiological advantage.

In considering the change from Hb F to Hb A, the problem of differentiation at the molecular level became apparent. This switch was important from a hereditary view since some hereditary hemoglobinopathies such as thalassemia resulted from an overproduction of the fetal protein.

Baglioni (1963) proposed an explanation of the fetal-adult hemoglobin switch mechanism based on the Jacob and Monod repressor and operator concept. Red blood cells arise from undifferentiated continuously dividing cells in the erythropoetic tissue. The stem cells then differentiate into erythroblasts which either continue to divide or mature into erythrocytes. Hemoglobin synthesis begins when the stem cell is transformed into the erythrocyte. Baglioni assumed that all hemoglobin genes are repressed in stem cells and that the alpha and gamma chain genes become activated as the cell differentiates. Derepression depends on the number of cell divisions previously undergone by the stem cell. In the normal adult, differentiated stem cells are sufficiently mature, thus derepressing beta and gamma genes in addition to alpha genes. In young stem cells a genetically controlled repressor substance inhibits the operator locus of both beta and delta genes while the alpha and gamma genes become derepressed, thus producing fetal hemoglobin. As the stem cell divides the concentration of repressor substance for the beta and delta chains subsides, and they are activated. It was also assumed that a new repressor for the gamma operator is stimulated or that beta chain messenger RNA molecules act to repress the gamma operator in a feedback inhibition mechanism.

Occasionally an adult produces excessive amounts of Hb F, as in thalassemia, which causes severe hemolytic anemia. The hemolytic situation triggers a demand for new red blood cells, which in turn stimulates maturation of stem cells and necessitates differentiation of young stem cells from erythropoetic tissues. These cells produced predominantly Hb F because the repressor substance for the beta and delta genes is not diluted from repeated cell division. A second possibility is that of mutation. One can assume that a genetic defect in the structural beta gene may result in defective beta chain messengers. The messenger might become defective in various ways. A point mutation leading to amino acid substitution or a more detrimental mutation, such as a deletion, could yield defective beta chain messengers which are unable to make beta polypeptides (Ingram 1963).

Fetal hemoglobin is also detected in the monkey, ox, goat, sheep and mouse (Ingram 1963). The ontogeny of multiple molecular forms of hemoglobin has also been studied in the developing chick embryo (Fraser 1964, Huisman and Schillhorn van Veen 1964, D'Amalio and Salvo 1961, Manwell et al. 1963, Manwell et al. 1966, and Simons 1966) and among adult and larval frog hemoglobins (Herner and Riggs 1963, Baglioni and Sparks 1963, Elzinga 1964, and Manwell 1966a).

Evolution of Hemoglobins

The study of the homology of different hemoglobin molecules provides a direct means of tracing evolution as it relates to gene action. The great similarity of hemoglobin from man down to the cartilagenous fishes suggests that one is dealing with an extremely well defined molecule. Even though certain changes in the peptides have taken place, there is

some evidence that there are so-called "basic centers" of the peptide chain that are very similar in the cow, horse, sheep, rabbit and goat. These centers are presumably needed to maintain the correct folding of the chains and thereby the proper functioning of the molecule (Ingram 1963).

Ingram (1963) proposed a scheme of evolution for the various chains found in man based on the degree of homology which existed between the different polypeptides. It was found that the alpha and beta chains had about one-fourth of their amino acid sequence in common while the gamma and beta chains had about two-thirds of their amino acid sequence identical. The delta and beta chains exhibited the greatest homology with 95% of their residues in an identical sequence. He postulated that the primitive hemoglobin molecule was once similar to the present day myoglobin molecule. The primitive heme protein gene presumably duplicated, and a subsequent translocation moved one of the duplicated segments to a different site. One of these changed very little and gave rise to the modern myoglobin gene while the other underwent extensive modification to produce the present day alpha chain gene. Among the changes which occurred was the ability to dimerize. Next, the alpha chain gene duplicated, evolved into the gamma chain gene and was translocated. The accumulated mutational changes in the dimers of these genes eventually interacted to form tetramers possessing even greater selective advantage as oxygen carriers. The gamma chain became responsible for the production of fetal hemoglobin. Later it was duplicated and evolved into the beta chain. Since both the gamma and beta chains linked with the alpha chain in tetramer formation, one would expect the alpha chain not to become altered any further because of the structural requirements demanded of it. As one would expect, the difference in the alpha

chains of various vertebrates has been very slight compared to variations in amino acid sequence of the beta and gamma chains.

A later duplication involved the beta chain. This led to the production of the delta chain. It is presumed to be the most recent because of its great similarity to the beta chain and because the beta and delta chains remain closely linked, as if they originated by unequal crossing over.

Zuckerkandl and Pauling (1962) have attempted to reconstruct the probable time of existence of the common molecular ancestors of the various polypeptide chains. The beta and delta chains differ by only ten sites and represented the most recent to arise. According to Pauling's calculations this implies a lapse of time roughly equal to 35 million years. The beta and gamma chains differ by 37 sites and were postulated to have originated by duplication some 150 million years ago. The beta and alpha chains differed by 76 sites, and their common ancestor was placed at 380 million years ago. If these rough approximations were valid, the genetic ancestor of the hemoglobin chains dated back to the Devonian period when amphibians first made their appearance. The differences in amino acid sequence between the hemoglobin and myoglobin chains are very numerous, and a common ancestor for these dates back approximately 650 million years to the end of the Precambian era. This was long before the appearance of the vertebrates, suggesting the possibility of finding in some living invertebrates a distant relative of the vertebrate hemoglobins and myoglobins.

A number of attempts have been made to construct a molecular phylogenetic tree based on the overall differences between homologous peptides. Although much of the information was incomplete, an examination of the

amino acid sequence in the alpha and beta chains of various species has shown many changes, ranging in number from species to species in a manner roughly correlated with taxonomic classification (Jukes 1966).

Hill, Buettner-Janusch and Buettner-Janusch (1963) have concentrated their efforts on the alpha and beta chains in primates. They observed less variation in the alpha chain than in the beta chain during primate evolution. The beta chain of lower primates was found to be identical in many loci with human gamma chains rather than with human beta chains, thus substantiating the belief that the beta chain was of more recent origin. Furthermore, some primates lack the A_2 type of hemoglobin, also emphasizing the comparatively recent origin of the delta chain.

Zuckerkandl, Jones and Pauling (1960) have shown that fingerprints of hemoglobin from the gorilla and chimpanzee were indistinguishable from human patterns. A greater variation was noted in peptide spots in the less closely related orangutan and rhesus monkey. As one moved further away from the primates the differences increased. Chickens showed considerable differences, while the fish hemoglobins gave drastically different fingerprints (Ingram 1963). It is also of interest to note that the primary structure of lamprey hemoglobin contains no sequence gaps, giving further evidence for its primitive nature (Braunitzer 1965).

Insect Hemolymph

Unlike the vertebrates, where the presence of hemoglobin appears to be the general rule, the occurrence of hemoglobin in the invertebrates is uncommon and haphazard when seen phylogenetically. Hemoglobin among the insects is exceptional. Insects generally respire with the aid of a trachael system which may ramify into individual cells where oxygen

diffusion is accomplished. Certain Hemiptera (backswimmers) and larvae of the dipteran genus Gastrophilus possess trachael cells which contained hemoglobin, and the Chironomidae (Diptera) include several forms whose larvae possessed the pigment freely dissolved in the hemolymph.

Wyatt (1961) has shown that there are characteristic patterns of protein synthesis during the metamorphosis of insects. During early larval life the concentration of protein increases, with a maximum concentration during the last instar. As one would expect, this increase parallels the growth of the insect. During pupation there appears to be little change in the types of proteins produced, although they are concentrated somewhat due to the general loss of water. During the transformation of a pupa into an adult there is a rapid decline in the types of protein and their concentration as the hemolymph proteins are being utilized in the formation of new adult structure.

Although metamorphosis in insects involves the alteration of larval structure into a mature, reproducing adult whose body structure and mode of life are completely different from its larval habit, it is assumed that every cell involved contains the same genetic information. Evidently the larval stage is an expression of one battery of genes and the adult stage another set of genes, except where corresponding body parts function in both stages. There may be many instances of repression as Jacob and Monod (1961) describe it for Escherichia coli.

As in the case of most higher organisms, the post-embryonic growth of an insect appears to be under the direct or indirect influence of hormonal control. A number of comprehensive review articles dealt with this aspect of metamorphosis (Bodenstein 1955, 1957, Butenandt 1959,

Campbell 1959, Karlson 1956, Novák 1959, Pflugfelder 1958, Schneiderman and Gilbert 1959, Williams 1952, and Wigglesworth 1954, 1957, 1959).

Beermann (1963) demonstrated in Chironomus that a puff formed at a locus of a chromosome in one cell may not occur on the same chromosome of an adjacent cell within the salivary glands. This suggested that the genes involved can be suppressed or activated. Becker (1962) shed further light upon the puffing phenomena. He placed a ligature behind the brain of Drosophila larvae. The anterior end containing the ring gland underwent metamorphosis whereas the posterior end remained unchanged. Becker had shown previously that an increased number of puffs normally occurred during metamorphosis. In the ligated animal he found puffs only in the anterior portion while the posterior portion remained quiescent. The hormone released by the ring gland was responsible for this reaction. In further transplant experiments he demonstrated that implanted chromosomes were induced to return to a puffing behavior they had previously passed through and that other chromosomes that had never reached the puffing stage were stimulated to do so. These experiments seemed to indicate suppression was not permanent.

The hormone of the ring gland which controlled molting and metamorphosis was a cholesterol derivative named ecdysone (Butenandt and Karlson 1954). Clever (1963) studied the effect of injected ecdysone upon the puff formation in the giant chromosomes of Chironomus tentans. The hormone appeared to stimulate puffing at two loci in chromosome one and later another puff was activated in chromosome four. His observation suggested that ecdysone acted directly on two loci of one chromosome and the products

which they produced triggered a sequence of events at other chromosomal loci.

Kroeger (1964), however, showed that Chironomus larvae injected with solutions of salts like zinc chloride will mimic the action of ecdysone with respect to puff formation. These observations suggested that ecdysone as well as other compounds produced their effect on an intermediate system which, in turn, activated a specific chromosomal locus.

It was of interest, therefore, to undertake a study of the developmental genetics of the hemoglobin molecule produced during the growth and development of the insect Chironomus tentans. This unique organism contained multiple hemoglobins freely dissolved in the hemolymph and, furthermore, the animal was adaptable to laboratory conditions with a minimum of expense and maintenance.

The first task was to characterize the hemoglobin pattern electrophoretically. Since it passed through six separate developmental stages, four larval instars, pupa and adult, one expected to find some reflection of this metamorphic change in the numbers or quantity of hemoglobin molecules produced. Furthermore, it seemed likely that different electrophoretic patterns were a possibility as a result of different populations arising from geographic isolation and mutation. The variant forms, if found and characterized electrophoretically, would lend themselves to hybridization experiments in an effort to elucidate the mode of control of the hemoglobin genes.

Analysis of the globin subunits of each molecular form was of interest for determining the distribution of similar and dissimilar polypeptides. If the molecule is a dimer, but with variant forms, it would be essential

to determine whether two or more of the hemoglobins had a similar subunit as in the case of multiple hemoglobins found in man. With the number of actual polypeptides known, the number of hemoglobin loci and their mode of control might be inferred.

Also of basic interest is the question of the site of hemoglobin synthesis. Eventual studies relating to the active sites of hemoglobin loci, their stimulation and repression and interaction with other genes hinged on the understanding of the fundamental hematopoetic process of this insect.

REVIEW OF EXPERIMENTAL PROCEDURES

Chironomus tentans, according to Johnnsen and Townnes (1952), is a very large species of midge with conspicuous mesoscutal stripes and dark abdomen. It is distributed in the New World from Quebec and Manitoba, Canada, to New York and west to British Columbia and Utah. It is a common species of the northern United States usually found breeding in polluted water.

The immature stages of the midge are aquatic and in nature the larvae overwinter until spring. There is a direct correlation between water temperature and the transformation of larvae to adults. By the time the water temperature reaches 10°C they begin to pupate and shortly thereafter adults appear. The time between emergence and oviposition varies from one to three days. The egg masses are a hollow C-shaped gelatinous mass 16 to 24 mm long and 4 to 6 mm wide. The eggs are imbedded in a flat gelatinous ribbon, which is wound back and forth in circular rows. Each female lays one egg mass consisting of 1,400 to 3,300 eggs with an average of 2,300. At a temperature between 20-22°C the eggs hatch out in three to four days (Sandler 1935).

The rate of mortality during the egg stage is exceedingly low. During larval stages, however, the mortality rate is high, although the controlling factor is unknown (Needham 1937). Experiments by Sandler (1935) indicated a loss of 15-30% with the major loss occurring during the first 8 to 10 days of larval life.

In general, the larvae ate whatever was offered them including powdered milk, chicken manure, dog food (Biever 1965), soybean meal, sheep manure and superphosphate (Sandler 1935). Clever (unpublished) used a

combination of ground nettle leaf, sterilized leaves, soft cellucotton and 0.06% NaCl solution.

When larvae first hatch they are transparent. They leave the egg mass after one day and begin tubule construction. The first instar lasts from five to nine days. Hemoglobin production begins during the second instar, which lasts from six to eight days. During this stage, the larvae feed voraciously and grow rapidly. After the second molt, the larvae are bright red and tend to avoid light by spending most of their time in their tubes. The third instar lasts from six to ten days. The final stage of larval life varies far more than any other stage, lasting from four to five days or up to three weeks. It is not understood why there is such a wide variation in the last instar, but it was probably due to a physiological phenomenon. The irregularity in maturation is desirable, however, since it ensures an overlapping of the life cycle and a constant supply of adults and eggs. The pupal stage is marked by a greatly enlarged thoracic segment and the cessation of feeding. This stage lasts only about three days, the first two of which are spent inside the tube. On the third day the pupae swim about and finally come to the surface, where their skin splits and the adults emerge. The adults do not feed before reproducing and live an average of three to five days (Sandler 1935).

Hemoglobin occurs free in the hemolymph of chironomid larvae. Since the larvae inhabit mud bottom pools where the environment is poor in oxygen (Miall, 1903), it was once thought that the hemoglobin of the larvae served as a store for oxygen during times of oxygen scarcity (Miall and Hammond 1900), but the amount of oxygen that is stored this way lasts the insect only about 12 minutes (Leitch 1916). At normal oxygen tensions the

blood is fully saturated and does not act as a carrier, and only at oxygen tensions less than one percent of the atmospheric pressure does the hemoglobin become partially reduced. Hemoglobin, therefore, acts as a carrier when the tension of oxygen becomes so low that a sufficient amount of oxygen is not supplied by the physiological environment (Wigglesworth 1965).

Chironomus kept under nitrogen for one-half hour contracted an oxygen debt which required one and one-half to two hours to pay off. The extra oxygen consumed during recovery about equaled the volume of oxygen used during deprivation. The ability of Chironomus larvae to live in anaerobic conditions depends not so much on their ability to exploit small amounts of oxygen in the water as on their ability to adapt to these conditions. Larvae of Chironomus thummi showed a tenfold increase in glycogen consumption when transferred to oxygen free water (Harnisch 1930).

The characterization of hemolymph proteins in general has depended heavily on the use of filter paper, starch gel and agar gel electrophoresis. Wyatt (1961) summarized much of this work. Generally, the conditions used with human serum have been applicable to hemolymph protein. Electrophoresis on paper resolved only a few protein bands which generally migrated at a velocity comparable to mammalian plasma globulins. The designation of these proteins as albumens, alpha- and beta-globulins as in human serum, based on their mobilities was unjustified, as pointed out by Denuce (1958). Agar and starch gel electrophoresis, based on the interaction of the blood protein with the medium as well as electrophoretic mobility, was first demonstrated by Smithies (1955). This technique gave sharper and greater resolution over other techniques used up to that time.

The patterns obtained with electrophoresis were generally characteristic of the species and were used as a taxonomic tool by van Sande and Karcher (1960) where nutritional and developmental stages of the insect were controllable. Differences were also observed between normal and lethal genotypes in Drosophila by Wunderly and Gloor (1953). Zone electrophoresis elucidated the presence of glycoproteins, lipoproteins, phospholipids and sterols by staining certain bands with selective reagents (Siakotos 1960, Whitaker 1959). The hemolymph of Hyalophora cecropia was studied antigenically by Telfer (1954) and Telfer and Williams (1953). Using rabbit antiserum in an agar diffusion technique, they found as many as nine bands were precipitated, seven of which were followed quantitatively through metamorphosis. There was a general increase in amount of protein during larval development until diapause and a decrease during adult development with individual protein bands exhibiting characteristic patterns.

A relatively new method called disc electrophoresis, developed by Ornstein (1964), takes advantage of the adjustability of the pore size of a synthetic gel and at the same time achieves a high degree of resolution in a short time. In contrast to starch gels, polyacrylamide gels are thermostable, transparent, strong, relatively inert chemically, can be prepared with a range of pore sizes and are non ionic. Starch gels actually carry a few anionic groups which result in some backward endosmotic flow.

Over 20 components were routinely resolved with human serum on disc gels, compared to 5 with the conventional Tiselius apparatus. Where only minute quantities of protein or other substances are available, the applicability of this method is particularly great. It has been possible

to use samples as small as one to five microliters and still attain a high degree of resolution. The versatility of this technique was demonstrated by Narayan, Narayan and Kummerow (1964) who used a modified disc electrophoretic system for a wide range of animal blood serums with excellent results. The application of disc electrophoresis to a variety of other materials has rapidly increased and many of these experimental results are cataloged and abstracted in the Disc Electrophoresis Newsletter published by the Canal Industries Corporation, Bethesda, Maryland.

The complex heterogeneity of hemoglobin in Chironomus thummi was reported by Braunitzer and Braun (1965), in Chironomus plumosus by Manwell (1966b) and in the same or additional species by Thompson and English (1966). Braunitzer and Braun reported that hemoglobin was the major soluable protein extracted from homogenized Chironomus larvae. They found four major hemoglobins separable by column chromatography, and by further chromatography and counter-current distribution, they were able to separate two of these hemoglobins into subunits which by amino acid analysis, finger-printing and N- and C-terminal determination were found to be polypeptide chains of different primary structure. Their experimental findings showed this insect hemoglobin molecule to be composed of dimers of different globins which were designated as alpha and beta chains as in humans. There were, however, striking differences when compared to human subunits. There were only three histidine residues per chain whereas humans have ten in the alpha and nine in the beta chain. Humans have no isoleucine, while Chironomus thummi had 16 residues. The phenylalanine content was nearly one-fourth higher in Chironomus and none of the N-terminal or C-terminal amino acids corresponded to mammalian hemoglobin.

The total number of residues in Chironomus thummi numbered 127 and 124 as compared to 155 for Lampetra whose hemoglobin was composed of a monomer and 141 and 146 residues in the alpha and beta chain of the human tetrameric molecule. In agreement with Svedberg and Eriksson-Quensal (1934), the dimers were found to possess two heme groups and a molecular weight of 31,400.

Thompson and English (1966) cited four major and four minor hemoglobins in Chironomus thummi. Unlike Braunitzer and Braun, they demonstrated the multiplicity of these forms in single individuals, rather than in mass homogenates. They found a general intensification of bands paralleling the superficial redding of larvae especially during the third instar where certain bands made their appearance well ahead of others. This sequential accumulation was compared to the fetal-adult transition in mammals. The entire pattern was found to disappear during the late pupal stage when the hemoglobins were degraded during metamorphosis. The consistency of the number of hemoglobins and their electrophoretic mobilities seemed to indicate a synchrony between polypeptide chain production and the activity of several different gene loci. An essentially co-dominant effect in the hybridization of races was reported.

Manwell (1966b) also used starch gel electrophoresis and found small larvae (1-4 mg) of Chironomus plumosus lacked two of the three major hemoglobins as well as a minor hemoglobin but possessed a minor-hemoglobin (Zone X) which was found only as a trace in larger larvae (8-11 mg). Therefore, a minimum of eight hemoglobins were found in small larvae, and a maximum of ten separate hemoglobins were found in larger larvae. In agreement with Braunitzer and Braun (1965), Manwell calculated that the

hemoglobin of a larva represented at least 90-95% of the hemolymph protein. The heterogeneity and the ontogenetic differences were not altered by the addition of mercaptoethanol nor by conversion of hemoglobin to globin by splitting the heme group off with acid acetone. Analysis of globins of isolated major and minor hemoglobins in gels made with HCl, formic acid or 8 M urea appeared not to produce separate polypeptide chains. It was suggested that a single polypeptide chain type made up each of the individual hemoglobins. This was in contrast to vertebrate hemoglobin which was broken down into alpha and beta chains under similar conditions.

The molecular weight of biological materials is conventionally determined by means of elaborate equipment, such as the ultracentrifuge equipped with Schlieren optics. This experimental method has not only been difficult in cases where the molecular weight is high, but also requires extensive purification. A more recent and simpler method of determining molecular weight has involved the use of Sephadex gels as chromatographic materials which possess the property of separating substances according to their size. The molecular weight of various proteins was shown by Andrews (1964) and Whitaker (1963) among others. These authors demonstrated that various grades of Sephadex separate molecules of similar shape, since the elution volume is approximately a linear function of the logarithm of the molecular weight. This method was used not only for globular proteins and a variety of peptides but was also used in the characterization of macromolecules other than protein by Laurent and Killander (1964) and Carnegie (1965). From a calibration curve produced by the chromatographic separation of proteins of known molecular weight, it is possible to estimate the molecular weight of an unknown molecule. This method has

been particularly useful because it is the only method of estimating molecular weight of proteins which does not require extensive purification and elaborate equipment.

The synthesis of hemoglobin can be followed by the use of several radioactive chemicals as labeled precursors. Walsh et al. (1949) showed electrophoretically that radioiron was assimilated in reticulocytes in the ferrous state three times as fast as ferric iron. DeCavalho (1955), using refined microspectroscopic techniques, demonstrated that heme granules appeared in the nuclei of bone marrow reticulocytes. Austoni (1954) used Fe^{59} in an autoradiographic demonstration of the presence of in vitro hemoglobin synthesis in the nucleus of rat bone marrow red blood cells. Krause and Morrison (1955) used Fe^{59} and autoradiography to show in vitro hemoglobin synthesis around the nucleus of the human bone marrow erythrocytes in contrast to Austoni.

Hammel and Bessman (1964) observed increased protein synthesis in avian erythrocyte nuclei incubated in the presence of added hemin and other tetrapyrols. Bruns and London (1965) used a rabbit reticulocyte system to reveal that hemin increased the incorporation of C^{14} valine hemoglobin production. Levere and Cranick (1965) cited evidence indicating that delta-aminolevulinic acid (ALA) had a significant effect on the rate of hemoglobin formation in the chick blastoderm, almost tripling the amount of hemoglobin found during periods of in vitro incubation up to 30 hours. With longer incubation the ALA effect was less pronounced. When actinomycin D, which was considered to block messenger RNA formation, was added with ALA, the rate of hemoglobin formation was the same as the controls. It was assumed that the mRNA was already present in the hemocytoblast

and did not inhibit hemoglobin synthesis. When puromysin, which blocks protein synthesis at the ribosome level, was added with ALA no hemoglobin was formed. It was postulated that the addition of ALA, which as a porphyrin precursor presumably increased heme formation, stimulated globin synthesis and thereby also increased hemoglobin formation at the ribosomal level.

MATERIALS AND METHODS

Culturing Technique

Two strains of Chironomus tentans were maintained in the laboratory. One strain was collected from several small ponds known as Jemmerson's slough west of Spirit Lake, Iowa. This strain is referred to as the Iowa strain. The second culture, called the Wisconsin strain, was collected near the campus of the University of Wisconsin.

Chironomus larvae were reared in five-quart polyethylene crispers. Approximately three centimeters of distilled water containing 0.06% NaCl and a 6 x 6 inch square of cellucotton or Kemwipe was placed in the bowl. Also added was a sprinkling of nettle powder (S. B. Penick Co.) and several leaves which had overwintered or were boiled to remove excess tannin. Every two weeks the cultures were fed more nettle powder, and new water was added if the culture turned dark or developed a foul odor, according to instructions of Professor Ulrich Clever, Purdue University.

Adults were collected and mated in shell vials which contained a little saline solution and were lined with a strip of Kemwipe. After the egg masses were laid, they were transferred to Petri dishes where they hatched in two or three days. The young larvae were then transferred to culture bowls and were not disturbed for three or four weeks. All cultures were grown under similar conditions except for temperature. Some cultures were reared at 18-20°C while the majority of them were reared at 20-22°C. A photoperiod of roughly sixteen hours of light and eight hours of dark was maintained.

Electrophoretic Methods

Cellulose polyacetate, starch gel, and polyacrylamide disc gel methods of electrophoresis were used. Electrophoresis on cellulose polyacetate strips was carried out using the Gelman Rapid Electrophoresis Chamber, model 51101 (Gelman Instrument Company) and a Heath power supply. Prepackaged high resolution buffer (Gelman Instrument Company) containing Tris-barbital-sodium barbital (pH 8.8) was idssolved in different volumes of distilled water and cooled before using. Sepraphore III cellulose polyacetate strips were soaked in the buffer for at least ten minutes before blotting and applying the hemolymph sample. A Gelman sample applicator was used. Four hundred to six hundred volts were applied to the strips until a two inch migration pattern was obtained. The procedure was carried out in a refrigerator at 0-5°C to prevent excess heating and evaporation. The strips were stained five minutes in Ponseau S stain (Hartman-Leddon Company) and destained in several changes of 5% acetic acid.

Of several starch gel systems tried, two gave satisfactory results. The first was a modified Kristjansson (1963) method. The gel buffer was made of two stock solutions. Stock solution A consisted of 0.2N HCl and stock solution B was a solution of 23 grams Tris(hydroxymethyl) aminomethane per liter of distilled water. Gels were prepared by mixing 22 ml of stock solution A with 7½ ml of stock solution B and bringing the volume up to 250 ml with distilled water (pH 8.5). A suspension of 33½ grams of starch in 65 ml of the cold gel buffer was prepared while the remaining buffer was heated to the point of boiling. The hot buffer was quickly mixed with the starch suspension and degassed under vacuum for approximately 45 seconds. The semisolid suspension was poured into an

18 x 18 x 0.6 cm mold and allowed to cool at room temperature for at least one hour. The vessel buffer consisted of 18.55 grams of boric acid plus 3.99 grams of NaOH per liter of distilled water (pH 8.5). After five to eight usages the vessel buffer was replaced. Usually the third to fifth usage gave optimal results.

The second starch gel used was Ferguson and Wallace's (1961) modified Poulik discontinuous buffer (gel pH 8.0). The gel buffer consisted of 90% V/V of 0.003 M citric acid and 0.016 M Tris(hydroxymethyl)aminomethane, and 10% V/V of 0.02 M lithium hydroxide and 0.076 M boric acid. Twenty-seven grams of starch were used to prepare the gels in a fashion similar to that already described. These gels were allowed to cool two to three hours for optimum results. The electrode buffer consisted of a solution containing 0.1 M lithium hydroxide and 0.38 M boric acid.

A slice was made across the solidified gel 5.7 cm from one end of the gel form, and samples of hemolymph inserted into the slice. Hemolymph samples were prepared by puncturing the larvae or macerating the smaller specimens on a 5 x 8 mm rectangle of Whatman number 43 filterpaper. One sample of cattle or human serum which contained bromophenol blue or a sample of RBY marker dye (Gelman Company) was used as an indicator. Once the samples were placed in the slice, it was closed and a sheet of Saran Wrap placed over the gel to prevent excessive loss of water. A 2.0 cm strip on each end of the gel was left uncovered for making contact with the bridge wicks. A glass plate or pressure plate was put on top of the wicks and gel surface to ensure good contact between wicks and gel.

Two electrophoretic systems were used. The first consisted of electrode vessels made of 9 x 5 x 2½ inch pyrex baking pans.

Nylon-reinforced cellulose sponge cloths were used as bridge wicks. This system was placed in a refrigerator at 0-5°C. A Heath power supply delivered the current. The initial power supplied was 165 volts for the first 15 minutes. The sample papers were then removed and an additional 15 minutes at 165 volts was administered. The remainder of the run was carried out at 350 volts. The run was stopped when the marker dye had reached a length of 6.0 cm. Runs beyond this distance tended to build up too much heat and the band diffused readily. If the gel was too warm to handle easily, a cooling period of five to ten minutes in the freezing unit of a refrigerator was used before slicing and staining.

The second electrophoretic system consisted of a water cooled pressure plate electrophoresis cell (EC Apparatus Corporation, model EC401). Separations were made rapidly with this system, since heating was no problem at higher voltages. Voltages from 350 to 500 V were used by connecting Heath power supplies in series. The details for gel preparation were the same as described previously. Some gels were given a 15-minute prerun before the samples were placed in the gels. This was the time required for the borate line to migrate to the insertion line. These runs were allowed to continue until the marker dye had migrated between 6.0 to 6.5 cm, the latter being preferred.

After the run, gels were transferred to a slicing form and were sliced with a large cheese cutter and the two halves stained. One half was usually stained either with buffalo black or nigrosin and the other half with benzidine. Buffalo black was prepared by adding 1 gram of the stain to a mixture of 450 ml distilled water, 450 ml methanol and 100 ml glacial acetic acid. Nigrosin was prepared by adding five grams of water soluable

nigrosin to 50 grams of $HgCl_2$ dissolved in 50 ml glacial acetic acid. This solution was diluted with distilled water to a volume of one liter. Gels were stained 15-20 minutes, then destained in several changes of 5 parts methanol, 5 parts distilled water and 1 part glacial acetic acid. Satisfactory gels were wrapped in Saran Wrap and kept refrigerated.

Benzidine stain was prepared by mixing one part benzidine in grams with nine parts glacial acetic acid in milliliters. This mixture was heated to 50°C and added to 36 parts of distilled water. To use this solution, equal parts of the stock solution and 3% H_2O_2 were mixed and poured over the sliced surface of the gel. One to three minutes were required for optimal staining, at which time a polaroid picture was taken. Overstaining with benzidine was reduced by running water over the gel. These gels were usually discarded because loss of clarity occurred rapidly. Semipermanent gels could be made by soaking the gels in 70% alcohol before wrapping in Saran Wrap; however, the resolution of the bands was poor after this treatment.

Disc electrophoresis was carried out with a Canalco model 6 apparatus (Canal Industrial Corporation). The gels were prepared in glass columns in the dimensions of approximately 4 x 165 mm. Lower gels or separating gels consisted of equal quantities of Canalco solutions A-1 and A-2 and an equal volume of catalyst (pH 8.8). The catalyst was prepared by dissolving 0.14 gms of ammonium persulfate in 100 ml of distilled water. Fresh catalyst was prepared weekly. Approximately 0.9 cc of the separating gel was used per column. This was layered over with a few drops of distilled water before polymerization for 45 to 60 minutes in front of a fluorescent lamp. After polymerization the water was removed and 0.1 cc

of full strength upper gel, (pH 6.9) was applied, layered again with distilled water and allowed to polymerize for 15 to 20 minutes. This functioned as the spacer gel. The water was removed after the appropriate time had elapsed, and 2 to 3 lambda of fresh Chironomus hemolymph was mixed with 0.1 to 0.2 cc of full strength upper gel and applied to the top of the spacer gel. Polymerization of the sample gel was allowed to take place for 15 to 20 minutes without the addition of a layer of water.

The columns were then fitted into the electrophoresis cell and buffer was added. The buffer consisted of 6.0 gms of Tris(hydroxymethyl)-aminomethane and 28.8 gms of glycine in one liter of distilled water (pH 8.4). Five millamps per column were applied until the tracking dye (bromophenol blue) in the upper resevoir had migrated 1.25 to 1.50 inches. All runs were made at 0-5°C.

The gels were removed from the glass columns in ice water and immediately fixed and stained in a 0.5% amino black solution in 7% acetic acid. Gels were stained for at least one hour and then destained electrophoretically in 10% acetic acid at 10 millamps per tube. When the sections of gel containing no protein were clear of stain, the gels were transferred to small storage tubes filled with 10% acetic acid and refrigerated. Gels which were specifically stained for peroxidase activity with benzidine were treated in the same manner as described for starch gels and then stored in 70% methanol and refrigerated to slow down deterioration.

Electrophoresis of the globin subunits of the multiple hemoglobins of C. tentans was performed in 8 M urea acid starch gels containing 2-mercaptoethanol. The method which gave the best results used an aluminum lactate containing urea gel (Poulik 1966). A stock solution of $AlLac_3$ was

prepared by dissolving 119.32 grams of $AlLac_3$ in two liters of distilled water. Gel buffer was prepared by diluting 50 ml of the stock solution to 300 ml and adding 2.5 ml of 2-mercaptoethanol. A thorough mixture of 63 grams of starch and 240 grams of urea was made, ensuring that the urea was in a granular form. The dry ingredients were slowly added to the buffer solution and continually mixed with a magnetic stirrer. The flask was heated in a water bath at 70°C for 15-20 minutes. Every three to five minutes the mixture was vigorously agitated and returned to the water bath. At the end of the procedure the starch became semifluid and clear. A final agitation was performed instead of degassing before pouring into a mold. Bubbles which remained in the gel came to the surface and did not interfere with the results. A glass plate was laid over the surface of the form and the gel was allowed to cool for 24 hours. The final gel pH was approximately 4.5.

The electrode buffer consisted of one part stock buffer solution in four parts water. The glass bread pan apparatus was used at a temperature of 0-5°C as described for the standard starch gels. A voltage of 100 V was applied for a period of 12 hours. At the end of the run the gels were sliced and stained with nigrosin or buffalo black.

Two different approaches were taken in the globin analysis. First, samples of hemoglobin were separated in LiOH gels in the usual manner and longitudinal sections of the pattern were cut out and treated in some cases with cold acid acetone for one hour before insertion into a urea gel (personal communication from Dr. Clyde Manwell). The other half of the sectioned gel was stained with buffalo black for a later comparison with

the urea gel. In the second method, the hemolymph was first run on a strip of Sepraphore III and the various bands were cut out and placed in the gel.

Molecular Weight Determination

A variety of proteins were used as molecular weight markers. Human hemoglobin (64,000) and sperm whale myoglobin (17,800) were obtained from the Mann Research Laboratories, horse heart cytochrome C (12,400) from the Sigma Chemical Company, beef pancrease ribonuclease A (13,700), swine stomach pepsin (35,000), bovine pancrease trypsin (22,500) and egg white ovalbumen (45,000) from the Worthington Biochemical Corporation. Trypsin and pepsin were run separately while all other proteins were used once in combination and once separately. Samples were prepared by dissolving 5 to 10 mg of the crystalline material in 3 mls of buffer. Sucrose was added to the sample until a total weight of 2.5 mg of protein per 3 ml was attained.

Chironomus tentans hemoglobins were prepared by cutting up 30 to 40 large larvae in 50 ml of 0.05 M phosphate 0.05 M NaCl buffer at pH 7.0. The mixture was immediately centrifuged at 45,000 g for one hour to remove large particulate matter. Further purification and concentration was accomplished by mixing coarse Sephadex G-25 with the solution in a 1:4 W/V ratio and centrifuging the mixture at 1,000 g for 10 minutes in a special test tube designed to separate the swollen gel from the supernatant. The process was repeated until an optical density of three was obtained.

A Sephadex column (Pharmacia Fine Chemicals, Inc.) with an internal diameter of 25 mm and a length of 45 cm was used. The gel slurry, which was allowed to stand in a phosphate-NaCl buffer at pH 7.0 for three days

with periodic decantation, was evacuated prior to pouring until air bubbles ceased to escape from the gel. The column was poured with G-100 gel medium in one pass, at an effective bed height of 5 cm and allowed to equilibrate overnight at a final operating height of 20 cm. The procedure yielded an overall gel bed height of approximately 40 cm. The column was allowed to equilibrate at 23±2°C, which was the operating temperature used throughout the experiment. A flow rate of approximately 30 ml per hour was produced.

Samples were applied with a syringe in 3-ml volumes beneath a layer of eluent on top of the column while the column system was closed. Samples consisted of the protein and sucrose mixture plus 0.1% blue dextran (Pharmacia Fine Chemicals, Inc.). One-milliliter fractions were collected in a Packard fraction collector employing a drop count method of collection. Fractions were collected as soon as the column was opened. Protein peaks were identified by their ratio at 412/280 mu. All readings were taken in a Beckman DU-2 spectrophotometer.

Autoradiography

Larvae selected for sectioning and autoradiography were injected with C^{14}-labeled delta-aminolevulinic acid (New England Nuclear Corporation) and periodically sacrificed at 1, 6, 24, 48, 72, 96 and 120 hours. Glass capillaries were drawn out and the injection of the label was made with the aid of a disecting microscope. A screw type micrometer syringe was used to force the label into the hemocoel of the larvae. Larvae were transferred to 0.06% saline solution until sacrificed.

At appropriate intervals the specimens were immersed in Bouins fixative. The presence of the label in the larvae could be detected by a Geiger-Mueller apparatus. After 18-24 hours the larvae were washed in 70% alcohol for 1 hour, and run up through a graded series of solutions containing water, ethyl alcohol, N-butanol and paraffin according to Stairs (1960). Specimens were finally embedded in paraplast possessing a melting point of 56-57°C (Scientific Products).

The paraffin blocks were sectioned at 8-10 microns, and run through xylene and 100, 95, 85, 75, 50, 35 percent ethyl alcohol and distilled water for 30 seconds each. Slides were stained in Mayer's acid hemalum for at least 4 hours and transferred to tap water for 15 minutes. Slides were taken to the darkroom and coated with Kodak Nuclear Track Emulsion type NTB-2 for two seconds according to the procedures of Kopriwa and Leblond (1962). A Wratten-2 red filter was used during the process. Slides were placed in small black slide boxes inside of which small packets of Indicating Drierite (W. A. Hammond Drierite Company) were placed. The boxes were taped shut and placed inside another, larger container which was stored in a refrigerator at about 4°C for a period of between 4 and 6 weeks.

After the exposure time had elapsed the slides were developed in a solution of one part Kodak Dektol to two parts water for two minutes, rinsed in distilled water for ten seconds and fixed in Kodak fixer for three minutes. After soaking in a running water bath for 15 minutes, the slides were run up the alcohol series into xylene, then made permanent by adding a drop of Permamount (Fisher Scientific Company) and a coverslip. After several days of curing the slides were cleaned and observed.

In an effort to determine time of actual incorporation into the hemoglobin molecule, a series of gels were prepared containing radioactive hemolymph and autoradiographs were subsequently prepared. Larvae which were injected with delta-aminolevulinic acid were allowed to metabolize from 1 to 120 hours after injection. After the larvae were sacrificed the blood was absorbed onto a section of Whatman filter paper number 43, placed in a LiOH gel and run in the usual manner. A count of the activity of the sample paper was taken both before and after electrophoresis to determine whether the label had moved from the origin. Upon completion of electrophoresis gels were fixed and stained with either buffalo black or benzidine. After wrapping in Saran Wrap the gels were placed in contact with a sheet of photographic paper and exposed for a period ranging from four to eight weeks. The photographs were then developed and observed for fogged areas which corresponded to specific bands on the gels.

RESULTS

Culturing

Chironomus tentans reared at a temperature of 21-23°C under laboratory conditions completed the life cycle in approximately eight weeks. At a slightly lower temperature of 18-20°C the life cycle was extended one to two weeks but the larvae did not otherwise vary greatly in morphology. There was a wide range in the size or stage of development of specimens in any particular culture. Some eggs hatched and developed into adults in six or seven weeks while other larvae of the same egg mass lagged behind in reaching maturity by several weeks. The cause of this variation remains unknown. It probably is related to competition within the confined limits of the culture. There were some indications that after several generations under laboratory conditions, the largest third and fourth instars were somewhat smaller than the specimens collected in the wild. It was assumed that this was due to nutritional deficiencies, although temperature or even genetic selection were possibilities.

Males and females were collected daily. Approximately fifty cultures were kept for maintenance purposes alone. From these cultures one expected to make six to eight pair matings daily. Since the adults were delicate organisms to handle, it was not unusual to find that they had drowned before laying an egg mass or had laid an unfertilized egg mass. The sex ratio of emerging adults was roughly 1:1. Some months the collection of males outnumbered the females rather noticably; however, over a long period this difference was balanced. The number of males to females over a five month period was as follows: 130:80, 262:236, 315:288, 227:255 and 165:188. This totaled 1099 males to 1047 females. The higher number of

males observed during some months was of interest since Miall and Hammond (1900) observed that the number of females in a swarm was never large and the number of females was often affected by the degree of wind. During calm weather mating was easily accomplished and the females soon left the swarm while high winds rendered mating more difficult and they remained in the swarm. They reported capturing 700 males and no females from a swarm on a calm day compared to 4,278 males and 22 females on a windy day. The data reported here would lead one to believe that there was no drastic difference in the sex ratio. A possible explanation of Miall and Hammond's work was that in the wild the males swarmed while females enter them only for breeding purposes and quickly left in preparation for laying the egg mass. This view has been taken by Thienemann (1954), who found females resting on vegetation with males swarming nearby.

It was noted that mating under laboratory conditions was a rapid process. Once the male had detected the female he danced about for a few seconds before mounting. The actual copulatory process lasted only four to six seconds. Once mated the female would not accept another male and responded by curling her abdomen beneath herself.

Egg masses averaged between 1,100 and 1,200 eggs for large masses and 450 and 600 for smaller (second) egg masses. Eggs were generally laid within 24 hours after mating and larvae began hatching from the gelatinous encasement 2 to 3 days later. In many cases the female laid a second smaller egg mass two to three days after the initial deposition. Some males were removed after the first mating and the second egg masses were observed for fertility. It was found that a single mating was sufficient for fertilization of both egg masses.

The hatchability of the egg mass was nearly 100% when fertilized. However, there was a very high percent of mortality in the early stages of development. As much as 50-80% mortality could be expected where subcultures were not made of young larvae. Apparently, competition played a large role in the efficiency of any particular culture.

Studies on Chironomus Hemolymph

General observations

Certain visual changes in pigmentation were noted in the hemolymph of Chironomus larvae, pupae and adults. The first instar lacked pigmentation and remained transparent for three to five days at which time the second instar developed and a light pinkish coloration appeared. The larvae gradually darkened in the third and fourth instars. There were variations in the intensity of pigmentation between specimens from different cultures at the same stage of development. It was assumed this difference was due to nutritional and environmental variations in the culture medium. The pupae gradually lost their red coloration and a greenish pigment became apparent. The increased presence of a green pigment and corresponding decrease in red pigment correlated with the breakdown of hemoglobin. The adults had very little hemolymph, and that remaining contained the greenish pigment characteristic of pupal hemolymph. Wigglesworth (1965) had noted these metamorphic changes in Chironomus plumosus. He reported the functional hemoglobin of this organism was broken down throughout larval life and inclusions of bilirubin and biliverdin were deposited in the fat bodies. The accumulation of the pigment biliverdin was responsible for the green color changes which began in the

early pupae and was observed in newly hatched adults. In a few instances during this study, exceptional adults were observed to exhibit a definite reddish coloration. The thorax and, in some cases, the wings retained this color until the specimen died.

Electrophoresis

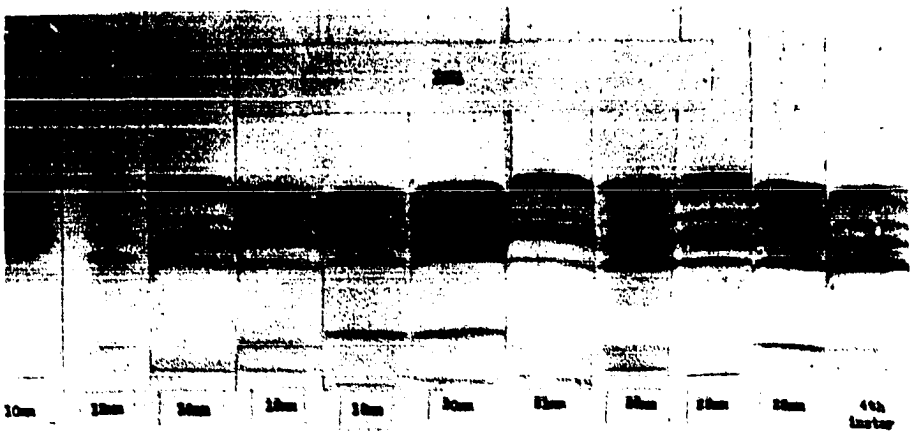
Cellulose polyacetate electrophoresis Sepraphore III cellulose polyacetate electrophoresis was carried out using a buffer prepared with one preweighed packet of dry ingredients per 1,000 ml of distilled water, as recommended by the Gelman Corporation. The resolution of the hemoglobins at this strength was not particularly good; therefore, a series of dilutions were prepared to determine the optimal buffer concentration. Buffers at 100 ml intervals were prepared ranging from 1,500 ml to 3,000 ml of distilled water per packet of ingredients. It was concluded that the *Chironomus* hemoglobin molecules separated most efficiently at 1,900 ml of distilled water per package of dry buffer chemicals. At one or two hundred milliliter of water either side of this dilution, the resolution of minor bands was definitely impaired.

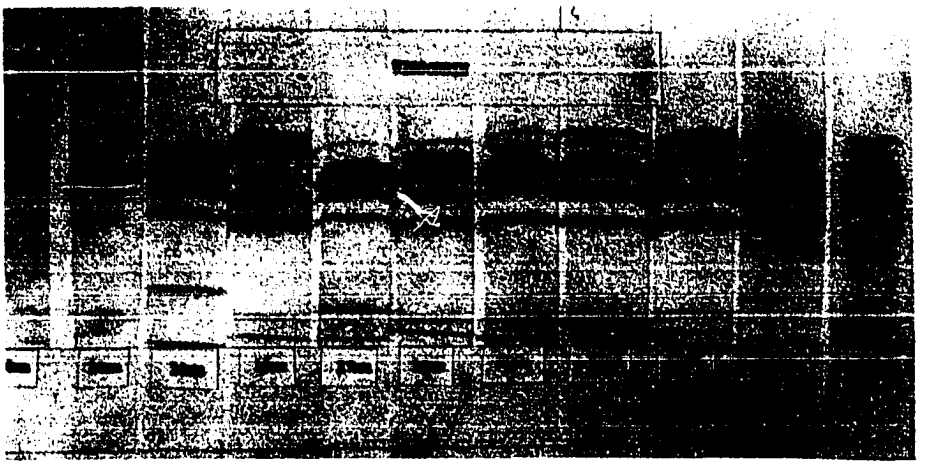
Cellulose polyacetate electrophoresis of hemolymph from different developmental stages of the Iowa and Wisconsin strains was compared (see Figures 1-3). In general, the same pattern was observed in both strains and only minor changes were noted between the different stages of development. The concentration of protein of any one band was variable between different specimens at the same stage of development, which probably reflected environmental conditions. The different stages of development also showed concentration differences with a general intensification of

Figure 1. Cellulose polyacetate electrophoresis of hemolymph from various stages of the Iowa strain. All samples were third instar larvae except the last sample which was a fourth instar. Prepackaged Tris-barbital-sodium barbital buffer (pH 8.8) in 1,900 ml distilled water was used at 5°C. Electrophoresis was carried out at 400 V until a two-inch migration was obtained. The proteins were stained with Ponseau S. All band migrate anodal.

Figure 2. Cellulose polyacetate electrophoresis of hemolymph from various stages of the Wisconsin strain. Similar conditions were used as described above.

Figure 3. A comparison of the Iowa and Wisconsin strains at various stages of development.





bands in the later stages. The pupae showed a slight decrease in concentration or a loss of bands.

The numbering system for all electrophoretic work began with the most distant band from the origin. Both strains of Chironomus exhibited a lead band of fairly consistent intensity in all stages. Hemoglobin band two, however, tended to become obscured by band three in the Iowa strain but vestiges of it were seen at the edge of the strips. Band three was a large rather diffuse hemoglobin in both strains and its inability to form a straight uniform band was characteristic.

Bands four and eight were quite variable in their expression and appeared only in the later stages of the Iowa strain. Since its observation was not consistent this suggested there was either a very short lived hemoglobin or that heat build up during the run was causing a degradation of one of the other hemoglobins. Band five was consistently present in all stages of larval development in both strains although its intensity was diminished in early stages. Bands six and seven appeared as a close doublet. In the Wisconsin strain they were usually found closer together at all stages when compared to the Iowa strain. Band nine was slightly more removed from the doublet of six and seven than was band five. Its presence in early stages was very faint. The last hemoglobin, band ten, was much further removed from the other nine hemoglobins. It was the only hemoglobin to remain close to the origin. Like band nine, its presence in the early stages of development was much reduced. Thus it appeared that there were eight major hemoglobin bands demonstratable by this technique and the Iowa strain exhibited two additional bands in the later stages of maturation.

It will be observed that there was considerable variation in the pattern obtained by this method. It was well established by others (Gelman Company) that a number of factors such as improper sample application, improper blotting, excessive evaporation, too high or too low milliamperage and improper tension on the strips, caused extensive modification of the pattern. This phenomena made the comparison of samples from the same run somewhat difficult and even more difficult between different runs. Nevertheless, a generalized pattern of migration was deduced. See Figure 4 for a summary of the electrophoretic patterns observed with cellulose polyacetate.

Starch gel electrophoresis Starch gel electrophoresis was a more reliable method for the comparison of different samples of hemolymph. This method offered the advantage of running a number of samples in a single medium and under identical conditions. Starch gel electrophoresis carried out in Tris-HCl buffer at 5°C was found less satisfactory than the LiOH buffer. Figures 5 and 6 demonstrate that the Tris-HCl buffer did not give as good a separation of bands as the LiOH buffer nor were the bands as sharp. The lack of resolution was due in part to the necessity of a cooling period for the gel after each run. The gels became quite warm and were practically impossible to handle unless given a five to ten minute cooling period in the freezing compartment of a refrigerator. This allowed diffusion of bands to take place, and a general loss of crispness of the pattern resulted.

The LiOH buffer used under the same conditions as the Tris-HCl method gave better results, as seen in Figure 6. Minor bands were resolved which

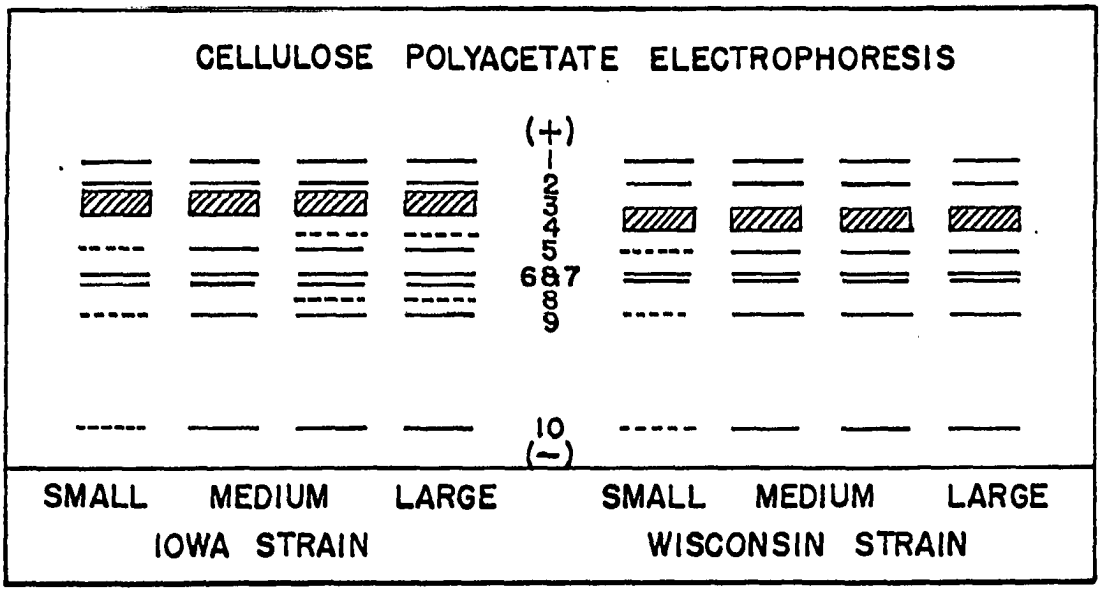


Figure 4. A diagram illustrating the hemoglobin pattern of the Iowa and Wisconsin strains at various stages of development.

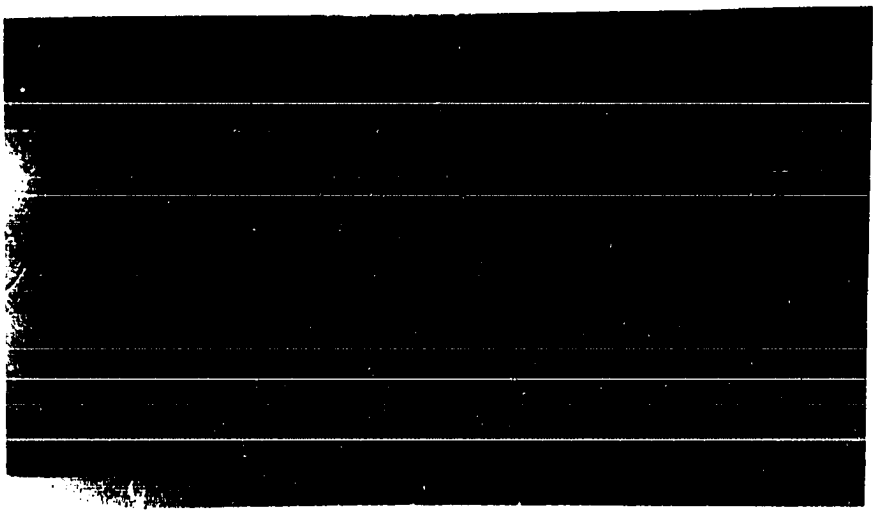
were not seen in the previous gel. There still remained a certain amount of diffusion during the cooling period.

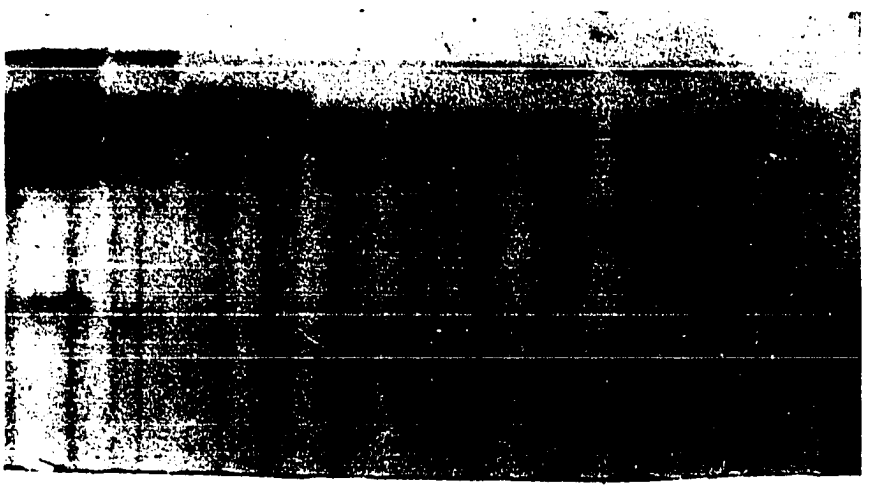
Gels run in a water cooled electrophoresis apparatus gave the best resolution of the three methods used. Gels were sliced and stained immediately at the end of a run since they did not heat up. It was also found that a prerun of 15 minutes before the samples were placed in the gel gave a sharper resolution. This was the time necessary for the borate line to migrate to the origin and as a result the samples began a distinct migration as this zone passed through the samples. Otherwise the samples tended to form a diffuse zone until the borate line reached the origin. It is evident from Figure 7 that the lead band was resolved into two

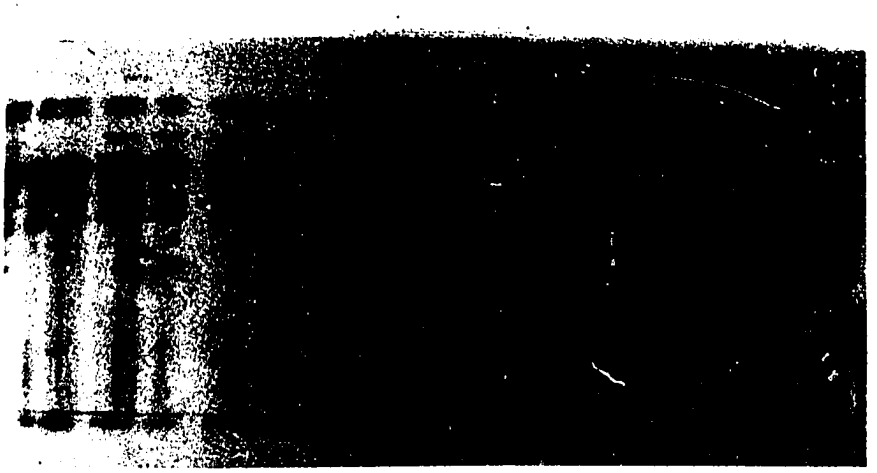
Figure 5. Tris-HCl starch gel electrophoresis (pH 8.5) at 5°C of hemolymph of the Wisconsin strain. Samples were run at 350 V until the bromophenol blue marker had moved 6 cm. Hemoglobins were stained with buffalo black. Larvae sizes were: pupa, cattle Hb, 6, 8, 8, 16, 18, 10, 17, 15, and 12 milimeters respectively.

Figure 6. LiOH starch gel electrophoresis (pH 8.0) of the Wisconsin strain at 5°C. Runs were carried out at 350 V until the marker dye traveled 6 cm. Proteins were stained with buffalo black. Sizes of the larvae were: 24, 22, 18, 14, 14, 12, 12, 9, 6, 8, 5 millimeters respectively, a two day old egg mass with small larvae and finally human hemoglobin.

Figure 7. LiOH starch gel electrophoresis (pH 8.0) of the Wisconsin strain. Runs were carried out at 500 V in a water cooled system until the marker dye migrated 6 cm. Hemoglobins were stained with buffalo black. Sizes of larvae were: 8, 9, 10, 12, 13, 12, 17, 18, 19, 17 (late 3rd instar), 20, 20, 21, 16, 16, 15, early pupa and 22 millimeters respectively.







distinct bands not observed previously. Other close bands and minor bands were generally much more visible. In the case of the LiOH buffer, a cathodal band was resolved which was not noted in the Tris-HCl buffer. Judging from the slight difference in pH of the two buffers, however, this band was probably the anodal band found very near the origin in the Tris-HCl gels.

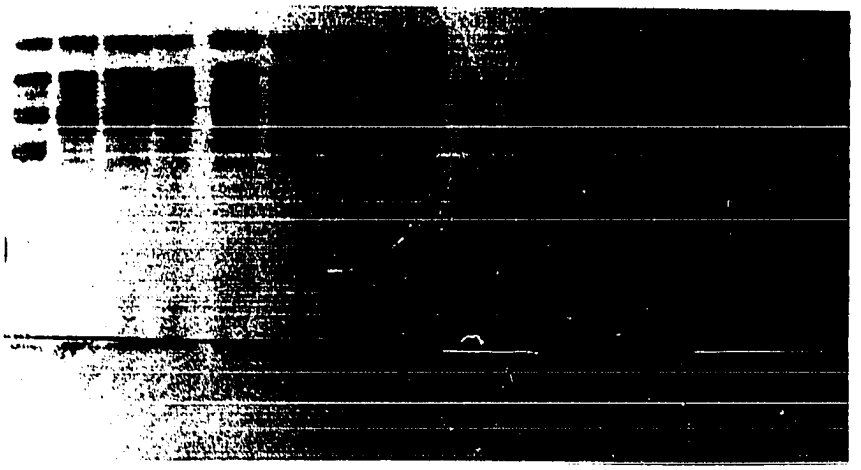
In Figures 8 and 9 a comparison is made between the general protein stain buffalo black and benezidine, which is a more specific stain for hemoglobin as dictated by its peroxidase activity. It was noted that the major bands and most of the minor bands stain in both halves of the gel. Some of the nonhemoglobin bands nearer the origin stained with the general protein stain but not with the more specific hemoglobin stain. Except for several of these bands near the origin, it was assumed that the banding patterns illustrated were, in fact, multiple hemoglobin forms.

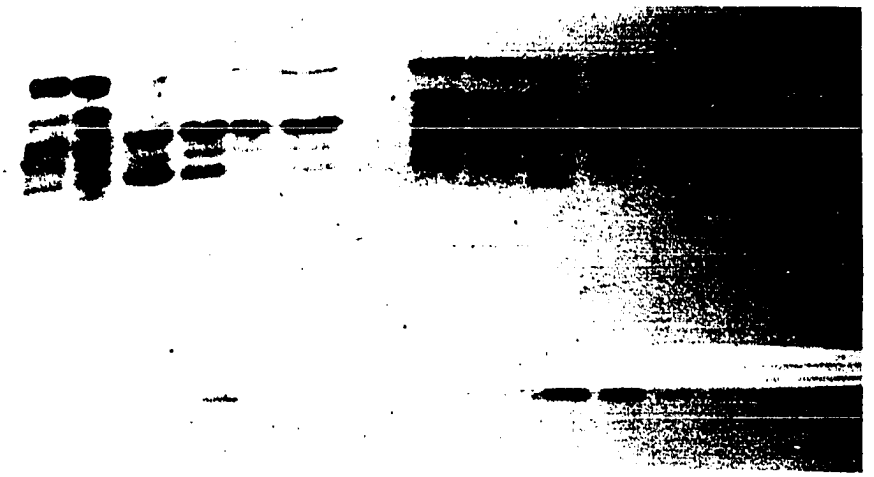
The variation in hemoglobin patterns at different stages of the life cycle is illustrated in Figure 10. The first two samples from left to right were adults and only a vestige of hemoglobin was observed in the first specimen. A diffuse protein or possibly a degradation product was seen near the origin in the adults. Samples three and four were pupae and, as one might expect, showed the degradation of hemoglobin during metamorphosis. The diffuse protein nearer the origin was found once again. The next six samples were mid to late third instar larvae. They showed the typical multiple hemoglobin pattern found in the Wisconsin strain. The last four samples were in the early third and second instars. It was observed that there was a progressive increase in the quantity of hemoglobin synthesized. During the later larval stages hemoglobin synthesis

Figure 8. LiOH gel electrophoresis (pH 8.0) of the Iowa and Wisconsin strains. The run was made at 500 V with the water cooled apparatus as previously described. Samples were stained with buffalo black. Sizes of larvae were: Iowa strain—10, 12, 10, 12, 18, 21, 22, 22 (dead), Wisconsin strain—small pupa, 15, 16, 20, 21, 19, 21 millimeters respectively.

Figure 9. The other half of the gel in Figure 8 stained with benzidine. The samples were in reverse order.

Figure 10. LiOH starch gel electrophoresis of the Wisconsin strain. The run was carried out at 350 V at 5°C until an 8 mm migration of the marker dye was accomplished and the gel was stained with nigrosin. Samples were: young adult female, old adult male, late pupa, late pupa, larvae 22, 24, 17, 17, 15, 18, 12, 5, 8, 9 millimeters respectively.





ceased, and the pigment was degraded in the pupal stage. This cyclic synthesis of hemoglobin coincided with the physiological needs and mode of life which is typical of the midge.

The gels illustrated in Figures 8, 9 and 11 demonstrated various stages of development in the Iowa strain. Figure 11 was stained with nigrosin and appeared to provide greater clarity of the hemoglobins. The first two bands consistently appeared as a close doublet in all samples, except in the case of the pupal material where only the second band was in evidence. Generally, the first band was more intense than the second. The next five bands were relatively close together. Band three was lacking or present in diminished quantities in the pupae and was slightly more concentrated in early stages of the life cycle. Hemoglobin four was slightly less concentrated, than number three, but was found in larval and pupal stages alike. There was a considerable amount of variation in intensity or occurrence of the next three bands. A hemoglobin labeled as band five in the Wisconsin strain was conspicuously absent in the Iowa strain. Bands six and seven usually appeared as a close doublet, while number eight was a little further removed from number seven. Band six was generally found in all stages while band seven was missing in the late third instar or fourth instar and the pupae. The loss of this band and number one in the later stages was assumed to represent a degradation of hemoglobins reflecting the metamorphic change. Band eight varied greatly in quantity at different stages. In very young larvae this band was scarcely distinguishable, but was easily observed in varying concentration in the third instar larvae. It was apparently degraded in the late third or fourth instars and early pupal stage and was missing in late pupae.

Band nine was found in all stages with a general increase in intensity in larger larvae, while degradation occurred during pupal development. Here again, however, there were individual variations. There was good evidence from other electrophorograms that this band was actually two very similar hemoglobins. Thus, it has been labeled as bands nine and ten. Band 11 was also found in all stages, but unlike region 9 it became less intense as the larvae increased in size. Band 12 was found in appreciable quantity only in the larger larvae and pupae. This suggests some breakdown product of hemoglobin, but it did, nevertheless, react with benzidine.

Band 13 was the only consistent cathodal band found with LiOH gels. It was lacking in the Tris-HCl gels, although a band very close to the origin on the anodal side possibly represented this band, as was noted previously. This band barely migrated cathodally and was very constant in its intensity in all stages of larval development.

A 14th band was found more cathodally in some cases. Its benzidine reactivity was very slight. It too, was found only in later stages of development and possibly represented breakdown products of hemoglobin during pre-metamorphosis.

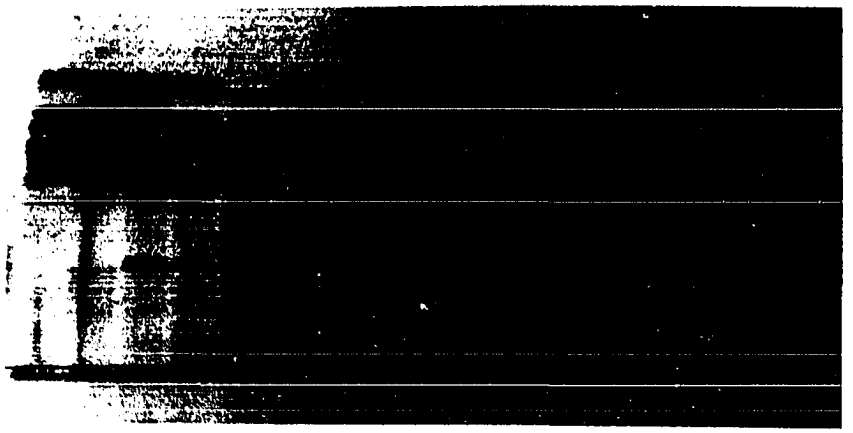
Figures 7, 10, 12 and 13 best demonstrate the banding pattern observed in the Wisconsin strain of Chironomus tentans. Once again a doublet was found in the most anodal position. Band one was usually a little more dense than band two and both were missing in pupal development.

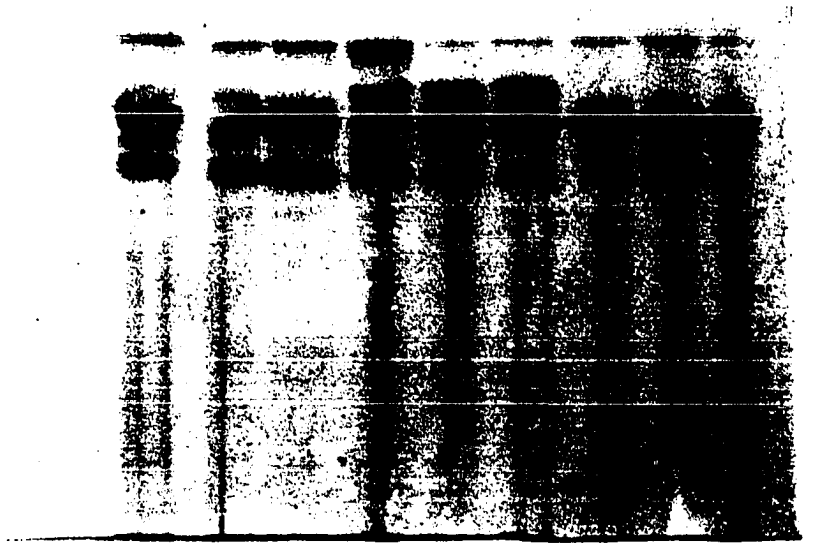
Bands three and four were not as prominent as that found in the Iowa strain and they were quite variable in intensity. Generally, however, they were very light in early stages of development and reached a maximum

Figure 11. LiOH starch gel electrophoresis of the Iowa strain of Chironomus tentans utilizing the water cooled apparatus. The run was made at 500 V until the marker dye migrated 6 cm. Hemoglobins were stained with nigrosin. Sample sizes were: 23, 15 (4th instar), 20, 23, late pupa, 23, 23, 21, 17 (late 3rd instar), 17, 16, 15, 15, 15, 10, 10, 9 millimeters respectively.

Figure 12. LiOH starch gel electrophoresis of the Wisconsin strain at 5°C, run at 350 V until a 7 cm migration pattern was achieved. Buffalo black was used as the stain. Sample sizes were as follows: 21, 22, 22, 17 (very dark), 17, 17, 13, 10, 5 millimeters respectively.

Figure 13. LiOH starch gel electrophoresis of the Wisconsin strain run at 500 V for a 6 cm migration pattern. The gel was stained with buffalo black. Sample sizes were: 21, 22, 21, 19, 17 (late 3rd instar), 16, 15, 12, 15, 12, 12, 10, 8, 10, 10, 8, 6 millimeters respectively.





intensity in the mid third instar (approximately 13-18 mm). Band four was commonly found in all stages, while band three was at a peak intensity around the 16 mm stage. Both of these bands became very diffuse in larger third and fourth instars, and during pupal development. A fifth band was found in the Wisconsin strain which was unique to this strain. It was prominent in all stages, increasing in intensity in the latter stages.

Bands six and seven tended to migrate slightly closer together than their counterparts in the Iowa strain. Band six was consistently more intense than band seven and diminished in quantity prior to band seven in the pupal stage. Band eight corresponded with band eight in the Iowa strain. It was present in consistent amounts in all stages until pupal development was reached, at which time it became diffuse. Bands nine and ten were similar to the two very close bands of the Iowa strain and were definitely distinguishable as a doublet. They were of equal intensity and reached a maximum concentration during the larger stages. During the fourth instar and pupal stages, they became less intense. Band 11 corresponded to band 11 of the Iowa strain. It was rather light and occupied the same position in both strains. As was characteristic of the Iowa strain, band 12 appeared only in the later 3rd and 4th instars.

Band 13 also corresponded to the 13th band of the Iowa strain and was usually the only distinct cathodal band observed. In a few instances a very light diffuse second cathodal region was observed, but it did not form a definite band.

A total of 13 protein bands were found in the Iowa strain, while one additional band was observed in the Wisconsin strain. Two of these bands, numbers 11 and 14, did not appear until late in the life cycle, were very

diffuse and may represent a breakdown of other hemoglobins which was characteristic of the metamorphic process. In any event, their mode of expression as determined by starch gel electrophoresis was uniquely different from the other protein bands. Other dissimilarities appeared to center around concentration, especially in the case of band three which was almost totally lacking in the later stages of development in the Wisconsin strain. See Figure 14 for a resumé of changes in degree of intensity of the multiple hemoglobins which were observed throughout the life cycle.

Two major observable differences were observed in the hemoglobin pattern of the two strains of Chironomus larvae. First, the heavy band three of the Iowa strain was virtually missing in the Wisconsin strain. On the other hand, band five of the Wisconsin strain was totally absent in the Iowa strain. The adoption of the symbols ($3^{+}5^{-}$) was used to represent the Iowa pattern, while ($3^{-}5^{+}$) was used to symbolize the Wisconsin pattern.

Hybrids produced by these two strains yielded an electrophoretic pattern which was, in effect, a superimposition of the two parental phenotypes. This pattern was designated as ($3^{+}5^{+}$). See Figures 15 and 16 for the type of pattern obtained from the parental and hybrid larvae.

Since the pattern observed in the parental lines was consistent it was assumed they represented a pure line. The hybrid individuals ($3^{+}5^{+}$) represented the heterozygous condition since they obviously combined the qualities of the Iowa ($3^{+}5^{-}$) and Wisconsin ($3^{-}5^{+}$) parents. Furthermore, from a genetic standpoint, the gene responsible for the production of hemoglobin observed in band three of the Iowa strain behaved as a dominant to its Wisconsin allele. In a similar consideration the gene for hemoglobin

Figure 14. A diagramatic representation of the intensity of the various multiple hemoglobin types found at different stages of the two strains of Chironomus tentans.

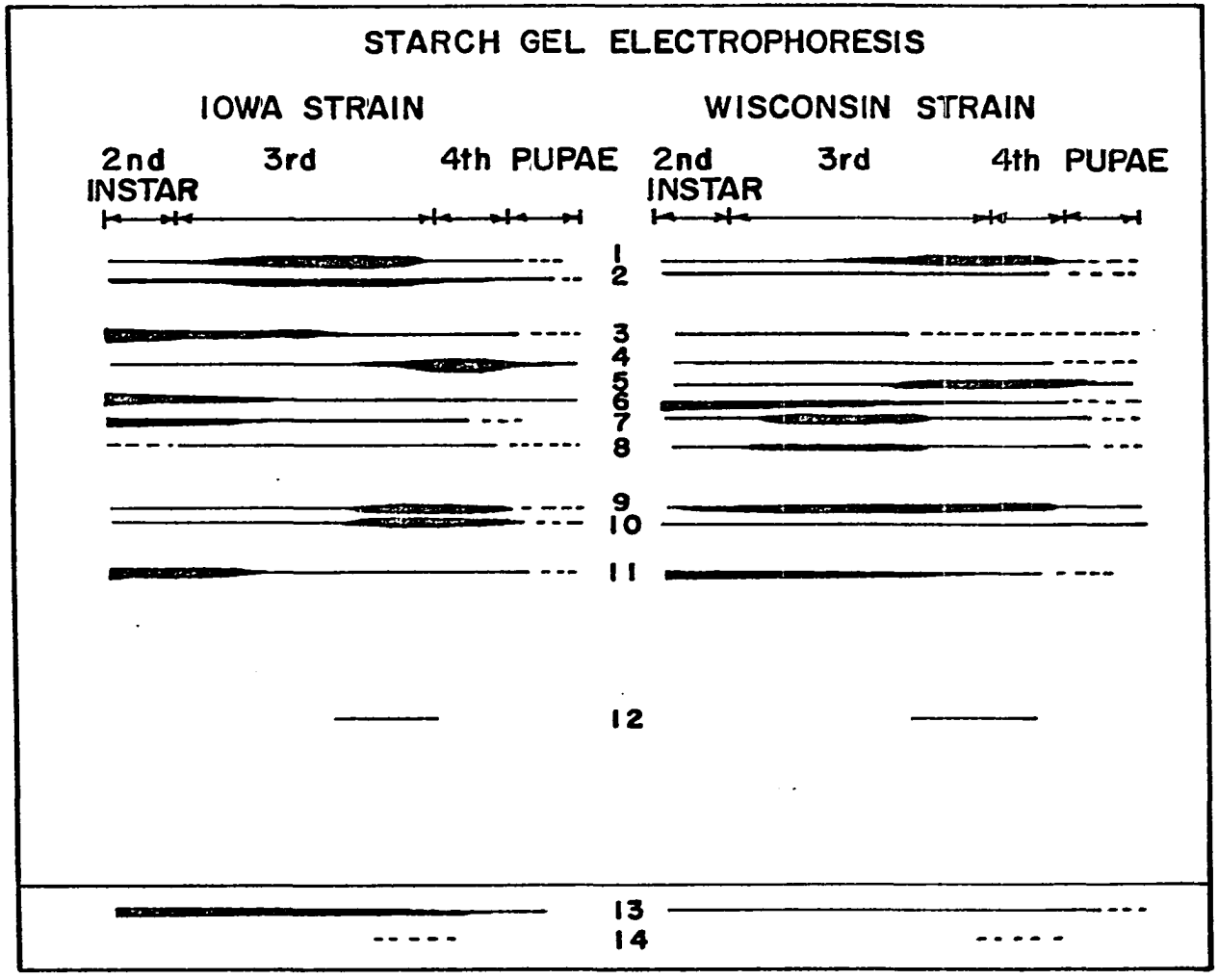
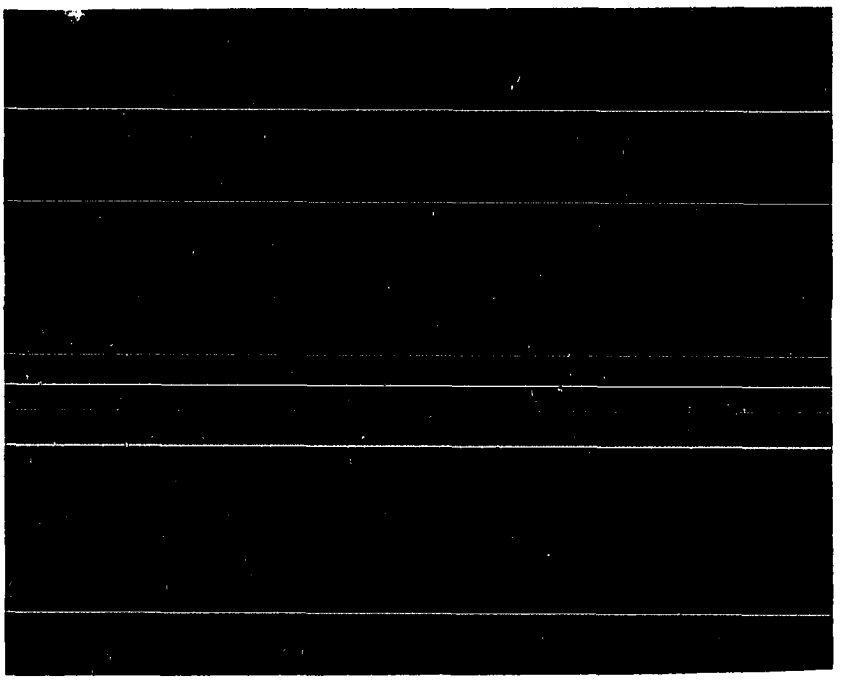

STARCH GEL ELECTROPHORESIS
IOWA STRAIN
WISCONSIN STRAIN
2nd INSTAR
3rd
4th PUPAE
2nd INSTAR
3rd
4th PUPAE
1
2
3
4
5
6
7
8
9
10
11
12
13
14

Figure 15. LiOH gel of pooled samples of Iowa, hybrid and Wisconsin samples respectively. A variety of larval sizes was used to make the pooled samples.

Figure 16. LiOH gel of the Iowa and Wisconsin parental types dispersed among the hybrid type. From left to right the samples were as follows: Iowa (21 mm), F_1 (21 mm), Wisconsin (23 mm), F_1 (22 mm), Iowa (18 mm), F_1 (21 mm), Wisconsin (17 mm), F_1 (16 mm), F_1 (23 mm), Wisconsin (22 mm), F_1 (20 mm).



five in the Wisconsin strain was dominant to its corresponding allele in the Iowa strain.

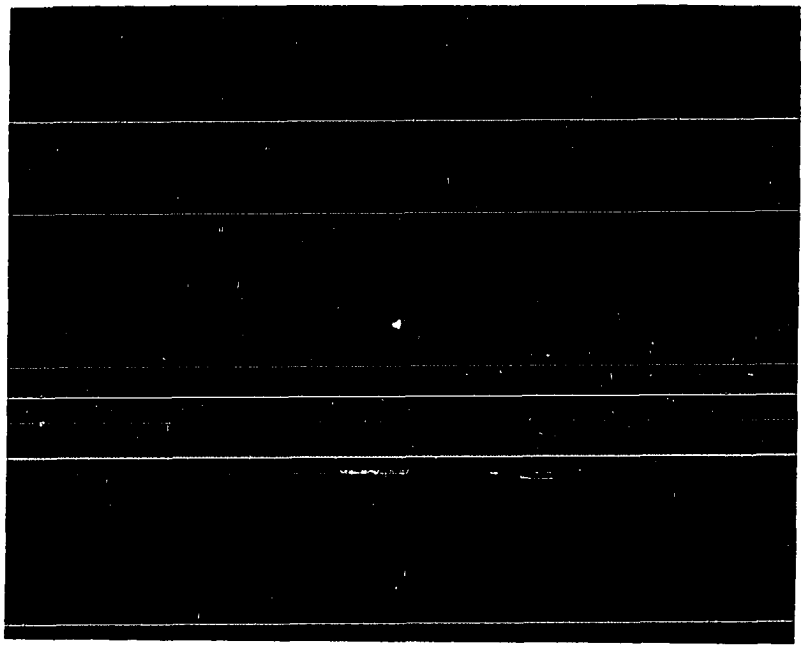
The logical question to ask at this point was whether these two loci were located on the same or different chromosomes. Hybrid offspring were mated in an effort to answer this question. Second generation offspring exhibited a variety of phenotypes. In most instances the F_1 pattern (3^+5^+) was obtained while a reduced number of the original parental types were recorded. A completely different fourth category was found in a very few instances. This phenotype lacked both the third and fifth bands and has been designated (3^-5^-). Of a total of 23 clearly distinguishable examples, 4 were of the Iowa phenotype, 4 were of the Wisconsin phenotype, 13 were the hybrid phenotype and 1 or possibly 2 showed the new phenotype. Figures 17 and 18 demonstrate the various phenotypes described in this experiment.

If the genes for hemoglobins three and five were in fact linked, the second generation would consist of three phenotypes, i.e. Iowa (3^+5^-), F_1 (3^+5^+) and Wisconsin (3^-5^+), in a 1:2:1 ratio. If, however, the genes were located on different chromosomes, one would expect random assortment of the genes in producing the F_2 generation. The expected ratio in this case would consist of approximately nine hybrid phenotypes to three Iowa phenotypes to three Wisconsin phenotypes to one which included neither the third or fifth hemoglobin bands.

The results mentioned provide two lines of evidence which suggest that there were at least two chromosome pairs involved in the synthesis of hemoglobin in Chironomus. First, the observed ratio of 13:4:4:1 approached the 9:3:3:1 ratio typical of a Mendelian dihybrid cross more nearly than a

Figure 17. A representative LiOH gel which showed the hemoglobin pattern of the F_2 generation.

Figure 18. A LiOH gel of the hemoglobin pattern exhibited by the F_2 generation. Arrows point out individual larvae which possibly exhibited the (3-5-) exceptional phenotype.



2:1:1:0 ratio. Qualitatively, however, the mere presence of the fourth class virtually eliminates the alterative of these genes being carried on the same chromosome pair. See Figure 19 for a diagramatic illustration of the various crosses and phenotypes described in this experiment.

Disc gel electrophoresis The results obtained with disc electrophoresis can be seen in Figures 20 and 21. Multiple runs of parental lines and hybrids gave consistently reproducible results. The best patterns were obtained when the tracking dye was allowed to migrate between 1.25 and 1.50 inches. Beyond this point diffusion became a problem, and prior to this time the bands near the marker dye were not easily discerned.

In many cases the sample gel failed to polymerize completely and care was taken not to disturb this area when the buffer was added to the upper reservoir. Although the reason for failure of polymerization was not understood, there are several factors which have been known to affect polymerization in human blood serum, such as slight hemolysis, protein concentration, aging of the upper gel solution, traces of acid, and increased amounts of specific proteins such as globulins.

The protein banding pattern exhibited by the Iowa strain was characterized by five major bands. The first, narrowest band was found to migrate at the same rate as the marker dye. It was followed closely by a fairly dense band. The third band was further removed from the leading edge and was broad, but much less intense. This band was followed by two very dense bands. In some instances minor bands were observed to be dispersed between the major bands. One of the most notable of these was a band between the fourth and fifth bands and to a lesser extent another minor band was occasionally found between the third and fourth bands and

Figure 19. A diagramatic representation of the hybrid analysis experiment as determined by the LiOH starch gel technique.

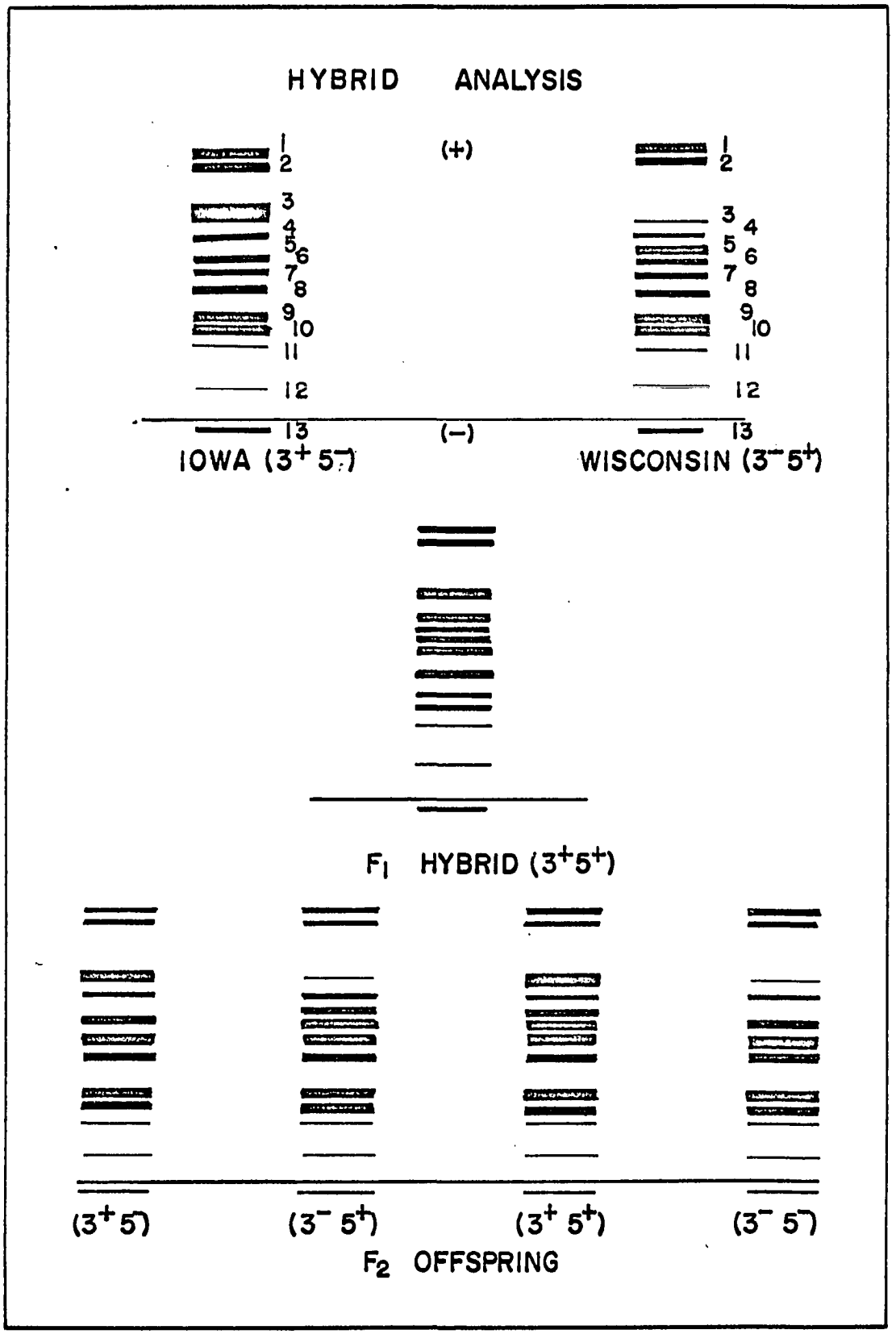
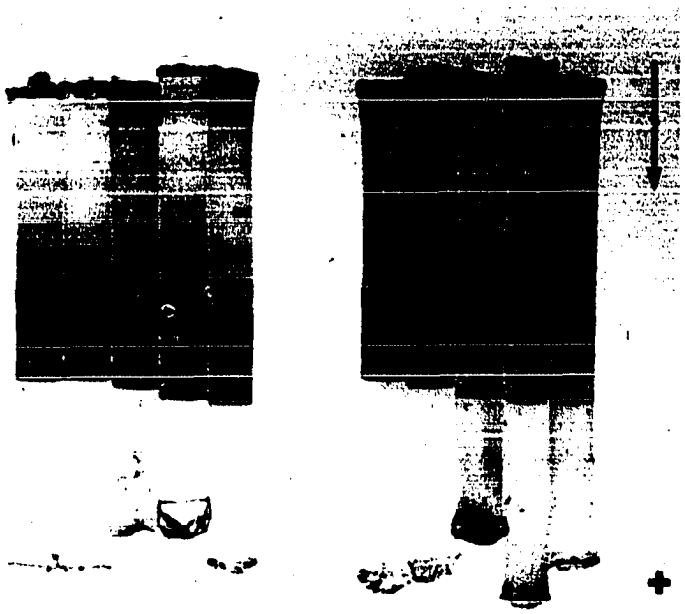

HYBRID ANALYSIS
(+)
1
2
3
4
5
6
7
8
9
10
11
12
13
(−)
IOWA (3+ 5−)
WISCONSIN (3− 5+)
F1 HYBRID (3+5+)
(3+ 5−)
(3− 5+)
(3+ 5+)
(3− 5−)
F2 OFFSPRING

Figure 20. Disc gel electrophoresis of the Iowa (left) and Wisconsin (right) strains. The hemolymph samples were pooled from larvae of various sizes.

Figure 21. Disc gel electrophoresis of pooled samples of hybrid hemolymph and of individual F_2 specimens. The F_2 gels were classified as follows: 1-2 Iowa, 3 Wisconsin, 4-5 hybrid, 6-7 exceptional class I and 8-9 exceptional class II.



several other minor bands were observed between the origin and the fifth band. The minor bands were quite variable in their expression and were not observed with the same reliability as the five major bands.

The pattern observed for the Wisconsin strain was not the same as the Iowa strain. The first band once again migrated at the same rate as the marker dye. The second band, however, was completely missing. Band three was generally more dense than the corresponding band found at this location in the Iowa strain, although it was found to be rather light. This variability made it difficult to draw any definite conclusions with respect to its degree of intensity. The fourth band was consistently lighter than its counterpart in the Iowa strain. Once again a minor band was often found between the fourth and fifth band but once again it appeared to be quite variable. The last major band had the same position and intensity as its counterpart in the Iowa strain. Several faint minor bands were observed between the origin and the fifth major band.

The differences in pattern between the two strains were largely the lack of band two in the Wisconsin strain and the greater intensity of band four in the Iowa strain. Other bands were either consistent in their presence and degree of intensity, or too variable to draw any conclusions from. It was therefore convenient to adopt the symbol of ($2^{H}4^{H}$) to represent the Iowa strain since it had both of these bands in a fairly heavy intensity and the symbol ($2\text{-}4^{L}$) for the Wisconsin strain, referring to the lack of band two and a light degree of intensity for band four. Since these two patterns were consistent, the two strains were considered homogyzous for the determining alleles. See Figure 20 for the pattern observed among pooled hemolymph samples of the two parental lines.

Only major bands were observed visually before staining. They were assumed to be hemoglobins because of their reddish coloration. To identify them more specifically and to check whether any minor bands were also hemoglobins, a number of gels were stained with benzidine for peroxidase activity. These tests verified the assumption that the major bands would produce a positive reaction to benzidine. In addition to these bands, the minor bands mentioned previously showed a faint reaction. Another band near the origin in both strains, not noted before, also produced a slight reaction. Benzidine-stained gels were only good for a couple of minutes, and preservation of the pattern proved to be very difficult.

Figure 21 illustrates the pattern exhibited by pooled hemolymph from hybrid specimens. The hybrid phenotype incorporated the features of both parental lines. The first band was found to migrate with the marker dye. The second band was typical of the Iowa strain. The third band once again showed a variability in intensity which was characteristic of the Wisconsin strain. Band four was fairly intense, as was the case for the Iowa strain, and was followed by a light minor band which was often observed. The fifth major band was observed to be deep staining, as was typical of both lines. Numerous minor bands nearer the origin, similar to the parental strains, were once again observed. In considering bands two and four, it was observed that the F_1 phenotype conformed to the Iowa parent and hence was also symbolized as $(2^{H}4^{H})$. These observations provided evidence for the Iowa hemoglobin genes being dominant to their alleles in the Wisconsin strain.

Once again the question arose as to whether the genes for these two hemoglobins were linked or acted independently of one another. If one

assumes the genes were linked, one would expect three-fourths of the F_2 generation to exhibit the Iowa phenotype ($2^{H}4^{H}$) and one-fourth to yield the Wisconsin phenotype ($2^{-}4^{L}$). On the other hand, if the genes were free to assort at random, one would expect the following phenotypic ratio: 9/16 Iowa ($2^{H}4^{H}$): 3/16 exceptional class I ($2^{-}4^{H}$): 3/16 exceptional class II ($2^{H}4^{L}$): 1/16 Wisconsin ($2^{-}4^{L}$).

A variety of phenotypic patterns was observed in the F_2 generation (Figure 21). Reading the gels of Figure 21 from left to right, the first two gels typify the Iowa pattern and the third gel resembles the Wisconsin type. The fourth and fifth gels combine the characteristics of the two parental lines and are typical of the F_1 pattern. The last four patterns represented exceptional cases. The sixth and seventh gels have the F_1 pattern at band four, but lack the second band. This type was referred to as exceptional class I ($2^{-}4^{H}$). The second exceptional case was a typical Iowa pattern for band two, but with a Wisconsin pattern at site four. This type was referred to as exceptional class II ($2^{H}4^{L}$).

In an analysis of 44 especially good gels, 28 were similar to the Iowa or F_1 phenotype, 2 were like the Wisconsin phenotype, 5 were typical of the exceptional class I and 9 had the exceptional class II phenotype. While the 28:5:9:2 ratio only vaguely approximates the expected 9:3:3:1 ratio typical of a dihybrid cross exhibiting random assortment, the mere presence of the two exceptional categories provided evidence that there are at least two chromosomes involved in the synthesis of hemoglobin.

Globin analysis The electrophoretic separation of the globin sub-units from each hemoglobin was carried out in urea-containing gels. Hemolymph samples were first run in a standard LiOH gel to affect a

separation of individual hemoglobins. A longitudinal section through the middle of this gel was then removed, placed at the origin of a urea-containing gel and run in the second dimension. Some of the LiOH gel sections were pretreated for one hour in cold acid acetone to separate the heme from the globin before the separation was made in the urea gels (Anson and Mirsky 1930). The pretreatment consistently gave more uniform results and the globins produced were not as diffuse. Figure 22 gives a typical representation of the two dimensional gel technique. The section of LiOH gel below the urea gel was stained to show the approximate positions of the hemoglobins before they moved into the urea gel. After a 12-hour run, it was observed that each of the hemoglobins had migrated between 2 and 3 inches, but still formed a single spot instead of 2 separate subunits. Cattle and human hemoglobins treated in a similar fashion were observed to separate into the characteristic alpha and beta polypeptide chains.

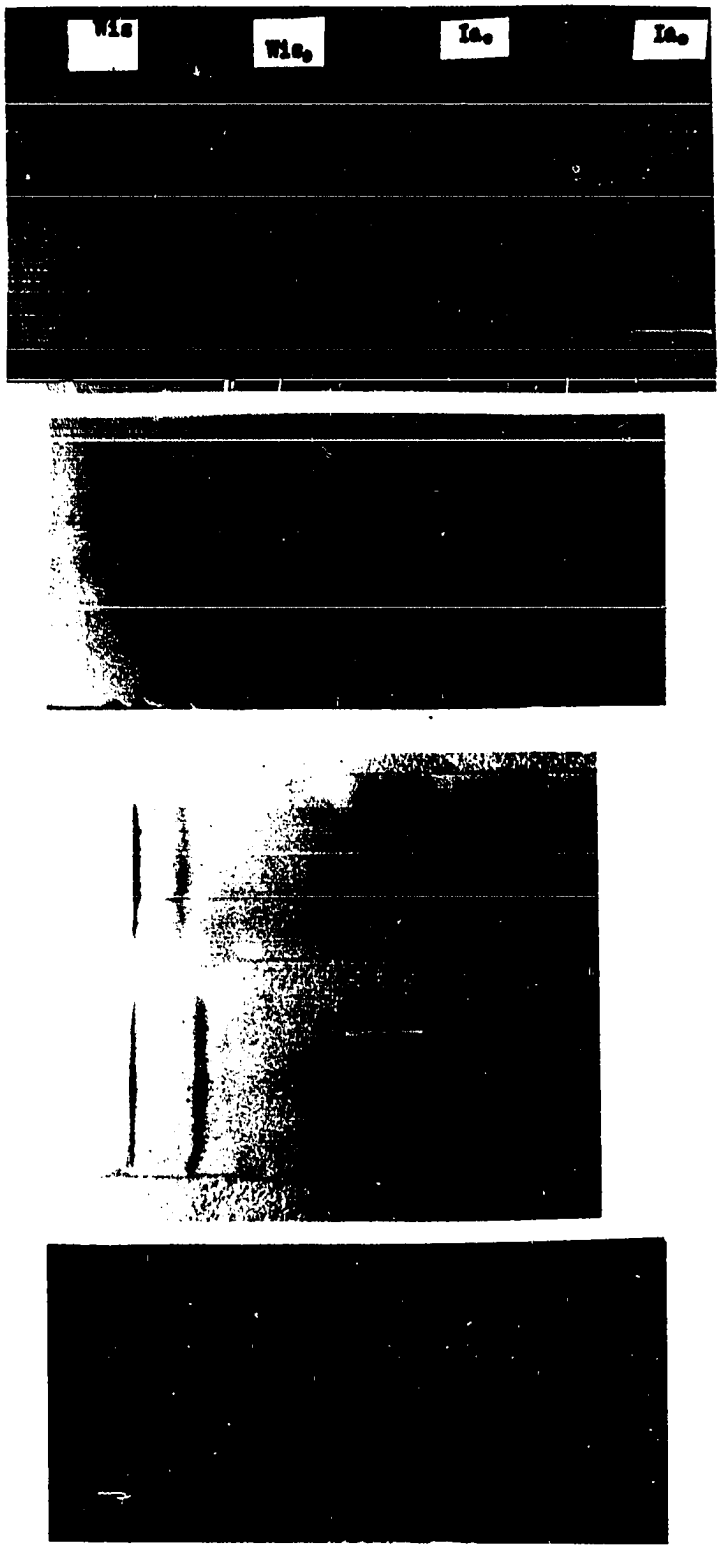
A second approach to the analysis of globin subunits involved the separation of the multiple forms of hemoglobin on cellulose polyacetate strips. Hemoglobins were first separated in one dimension on Sepraphore III, and the various hemoglobins were cut out and inserted in urea gels. Since the hemoglobins diffuse readily on the cellulose polyacetate strips and were unstained, some difficulty was encountered in selecting individual bands without contamination from neighboring hemoglobins. Figure 23 and the upper half of Figure 24 show results observed when using the Iowa strain. The lower half of Figure 24 and Figure 25 were typical results observed with the Wisconsin strain. The hemoglobins of the Iowa strain did not separate into two globin subunits. The double band which appeared in

Figure 22. Two-dimensional gel electrophoresis of individual globins. The first electrophoretic run was on the usual LiOH medium. Note a section of this run at the bottom of the photograph. The second dimension was carried out in 8 M urea gels containing 2-mercaptoethanol at a pH of 4.5. The run was made for 12 hrs. at 100 V.

Figure 23. Two-dimensional electrophoresis of individual Iowa hemoglobins. The first dimension was on cellulose polyacetate and the second dimension in an 8 M urea gel as described above.

Figure 24. Typical separations of Iowa (top) and Wisconsin (bottom) hemolymph, as determined by cellulose polyacetate electrophoresis. These bands and interbands were used in 8 M urea gels to determine the nature of the subunits.

Figure 25. Two-dimensional electrophoresis of Wisconsin hemolymph, using cellulose polyacetate electrophoresis for the first dimension and 8 M urea gels for the second dimension. The conditions were the same as described above.


W1s
W1s,
Is,
Is,

the middle of the gel illustrated in Figure 23 was the result of cutting the two closest bands (upper Figure 24) out together, since it was very difficult to see these as separate bands on the unstained cellulose polyacetate strips. These two bands when stained were seen as separate hemoglobins in the Iowa strain, but not in the Wisconsin strain. The corresponding Wisconsin hemoglobins were in every case observed to move as single bands in the urea gels, including the hemoglobin corresponding to the close doublet in the Iowa strain. This was consistent with the results previously obtained on Sepraphore III analysis. Slight contamination of some bands was suggested by the diffuse areas around the primary band.

The globins displayed differences in their speed of migration in the urea gels, but there appeared to be no direct correlation between the position of the band in the cellulose polyacetate strip and the urea gel. Neither was this the case with the LiOH gel sections and the urea gels, as might be expected from extensive differences in pH and other factors.

Thus, it appears at this time that each of the multiple hemoglobins of Chironomus tentans isolated by medium voltage electrophorsis was composed of a single polypeptide chain in contrast to the typical vertebrate hemoglobin. There remains the possibility, however, that the molecules were dimers composed of identical or very similar peptides which were not separable by these methods, or that a very firm bond existed between the polypeptide chains. This seemed unlikely, however, because a weak binding between chains is known to exist in human and other vertebrate hemoglobins.

Molecular weight determination

The proteins listed in Table 1 were used in various combinations or alone, as in the case of the digestive enzymes, together with 0.1% blue dextran and sucrose. The elution volumes for all reference proteins and Chironomus hemoglobins were measured on the same column. In addition, the void volume (the elution volume of substance excluded from the gel pores as determined by the blue dextran) of the column was measured for each run. The observed separation of the various reference proteins was used to determine the molecular weight of Chironomus hemoglobin by first calculating the ratio of the elution volume to the void volume (see Table 2). A plot was made using the value for V/Vo as the abscissa and the log of the molecular weight as the ordinate. A line was fitted to these points on the graph and the molecular weight of Chironomus hemoglobin extrapolated from the curve. These data suggested that the molecular weight of Chironomus hemoglobin was approximately 19,500 (see Figure 26). If the molecule is highly asymmetrical, as has been suggested, a somewhat lower estimate would be in order.

One of the errors encountered in this experiment was in the accuracy of locating peaks with the spectrophotometer. It is assumed that the column was uniform throughout the experiment, on the basis of the similarity in the values obtained for ovalbumin in the first and tenth experiments. The principle errors were then due to the size of the drops and variable accuracy in reading the optical density. During any given run, the drop size was probably uniform except in fractions with high protein concentration. This would result in a higher surface tension and a larger drop size. For a given peak, this displacement was not more than

Table 1. Reference proteins and Chironomus hemoglobin V/Vo value on Sephadex G-100

Protein	Mol. wt.	V/Vo	log. mol. wt.
Cytochrome C	12,400	2.00	4.09
Ribonuclease A	13,700	1.98	4.14
Myoglobin	17,800	1.93	4.25
Trypsin	22,500	1.82	4.35
Pepsin	35,000	1.29	4.54
Ovalbumin	45,000	1.47	4.65
Human hemoglobin	64,000	1.50	4.81
Chironomus hemoglobin	unknown	1.88	

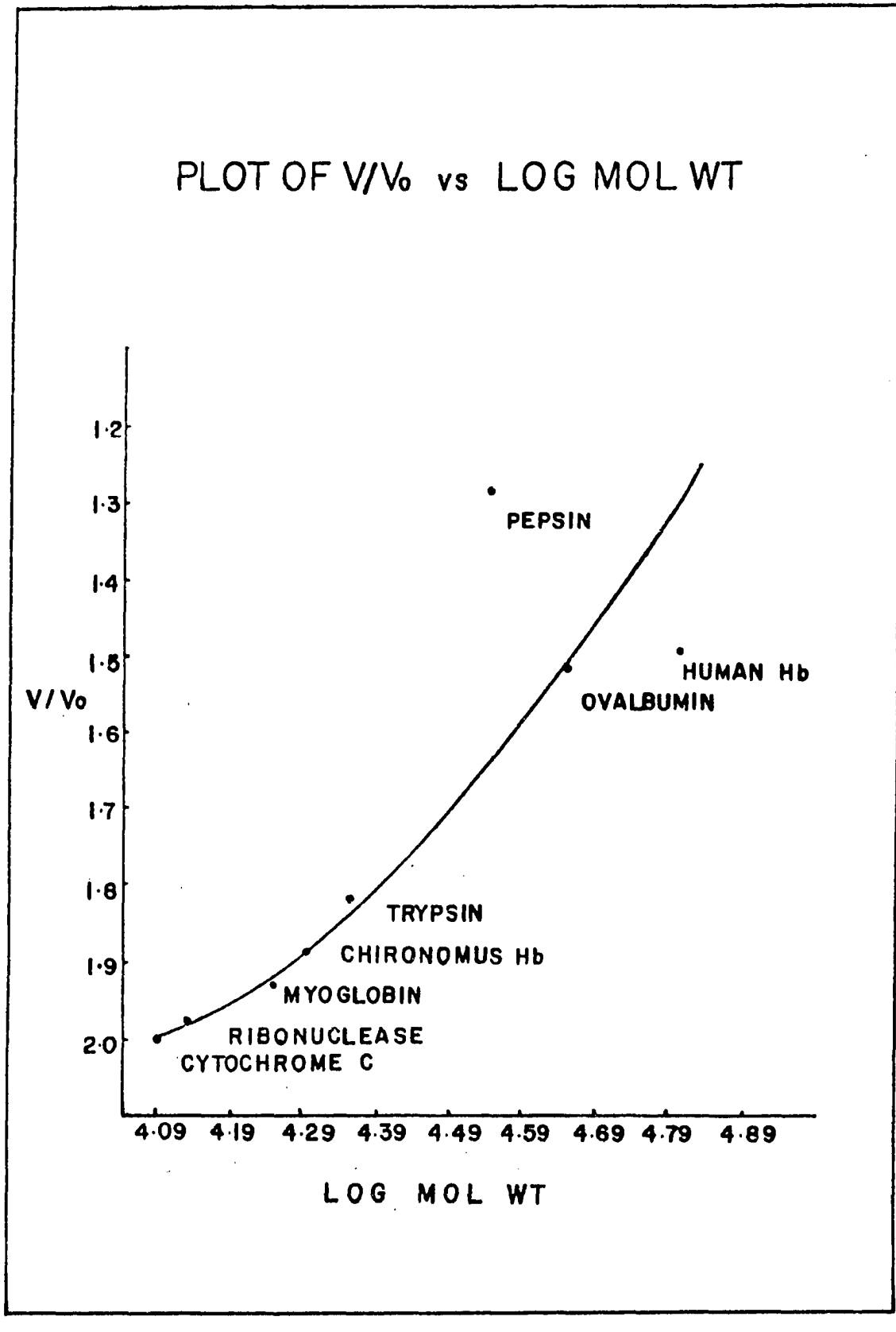
one milliliter and would not shift the peak more than one tube earlier. As far as optical density readings were concerned, absolute accuracy was not required, only reproducibility. This requirement was apparently met, since all proteins used, except human hemoglobin, did not deviate more than 0.02 in their V/Vo values.

The aberrant position for human hemoglobin was encountered in previous experiments. This suggested that some interaction was possibly taking place between the Sephadex system and the tetrameric molecule. The reason for the anomalous positioning of pepsin was not easily explained, except that it is considered to be less globular than the other proteins. Repositioning of this molecule on the curve by its V/Vo value yielded a molecular weight of 74,000 which indicated the possibility of dimerization.

Table 2. The determination of V/Vo for various reference proteins and Chironomus hemoglobin on Sephadex G-100

Experiment	Substance	Tube number	V/Vo
1	Dextran	77	
	Ovalbumin	113	1.48
	Cytochrome C	155	2.01
2	Dextran	79	
	Human Hb	117	1.48
	Cytochrome C	158	2.00
3	Dextran	77	
	Human Hb	117	1.52
	Myoglobin	149	1.94
4	Dextran	78	
	Myoglobin	150	1.92
5	Dextran	74	
	Pepsin	96	1.30
6	Dextran	74	
	Pepsin	95	1.28
7	Dextran	73	
	Trypsin	133	1.82
8	Dextran	76	
	Trypsin	139	1.83
9	Dextran	77	
	Ribonuclease	152	1.98
10	Dextran	76	
	Ovalbumin	111	1.46
	Chironomus Hb	142	1.87
11	Dextran	75	
	Chironomus Hb	141	1.88

Figure 26. A graphic illustration of the relationship between seven proteins of known molecular weight and Chironomus hemoglobin. The elution volume divided by the void volume as the abscissa was plotted against the log of the molecular weight on the ordinate. An approximate molecular weight of 19,500 was calculated for Chironomus hemoglobin.


PLOT OF V/Vo vs LOG MOL WT
V/Vo
1·2
1·3
1·4
1·5
1·6
1·7
1·8
1·9
2·0
PEPSIN
HUMAN Hb
OVALBUMIN
TRYPSIN
CHIRONOMUS Hb
MYOGLOBIN
RIBONUCLEASE
CYTOCHROME C
4·09
4·19
4·29
4·39
4·49
4·59
4·69
4·79
4·89
LOG MOL WT

Autoradiography

Histological sections from larvae injected with delta-aminolevulinic acid for various periods of time were examined by means of liquid emulsion autoradiography. The uptake of the label by a specific organ or tissue was not apparent until 24 hours after injection. Prior to this time, there was a general dispersal of the label in all tissues with slightly higher concentration found in the salivary glands, the outer intestional epithelium and to a lesser extent in the muscle and nervous system.

Twenty-four hours after injection, there was some indication that a group of cells along the dorsal blood vessel called the pericardial cells and the Malpighian tubules were accumulating the precursor in visibly greater quantities. Other tissues were observed to be tagged as well. By the end of the fifth day after injection, however, these same two sites were obviously more heavily labeled than the surrounding tissues. The intestional epithelium and the salivary glands also appeared to have a slightly higher concentration of label by this time, but not to the extent that was found in the Malpighian tubules and pericardial cells.

Figures 27 and 28 illustrate the accumulation of the precursor in the pericardial cells. It was noted that in these sections the intestinal epithelium showed some evidence of accumulation of the label, while little of the material was found in the hemocoel and other tissues. The uptake of the label was also shown in the Malpighian tubules, hind gut and in the salivary glands (see Figures 29 and 30).

The mere accumulation of the radioactive precursor did not necessarily mean that the site of uptake was active in hemoglobin synthesis. Cuénot (1895) suggested that the hemocytes were possible candidates for this

Figure 27. Autoradiography of a longitudinal section of a third instar Chironomus tentans larva. The parts labeled represent: C, coecum; D, dorsal ganglia; E, esophagus; H, head; P, pericardial cells and V, ventral ganglia.

Figure 28. Higher magnification of the pericardial cells after exposure to radioactive delta-aminolevulinic acid.

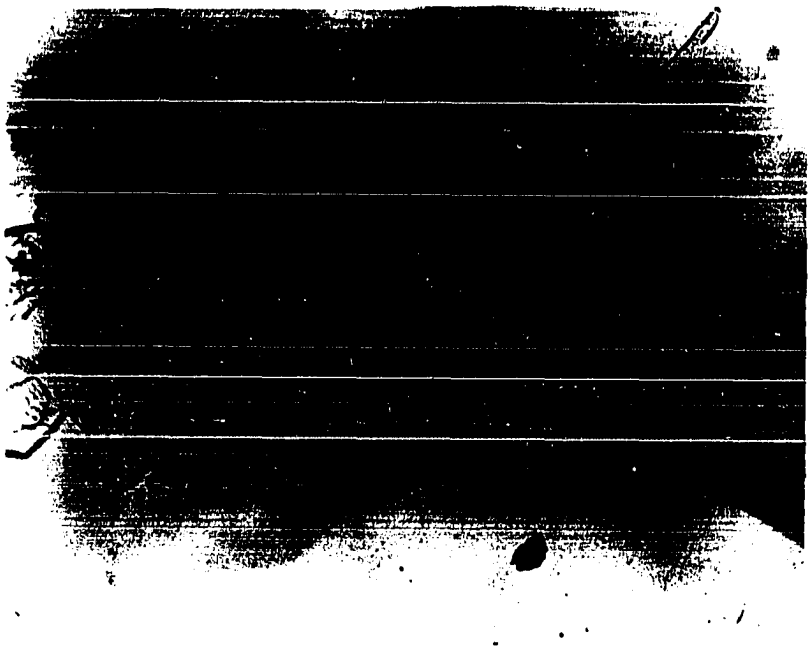


Figure 29. Photomicrograph of the accumulation of delta-aminolevulinic acid in the hind gut (H) and Malphigian tubules (M).

Figure 30. Photomicrograph of the dense labeling found in the salivary glands (S) after the injection of radioactive delta-aminolevulinic acid.

function. These cells, however, appeared to carry out a variety of other activities during metamorphosis including the transfer of nutrients, glycogen production and fat storage. Wigglesworth (1965) noted that there was little doubt from C^{14}-tyrosine injection experiments that the hemocytes were concerned with tyrosine metabolism in the process of melanization and sclerotization of the cuticle, but their actual involvement in hemoglobin synthesis remained uncertain.

Hollande (1922) studied the function of the pericardial cells extensively and found that, unlike the hemocytes which were found free in the hemolymph, these cells were incapable of migrating and were found in close association with the heart. The cells often contained yellow, brown, red or green vacuolar inclusions. The actual involvement of these cells in hemoglobin synthesis was not conclusively demonstrated.

The current view of the function of the pericardial cells, as stated by Wigglesworth (1965), suggests that they are similar to the reticulo-endothelial system of vertebrates. These cells, like the hemocytes which were believed to be concerned with the removal of large fragments in the blood, were responsible for microphagocytosis of colloidal particles. Ammonia carmine, hemoglobin, chlorophyll, egg white and other foreign materials when injected into the hemolymph, were accumulated in the pericardial cells of insects. In Rhodnius the pericardial cells were shown to become filled with the blue-green deposits of biliverdin which came from hemoglobin when it was absorbed into the blood without being further digested. Wigglesworth (1943) further observed the transition of hemoglobin to hematin, to a verdoheme pigment, to biliverdin and then finally the accumulation of free iron in Rhodnius. The same sort of

changes occurred with hemoglobin when ingested by the Kupfer cells of the reticulo-endothelial system in the liver of higher vertebrates.

It was, therefore, difficult to ascertain with certainty whether a specific site of hemoglobin had been located. Only two tissues, the pericardial cells and Malpighian tubules, appeared to take up the label in any significant quantity. Since the primary function of these two tissues appeared to be that of adsorption and excretion it was difficult to determine whether the abundance of the labeled compound in the hemocoel was merely a function of elimination or whether active hemoglobin synthesis was taking place at these sites.

Autoradiographs of LiOH gels after the electrophoresis of samples of labeled hemolymph showed some indications of localized activity. The majority of the samples, however, failed to provide any positive results. This was probably due in part to the fact that many of the larvae died as a result of the injection technique or were in poor condition at the time the hemolymph sample was collected.

Of the several positive indications of label uptake, the heaviest concentration of label was found in specimens which had metabolized the precursor for two days after injection. The area of fogging was found to coincide with bands five to seven of the typical Wisconsin LiOH pattern. The exposed areas were nearly always associated with the darkest staining bands. Other samples which showed fogging of the photographic film were specimens sacrificed one and three days after injection. The filter paper upon which the labeled hemolymph was absorbed was checked both before and after the run. In all cases the label was observed to be absent from the sample paper and had moved away from the origin.

In no instance was there evidence of uptake before 24-hours. Many of these specimens were healthy and otherwise normal in appearance. Neither were there any cases of uptake in specimens older than 96 hours. The major problem in this case was keeping the larvae alive for so long a period of time. Two other factors were likely to impair the observation of activity in the autoradiographs. First, a sizeable quantity of material was necessarily injected into the larva in order to make it detectable in the gel. The hemoglobins tend to diffuse some during the run, and an otherwise detectable amount might not show up as a descrete area. Furthermore, if the labeled area became localized in a gel there was the problem of self adsorption within the medium. A small sample would thus go undetected because only a small fraction of the radioactivity would actually reach the photographic emulsion of the film.

Although the evidence relating to site of synthesis was rather inconclusive, there was evidence that the labeled molecules were in fact synthesized. It appeared that synthesis began after a 24-hour period and depended on the health of the animal.

DISCUSSION

Chironomus tentans was adapted to laboratory conditions with relative ease. Although not as well-known an organism as Drosophila, this animal possessed a number of unique characteristics which made the effort worthwhile. The mere possession of hemoglobin made it a unique organism in the insect world, and the fact that there were multiple forms of hemoglobin increased its usefulness.

Unlike other closely related species, Chironomus tentans adults can be mated as single pairs in the confines of a shell vial. The adults do not feed before mating and are very prolific, producing over one thousand eggs per female. The life cycle lasts from six to eight weeks, depending on the conditions of the culture. Since the maturation of adults is not synchronous, the life cycles tend to overlap, which increases the probability of successful matings. Although considerable time and space was required to maintain the cultures, they were extremely economical to rear.

Few other organisms offer such a diverse number of molecular forms of hemoglobin. The ease with which the hemoglobins were obtained was enhanced by the fact that this pigment is freely dissolved in the hemolymph. This eliminates several preparatory steps in its isolation. Since hemoglobin comprises over 90% of the total protein of the hemolymph, this circumvented a purification step usually required in the analysis of vertebrate hemoglobins. Because of the high concentration of hemoglobin, it was possible to verify the electrophoretic pattern with a specific but less permanent hemoglobin stain and then utilize a generalized protein

stain for the analysis and storage of most material. Full grown larvae were large enough to provide a more than adequate sample of protein for electrophoretic purposes. It was entirely feasible to remove a small sample of blood without killing the larva, making it possible to carry out later genetic analysis of an individual whose pattern had been determined.

There were some disadvantages in using Chironomus as a laboratory organism. The life cycle was longer than one might desire, and it was easily influenced by the temperature and other environmental conditions of the cultures. The adults did not live more than three or four days, which necessitated their immediate use. Adults were rather delicate to handle and were easily injured or drowned. The mortality rate of the larvae was high in the early stages, and when this was coupled with the asynchrony of emerging adults, the continuance of the cultures required daily attention.

Contrary to Sandler (1935), these tentans strains were found to frequently lay a second smaller egg mass of approximately 500 eggs. Furthermore, the number of eggs per initial egg mass was found to average about 50% less than that reported by Sandler who reported a range of 1,400 to 3,300 eggs per mass with an average of 2,300 eggs. Unlike Sandler's experiments, the specimens used in the present study were confined to limited space and a certain amount of inbreeding took place. Whether these factors are enough to account for this discrepancy has not been determined. The rate of development and the mortality rate were roughly in agreement with other investigators.

Hemoglobin is known to exist in nearly all vertebrates as well as some invertebrates and microorganisms. In many instances, two or more

distinct hemoglobins were found in a single species. The incidence of hemoglobin polymorphism has varied from race to race, and sometimes was subject to intense selection. Recent investigations defined the effect of mutation in most variant human hemoglobins as being due to a single amino acid substitution. These results led to a better understanding of the genetic control of protein synthesis.

Only a few invertebrate organisms possess hemoglobin. Although it may have originated several times independently in the course of evolutionary development, all of the hemoglobins of vertebrates and invertebrates alike are remarkably similar, illustrating the success of this molecule in a diverse number of organisms.

The primitive hemoglobin molecule and the myoglobin molecule were believed to have a common ancestry. Supposedly, different hemoglobins arose when the primitive form duplicated and a subsequent translocation separated the two genes. Each evolved independently and with further duplications and translocations it was possible for many molecular forms of hemoglobin to evolve. This process apparently is a continuing one, since the close linkage of the beta and delta chain in humans was interpreted as the formation of a relatively recent duplication by unequal crossing over, without the translocation to a distant locus having taken place.

The process of dimerization and tetramerization had an even greater selective advantage in the transportation of oxygen. Even in the more primitive cartilagenous and bony fishes, the tetrameric molecule evolved and was successfully continued in nearly all higher forms over the many millenia of evolutionary development.

The undertaking of a study of the hemolymph of Chironomus tentans lent itself very well to several different types of electrophoresis. Of the three types of electrophoresis used, the cellulose polyacetate method provided the least information. In most cases the pattern for a single strain was the same at different stages of development, although the intensity of the bands varied. The Iowa and Wisconsin strains did not differ greatly, the primary differences being a slightly greater mobility of the first bands of the Wisconsin strain, a greater spread of bands six and seven in the Iowa strain and two additional bands (bands four and eight) in the Iowa strain. It was very difficult to get consistent results, and this system was apparently more sensitive to temperature changes and diffusion. Each sample strip was essentially a separate run which was under the influence of numerous variables already mentioned. This reduced the accuracy of comparing strips of the same run and further reduced the validity of comparison between different runs. The technique, although valuable in some applications, was not found as dependable as starch gel electrophoresis.

Of the two starch gel media used, the LiOH buffer gave superior results. This was particularly true when excessive heat generated during the run was removed by the water cooled apparatus. The starch gel system indicated as many as 13 different hemoglobins. The amount of protein produced in a specific band depended on the stage of development. In most cases, traces of each band were found at any stage of development. Exceptions were observed in very early and very late stages of the life cycle when synthesis and degradation of hemoglobin was occurring. A comparison of the general protein stain with the more specific benzidine

stain revealed that practically all of the major proteins were hemoglobin. This was in agreement with Manwell (1966b), who estimated that the hemoglobin of Chironomus plumosus comprised at least 90-95% of the hemolymph protein. Polyacrylamide disc electrophoresis likewise gave consistently good results although fewer bands were resolved. The Iowa strain produced five bands compared to four in the Wisconsin strain, with band two missing in the Wisconsin strain. Stage differences were not studied with this technique.

All electrophoretic methods indicated that differences existed among the hemoglobin patterns of different species, as well as strains. The complex heterogeneity of hemoglobins in an organism as small as Chironomus was surprising, but numerous reports bear this out. Stich (1964) reported from six to ten major proteins in various species of Chironomus. Braunitzer and Braun (1965) reported four major hemoglobins separable by column chromatography in mass homogenates of Chironomus thummi. Several additional minor hemoglobins were also noted. Thompson and English (1966) reported variation in the patterns and numbers of hemoglobins of several species, ranging from two for C. atrella to six or more for other species. Manwell (1966b) observed eight different hemoglobins in young C. plumosus larvae and ten in large larvae. These species differences undoubtedly have their basis in the duplication and accumulated differences in an ancestral genotype. Over the centuries substitutions, deletions and translocations were incorporated, thus producing slightly different genotypes and possibly through isolation due to geographic and environmental barriers, numerous species were able to successfully adapt variant hemoglobins to their respective environments.

The same influences were, no doubt, responsible for the minor differences observed within the same species, Chironomus tentans. Thompson and English (1966) reported differences in the hemoglobin pattern of the C. tentans population sampled from Lost Lake near Boone, Iowa, (American race) and specimens obtained from Germany (Professor U. Clever). These accumulated changes, although not as extensive as those observed between species, nevertheless were typical of a particular strain. Eventual amino acid analysis, finger-printing, etc., will undoubtedly confirm these variations as resulting from different numbers and types of residues contained within the variant forms of hemoglobin.

Stage differences in the production of hemoglobin were also noted throughout the life cycle of both the Iowa and Wisconsin strains. Thompson and English (1966) were the first to report these ontogenetic differences. They noted that certain molecular forms appeared earlier than others during larval development. Manwell (1966b), likewise, reported eight hemoglobins in young C. plumosus larvae and ten hemoglobins in large larvae, seven of which were unchanged throughout the life cycle.

Unlike the above finding, the two strains of C. tentans in this study appeared to produce their different hemoglobins throughout the majority of the life cycle, but in varying quantities. Exceptions, of course, were noted in very early and late larvae when synthesis was starting or ending.

Since different hemoglobin polypeptide chains are the eventual products of different genes, it appeared that the abundance of certain hemoglobins at specific intervals in the life cycle indicated a switch in the activity of corresponding hemoglobin genes during the normal developmental process. In Chironomus it appeared that some genes were more

active in early stages, while others were more active in the middle and late stages. Still other genes appeared to function equally at all stages of development. This constant activity of some genes and the modulated activity of others, is suggestive of the hemoglobin polypeptide control found in amphibians and humans.

In Rana catesbeiana the tadpole had from three to five separable hemoglobins, while the adult had three (Elzinga 1964). There were no hemoglobin bands found in both adult and tadpoles which migrated at the same rate. This suggested that certain structural genes in the young were under a regulatory system which switched them off when the adult genes were activated. A similar situation is found in humans. Fetal and adult hemoglobins involve a switch from the expression of the gamma chain gene in early life to the beta chain gene in adult life, while the alpha chain gene remains constant throughout development.

The analysis of separate polypeptide subunits of hemoglobin involved the ability of urea to dissociate individual chains making up the hemoglobin molecule (Wilson and Smith 1959). The addition of 2-mercaptoethanol acted as a block at reactive -SH sites which could in some instances cause disulfide bridges resulting in altered polypeptide mobility and polymerization (Smithies 1955). The acidity of the medium also promoted chain separation (Muller 1960).

In contrast to the typical vertebrate hemoglobin, electrophoresis of globins prepared from each of the isolated Chironomus hemoglobins suggested that there was only a single polypeptide chain type for each molecular form. Each multiple hemoglobin type, however, appeared to possess its own unique set of characteristics. That the hemoglobins were

dimers consisting of identical subunits was, nevertheless, a possibility.

According to Elzinga (1964) and Manwell (1966b) the analysis of polypeptides in urea gels was more successful than the separation of non-mammalian subunits by column chromatography, which was a procedure usually used on human hemoglobins. In this study, the urea gel method readily separated individual human hemoglobins, which were used as controls, into individual polypeptide chains. The addition of mercaptoethanol to investigate the possibility of disulphide bonds had essentially no effect on the patterns produced. Recently it was shown by Riggs (1965) and Trader and Frieden (1966) that mammalian and amphibian hemoglobin heterogeneity was due to altered -SH groups. Treatment of vertebrate hemoglobin with mercaptoethanol often altered the electrophoretic mobility and heterogeneity. In the case of Chironomus hemoglobin, there was no modification of sulfhydryl groups. Manwell (1966b) obtained similar results with Chironomus plumosus, and the lack of active sulfhydryl groups was further established by Braunitzer and Braun (1965). They reported only one cysteine residue per chain for one of the peptide chains of Chironomus thummi. This indicated that there were very few sites which might react with mercaptoethanol. The results reported here were also consistent with these observations.

The absence of subunit structure led to the postulation of a single chain or identical polypeptide chains for each hemoglobin. In either case, the data suggested that each hemoglobin is under the control of a separate gene. This would necessitate as many as a dozen different loci, each transcribing for a different hemoglobin and many under separate ontogenetic controls. Further evidence from hybridization experiments

supports this concept. In starch gel patterns, those hemoglobins not shared by both strains appeared to be less concentrated in the hybrid. This suggested that only a single gene was active and half as many protein molecules were produced in comparison with hemoglobins shared by both parents (Figures 15 and 16).

Evidence for a simplex molecular form was also obtained by Manwell (1966b) in studying the eight hemoglobins of Chironomus plumosus. Braunitzer and Braun (1965), on the other hand, reported finding four major hemoglobins in Chironomus thummi, two of which were separated into polypeptides of different primary structure. Although the latter situation is similar to that found in higher vertebrates, a high degree of multiplicity might lessen selective pressures to the point of tolerating quite different molecular forms.

The genetics of Chironomus has not been studied previously. Thompson and English (1966) eliminated genetic heterogeneity as a primary factor in the multiplicity of hemoglobins, on the basis of the consistency of pattern within various species of Chironomus. Likewise heterozygosity was not a factor, since this would depend on an elaborate system of balanced lethals to maintain consistency. Finally, the possibility that multiple hemoglobins were generated by a single locus system, as found in the haptoglobins, also appeared unlikely. The haptoglobin factor may produce several molecular types, depending on the degree of polymerization, with each polymer exhibiting its own distinctive electrophoretic mobility (Smithies and Connell 1959). There is no evidence for molecular weight variations of this kind in the present case.

Hybridization experiments involving crosses between variant forms of Chironomus tentans likewise offered some insight into the genotypic composition of the hemoglobin loci. The cross between the Iowa and Wisconsin strains produced a hybrid which characteristically displayed intermediate patterns. Many hemoglobins were the same in both strains, but where differences did exist the hybrid showed a co-dominant pattern. If one assumes the molecule is a dimer, two alternative mechanisms could account for multiple hemoglobin types. First, several genes might be controlling the synthesis of several different chain types, which combine at random or in specific pair-wise associations to form the multiple hemoglobins. If this were the case, the F_1 would show a number of new bands not previously observed. Such new bands were not seen. Under the second alternative, the same two identical chains would always associate in a specific manner. This possibility could account for the patterns actually obtained. Another possibility which might account for the hybrid patterns, however, is the exclusive occurrence of monomeric forms of hemoglobin. In this case each hemoglobin would differ only in the primary structure of its single polypeptide.

The production of several different hemoglobins has suggested one or more nests of hemoglobin loci. It was noted in the human situation that there are at least three general sites of chain synthesis. These are the alpha, gamma and beta-delta sites. The latter genes appear to be closely linked. Observations made on the F_2 generation of Chironomus hybrids suggested that there are at least two linkage groups. The possibility of more linkage groups was definitely not ruled out. Therefore,

through a process of gene duplication and translocation, at least two of the four chromosome pairs have become involved in hemoglobin synthesis.

In an effort to establish whether Chironomus hemoglobin molecules were composed of monomers or dimers, molecular weight determinations were made by filtration on a Sephadex G-100 column using reference proteins. The molecular weight of Chironomus hemoglobin was calculated at approximately 19,000 by this method. This suggested the molecule was behaving more like a monomer of 17,000 than a dimer of 34,000 as calculated by Svedberg and Eriksson-Quensal (1934).

The excess of 2,000 over the expected 17,000 molecular weight for a monomer leaves some doubt for believing the molecule was actually a monomer in the native state. The same dissociation-association phenomenon often experienced with human hemoglobin might possibly have been taking place. On the other hand, molecular asymmetry could have produced a high estimate in the column study.

In this same regard, two of the reference proteins gave anomolous results. Pepsin, for example, did not fall in line with the other proteins. It behaved as though it had a molecular weight of 74,000 on Sephadex G-100, which was twice the molecular weight given by the manufacturer. The possibility exists that under column conditions pepsin associated into a dimer. Another possibility for this abnormal behavior is that pepsin might form a complex with other materials in the columns. Since pepsin was run without other proteins, to avoid the complication of its enzymatic activity, the only complexing possible was with dextran or sucrose. Gelotte (1960) and Porath (1960) reported that phenylalanine,

tyrosine and tryptophan were adsorbed to dextran gels. Thus, a protein high in content of these amino acids might behave anomalously on Sephadex columns.

Human hemoglobin also behaved abnormally. It was retarded on the Sephadex G-100 and acted as if its molecular weight were about 46,500. Whitaker (1963) showed that both chicken and bovine hemoglobin were retarded on Sephadex G-75 and behaved as if the molecular weight were 40,000. On Sephadex G-100, the bovine hemoglobin separated into two approximately equal components with molecular weights of 55,000 and 43,000. Gutfreund (1949) reported that hemoglobin dissociates into subunits of 34,000 molecular weight in very dilute solutions and Andrews (1962) also reported a molecular weight of 32,000 for ox blood hemoglobin on agar gel columns. Thus it appeared that hemoglobin, like beta lactoglobulin which also showed marked dissociation (Andrews 1964) and lysozyme which appeared to interact with the gel filtration medium (Whitaker 1963), was unsuitable as a reference protein. Pepsin was used successfully by Whitaker (1963), and its abnormal behavior in this study is not completely understood.

Because Chironomus is noted for its time-specific alterations in the puffing patterns of the giant polytene chromosomes as sites of high genic activity, the exciting possibility of identifying hematopoetic tissues was pursued. Beerman (1952) and Clever (1964) noted striking puff changes during the middle of larval development or approximately at the end of the third instar, and Manwell (1966) has proposed that such a chromosomal change possibly accompanies the switchover from the small to the large larval hemoglobin pattern.

An attempt to answer this question was undertaken with the use of radioactive labeled hemoglobin precursors. Delta-aminolevulinic acid appeared to concentrate to some degree in the pericardial cells and to a lesser degree in the Malpighian tubules, salivary glands and intestional epithelium. It is doubtful whether any of these sites are involved in hemoglobin synthesis. The accumulation in the intestinal epithelium might be explained by simple adsorption, and since the function of the Malpighian tubules is excretion there is no particular interest in its accumulation in this organ. The greater accumulation in the pericardial cells was of interest. Whether complete hemoglobin molecules were synthesized here cannot be determined since only the heme group would carry the C^{14} label. The eventual production of labeled hemoglobin was established, however, since there was evidence of labeled hemoglobin bands on autoradiographed gels.

The possibility of correlating hemoglobin synthesis with polytene patterns still remains. As a matter of fact, the use of delta-aminolevulinic acid to label the site of synthesis is itself not completely satisfactory, since it identifies only heme in the completed molecule rather than a globin chain. If hemoglobin is quantitatively so predominant among proteins, it seems possible that use of one of the labeled amino acids, and comparison with the delta-aminolevulinic acid results, might provide further evidence concerning the site and time of globin synthesis. Such experiments have been projected for the future.

SUMMARY

1. Chironomus tentans was successfully used as a genetic organism for the study of multiple hemoglobins. Rearing of these organisms was feasible, although considerable space and time was required to maintain the cultures. Unlike other species of Chironomus, C. tentans will mate as pairs in shell vials. Each pair produced over 1,000 eggs, although a high mortality rate was observed in the young larval stages.

2. Cellulose polyacetate electrophoresis (pH 8.8) revealed eight major hemoglobins in the Wisconsin and Iowa strains with two additional bands in the later stages of the Iowa strain. This technique showed a great deal of variability and was abandoned for the more reliable and informative starch gel technique.

3. Maximum clarity of hemoglobin patterns on starch gels was obtained with a LiOH buffer (pH 8.0) used in a water-cooled apparatus. The results indicated ontogenetic variations in the concentration of hemoglobin produced throughout the life cycle. As many as fourteen different hemoglobins were observed in the late fourth instars. The two strains also exhibited genetically different patterns which were followed in the F_1 and F_2 generations. The genetic data suggest that at least two chromosome pairs contain genes for hemoglobin chain synthesis. Polyacrylamide disc electrophoresis (pH 8.8) revealed fewer bands, but likewise indicated that two chromosome pairs are involved in the production of hemoglobin chains.

4. An aluminum lactate acid gel containing urea (pH 4.5) was used to study the subunit composition of Chironomus hemoglobins. This technique

separated the chains of human and bovine hemoglobin, but failed to show any duality in the polypeptide chain make up of any one of the numerous Chironomus hemoglobins. These data suggested that each hemoglobin contains either identical subunits or a single 17,000 molecular weight polypeptide. Each hemoglobin, nevertheless, possessed a distinctive set of electrophoretic characteristics.

5. Molecular weight determination on Sephadex G-100 indicated the molecules had a weight of about 19,500. This implies monomeric structure, although the heavier than anticipated molecular weight did not conclusively rule out the possibility of an equilibrium with some dimers. A dissociation-association phenomenon commonly experienced with human hemoglobin was a possibility.

6. A radioactive labeled precursor of heme, delta-aminolevulinic acid, when injected into larvae and studied in histological sections, suggested that the pericardial cells and Malpighian tubules concentrated greater amounts of the compound than surrounding tissues. These tissues were possible sites of hemoglobin synthesis, although their excretory function was still a likely explanation for the accumulation of this compound. Autoradiographs made from gels containing labeled hemolymph, as well as histological sections of larvae containing the radioactive precursor, both indicated that a detectable amount of labeled hemoglobin was synthesized by the 24th hour after injection.

ACKNOWLEDGMENTS

The author wishes to express his appreciation to his advisor, Dr. Peter E. Thompson, for the suggestion of this problem and for his guidance and encouragement during the author's program of graduate study and research.

The author wishes to thank Dr. Lou Baker and Dr. Wilmer Miller for their advice concerning electrophoretic procedures and Mr. Walter Bleecker for his help in the molecular weight determinations. The author was also indebted to Mr. Wayne Heimermann for his technical help in maintenance of the cultures. Financial assistance was provided by the Agricultural Station, Iowa State University, the President's Permanent Objectives Fund, Iowa State University, and grant GB-6237 of the National Science Foundation.

Finally, the author wishes to acknowledge the encouragement given to him by his wife, Yvonne, during his graduate study and research, as well as her assistance in the preparation of this thesis.

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