Ontogenetic changes in hemoglobin synthesis of two strains of *Chironomus tentans*

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Differentiating cells can be characterized by the number and types of proteins they produce (Markert, 1963). In man and other organisms the diversity of hemoglobins has been correlated with ontogenetic changes which reflect the alterations of gene activity (Ingram, 1963). Furthermore, the types of proteins produced are generally specific for the species.

The study of insect coelomic proteins has followed closely that of mammalian serum protein analysis; however, profound differences exist between human blood and insect coelomic fluids. For this reason, the term ‘hemolymph’, rather than blood, has been used to designate these fluids. Svedberg & Eriksson-Quensal (1934) first studied the ultracentrifuge properties of *Chironomus plumosus* hemoglobins and determined them to have a molecular weight of 34000. Braunitzer & Braun (1965) described the multiplicity of hemoglobins in *Chironomus thummi* and claimed two subunits per molecule. Manwell (1966) suggested the hemoglobins of *Chironomus plumosus* possessed a monomeric structure. Thompson, Bleecker & English (1968) demonstrated the monomeric nature of *Chironomus tentans* hemoglobins and calculated a molecular weight of 15900 and suggested the hemoglobins were more asymmetrical than those of myoglobin.

Unlike vertebrate hemoglobins, this insect protein is dissolved in the body fluids and constitutes over 90% of the proteinaceous material (Manwell, 1966). Thompson & English (1966) have demonstrated the species-specific patterns for several species of *Chironomus* and noted a general intensification of individual larval hemoglobins during the later stages of development. Manwell (1966) observed similar ontogenetic changes in small and large larvae of *Chironomus plumosus*. These differences in the sequential development of multiple hemoglobins suggested a ‘switchover’ in activity of certain hemoglobin genes.

The following data give a more complete description of the ontogenetic changes in hemoglobin synthesis in *Chironomus tentans* and provide further evidence for the monomeric nature of this molecule. Such information may

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have profound future implications when considering the possibilities of correlating synthesis patterns with morphological changes in chromosomes. Similar observations of gene activity (puffing) and protein synthesis have been made by Beermann (1952), Clever (1964a, b), Laufer & Nakase (1965) and Kroeger (1968). Also reported are the first observations of intraspecific variations of hemoglobin patterns between two widely dispersed populations of *Chironomus tentans*. The genetic and evolutionary implications of these isolated populations are discussed.

**Materials and Methods**

*Chironomus tentans* larvae were collected from the states of Wisconsin and Iowa. Adults were reared and mated to maintain the larval cultures. Hemolymph samples were obtained by puncturing the cuticle of larger larvae or by mascerating several small larvae and collecting the pooled sample.

Sepraphore III cellulose polyacetate electrophoresis (Gelman Corporation) was used with a modified tris-barbital-sodium barbital buffer (pH 8.0) at 5 °C. Four to six hundred volts were applied until a 5 cm pattern developed. Ponceau S and benzidine stains were used. Starch gel electrophoresis after Ferguson & Wallace (1961) at pH 8.0 was carried out in a water cooled horizontal electrophoresis apparatus, using 350–500 V until a 6.0–6.5 cm pattern was produced. Benzidine and Buffalo Black or Nigrosin stains were used. Polyacrylamide disc electrophoresis (Ornstein, 1964) at pH 8.8 was run at 5 mA/tube until the tracking dye migrated 3.3–3.8 cm. All runs were made at 5 °C. Gels were stained with Amido Black or benzidine.

Electrophoresis of globin subunits of individual multiple hemoglobins was performed in 8 M urea starch gels containing 2-mercaptoethanol (pH 4.5) after the methods of Poulik (1966). Individual hemoglobins were first separated on cellulose polyacetate strips or in starch gels, treated in cold acid acetone for 1 h to aid in the dissociation of the heme and globin, inserted in the urea gel and run at 100 V for 12 h at 5 °C. Globins were stained with Buffalo Black.

**Results**

*Electrophoresis of Chironomus tentans hemoglobins*

Cellulose polyacetate electropherograms of hemolymph from different developmental stages of the Iowa and Wisconsin strains are shown diagrammatically in Fig. 1 and photographically in Fig. 3A, B. The different developmental stages contain essentially the same patterns except in very young and old larvae where synthesis and degradation take place respectively. There is a general intensification of bands in the later stages. This technique resolved eight major bands in the Wisconsin strain and two additional bands in the Iowa strain. The position of the bands was characteristic for the individual strains. Considerable variation in the pattern for a particular size of larva was
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noted. This may reflect environmental differences or probably the difficulty in duplicating identical conditions from run to run.

Starch gel electrophoresis of larvae at different stages of development was much more reliable since a series of samples from the same or different strains could be run simultaneously and under identical conditions. A total of thirteen protein bands, which were also benzidine positive, were found in the Iowa strain and one additional band identified as band five was observed in the Wisconsin strain. Generally, the various hemoglobins were present in the

![Figure 1](image1.png)

**Fig. 1.** A diagram illustrating the ontogenetic changes in hemoglobin pattern of the Iowa and Wisconsin strain as observed by cellulose polyacetate electrophoresis.

![Figure 2](image2.png)

**Fig. 2.** A diagrammatic representation of the ontogenetic changes observed in starch gel electrophoresis of the various multiple hemoglobins found in two strains of *Chironomus tentans*. Note the differences between the two strains as illustrated at bands three and five.
second instar, the stage during which hemoglobin synthesis is visibly initiated. An exception was band eight in the Iowa strain which was delayed. Two bands, numbered 12 and 14, did not appear until late in the life cycle and were quite diffuse. These bands, although showing a benzidine positive reaction, may represent a breakdown of other hemoglobins since there is a general disintegration of hemoglobins during metamorphosis. Major pattern differences between the two strains can be summarized as follows: (1) the absence of band five in the Iowa strain, (2) the lack of an intense band three in the Wisconsin strain, (3) slight shifts of certain bands as in the case of closer doublets of one to two and six to seven in the Wisconsin strain and (4) major alterations in the concentration of hemoglobins at different stages of development. Only two cathodal components were observed, the furthest from the origin was very diffuse and present only in late stages of larval development. The ontogenetic differences in hemoglobin production and the variations in overall pattern morphology are illustrated in Fig. 2 and photographically in Fig. 3 C, D.

The results obtained with disc electrophoresis are illustrated in Fig. 4 A. Reproducible results were obtained using this method. Protein banding patterns exhibited by the Iowa strain were characterized by five major bands. Minor bands were often observed dispersed between major bands but were not consistent in their appearance. The pattern observed for the Wisconsin strain varied from the Iowa strain in that the second band from the anodal end was completely missing and band four was generally less intense than its counterpart in the Iowa strain.

Hybrid adults produced from the Iowa and Wisconsin strains were completely viable and fertile. Both starch and disc gel electrophoretic patterns prepared from hybrid larval hemolymph expressed an intermediate or codominant pattern between the two parents (see Fig. 4 B, C). In starch gels, bands three and five were present while band two and an intense band four were observed in hybrid disc gels. Preliminary data suggest the possibility of bands three and five and bands two and four behaving independently in starch and acrylamide gels respectively.

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Fig. 3(A). Cellulose polyacetate electrophoresis of hemolymph from various stages of the Iowa strain. The run was made in a modified Tris-barbital-sodium barbital buffer (pH 8-8) with 400 V at 5 °C. Strips were stained with Ponceau S. Reading left to right, larval sizes were: 10, 12, 14, 15, 18, 20, 21, 22, 23, 25 mm and 4th instar. All bands migrated anodal. (B). Cellulose polyacetate electrophoresis of hemolymph from various stages of the Wisconsin strain. Similar conditions were used as described above. Larval sizes were: 8, 10, 14, 15, 17, 18, 20, 22, 23, 25 mm and 4th instar. (C) LiOH starch gel electrophoresis of the Iowa Strain (pH 8-0) at 500 V as described by Ferguson & Wallace (1961). The total protein stain Nigrosin was used. Larval sizes were: 23, 15 (4th instar), 20, 23, late pupa, 23, 23, 21, 17 (late 3rd instar), 17, 16, 15, 15, 10, 10, 9 mm respectively. (D) LiOH starch gel electrophoresis of the Wisconsin strain as described above but stained with Buffalo Black. Larval sizes were: 21, 22, 21, 19, 17 (late 3rd instar), 16, 15, 12, 15, 12, 12, 10, 8, 10, 10, 8, 6 mm respectively.
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Analysis of globins

Electrophoresis of globins of major hemoglobins of *C. tentans* in gels containing a combination of 8 M urea, low pH and mercaptoethanol is shown in Fig. 4D, E. The globins migrated as individual bands with or without mercaptoethanol although traces of other protein material, presumably contamination from neighbouring bands or non-hemoglobin protein, were observed around the major band. The single bands displayed characteristic mobilities although their intensities varied. The control separation of human and bovine globins into α- and β-chains was consistent.

DISCUSSION

The electrophoretic study of *Chironomus* hemolymph has revealed a surprising number of hemoglobin types. Analysis of hemoglobins from various stages of development confirms and extends the observations of Thompson & English (1966) and Manwell (1966). Second, third and fourth instar and pupal hemoglobins were easily resolved with a variety of electrophoretic techniques (cellulose acetate, starch and acrylamide). The results indicated ontogenetic variations in the amounts of hemoglobin produced throughout the life cycle. As many as 12–14 different hemoglobins were detected in the late fourth instar larvae. The two strains also exhibited genetically different patterns. Alterations in the hemoglobin pattern at various stages of development suggest a 'switchover' mechanism in the activity of certain hemoglobin genes. Similar changes in development of large and small larvae were observed by Thompson & English (1966) and Manwell (1966). These switches in gene activity are reminiscent of the changes from γ- to β-chain production in the fetal–adult hemoglobin transition of higher vertebrates.

There is good evidence to assume that each hemoglobin is the expression of

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Fig. 4 (A). Disc gel electrophoresis of the Iowa (left) and Wisconsin (right) strains. Pooled hemolymph of various sized larvae was used. Gels at pH 8·8 were run at 5 mA/tube at 5 °C. Gels were stained with Amido Black. The bands migrated anodal. Note the strain differences at band two and four from the anodal end. (B) Disc gel electrophoresis of pooled samples of hybrid hemolymph. Note the codominant appearance of the pattern. (C) LiOH starch gels of pooled samples of various sized Iowa, hybrid and Wisconsin larvae respectively. Most bands migrated anodal. Note the codominant effect of bands three and five from the anodal end. (D) Two-dimensional gel electrophores of individual globins. The first dimension, seen at the bottom of the gel, was run in the LiOH gel as described previously. The second dimension was carried out in 8 M urea gel containing mercaptoethanol at pH 4·5. The run was made at 100 V for 12 h. Note the single spot which corresponds to an individual hemoglobin separated in the first dimension. (E) Two-dimensional electrophoresis of Wisconsin hemolymph using cellulose polyacetate electrophoresis for the first dimension and 8 M urea gels for the second dimension. Note the single band which corresponds to a single band sectioned from the cellulose polyacetate strip.
an individual gene. Thompson & English (1966) observed the consistency of electrophoretic patterns which apparently eliminates genetic heterogeneity as a primary factor in multiplicity of hemoglobins. Likewise, heterozygosity was not likely since this would necessitate a rigid system of balanced lethals; the possibility of a single locus generating the multiple hemoglobins like that of the haptoglobins seemed unlikely based on the lack of evidence for several degrees of polymerization. The use of different amounts of starch in gels by Manwell (1966) showed no differential retardation in electrophoretic mobility of *C. plumosus* hemoglobins. This suggests that none of the hemoglobins are polymers of the other (Smithies, 1962; Ornstein, 1964). Likewise, Weber (1965) observed a single peak in *C. plumosus* hemoglobins in the analytical centrifuge. Therefore, the heterogeneity of *Chironomus* hemoglobin is not the result of different degrees of stabilized molecular aggregation. Manwell (1966) also studied the effects of various -SH and -S-S- reagents on *Chironomus* hemoglobin without effect. This proves that the heterogeneity of *Chironomus* hemoglobin does not involve chemical alterations of sulphhydryl groups and that there are no readily reactive -SH groups in *Chironomus* hemoglobin. Braunitzer & Braun (1965) reported only 0–1 cysteine per chain for one of the multiple hemoglobins of *C. thummi*.

Further evidence for the presence of multiple but distinct hemoglobin genes comes from studies of globins prepared from each of the isolated hemoglobins. Manwell (1966) suggested that the hemoglobin of *C. plumosus*, in contrast to the typical vertebrate hemoglobin, consists of single polypeptide chain types in each of the multiple hemoglobins. Each multiple hemoglobin probably possesses a unique chain type. The present study confirms the observations of Manwell's globin analysis for *C. tentans*. Each major hemoglobin failed to separate into different subunits; however, individual globin units exhibited characteristic mobility patterns. Additional evidence for the monomeric nature of *C. tentans* hemoglobins was made by Thompson, Bleecker & English (1968). Their studies indicated *C. tentans* hemoglobins to be exclusively monomeric with a molecular weight of approximately 15900. These findings were based on sedimentation velocities at various concentrations and with agents which separate subunits, together with determinations by sedimentation equilibrium. Gel filtration studies suggested hemoglobins were more asymmetrical than myoglobin.

This evidence establishes a direct correlation between the nature of the hemoglobin molecule and the number of globin genes. Therefore, it appears that *C. tentans* may possess as many as 12–14 different hemoglobin loci. Similar results were envisaged for *C. plumosus* by Manwell (1966) with a minimum of eight to ten loci in the large larvae.

In the vertebrates, hemoglobin is a ubiquitous protein. The hemoglobins of the vertebrates are remarkably similar in overall architecture down to the cartilaginous fishes. They contain four polypeptide chains, each with a molecular
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weight of approximately 17000. In species lower down the evolutionary scale than the fishes, the hemoglobins are different. The lamprey hemoglobin is a single polypeptide of molecular weight 17000 and the hagfish hemoglobin appears to be similar or possibly a dimer of 34000 molecular weight. The possession of hemoglobin in the invertebrates is frequent but erratic. Much remains to be learned about the complexity of invertebrate hemoglobins.

Hemoglobin and myoglobin are thought to have a common ancestry. The accumulation of duplicated hemoglobin genes, subsequent mutation and translocation has resulted in several distinct vertebrate polypeptide types (Ingram, 1963). Apparently a similar situation has occurred in Chironomus. The multiple hemoglobins observed may have resulted from the duplication of a basic Chironomus hemoglobin locus. The observation of strain differences suggests that an alteration in phenotypic patterns as determined by changes in the genotype is a continuing one. It is unclear why this insect should possess so many different hemoglobins. If the Chironomus gene was able to incorporate hemoglobin mutations more frequently than the vertebrate gene this could account for the multiplicity of hemoglobin loci in this genus. The selective advantage of several hemoglobins may provide a certain amount of stability; however, the physiological role of hemoglobin in Chironomus appears to be significant only at very low oxygen tensions.

Hybrid studies show codominant electrophoretic patterns. Preliminary examination of crosses between hybrids produces a preponderance of the hybrid phenotype over the Iowa and Wisconsin types and also the appearance of new phenotypes. This suggests the possibility that the two major differences (noted in the starch gel and acrylamide gel) may be inherited as two independent linkage groups, i.e. in the typical Mendelian dihybrid fashion. The possibility of linked genes and crossing over must, however, not be ruled out at this time. Additional data and more precise means of classifying the electropherograms are being used to clarify this point. The observation of several different hemoglobins localized in one or more nests of hemoglobin loci is a possibility and has been postulated in the vertebrates.

Of particular interest is the possibility of correlating the time-specific alterations of chromosome puffs with the changes in hemoglobin synthesis. Manwell (1966) has noted that several striking puff changes occur in the middle of larval development which coincides with the switch from small to large larval hemoglobin patterns.

SUMMARY

1. The study of hemoglobins in Chironomus tentans by cellulose polyacetate, starch and acrylamide disc electrophoresis, illustrates the multiplicity of hemoglobin types. As many as 12–14 distinct hemoglobins were observed.

2. Ontogenetic changes were observed for each hemoglobin with all hemo-
globins generally being present to some degree. An exception to these observations occurred in very early second instar larvae when synthesis began and in late fourth instar larvae when degradation of hemoglobin occurs. The ontogenetic sequence of hemoglobin production in Chironomus was reminiscent of the fetal–adult switchover which occurs in man.

3. Globin analysis of major hemoglobins in 8 M urea gels confirmed the hypothesis that, in contrast to higher vertebrate hemoglobins, Chironomus hemoglobins are composed of a single polypeptide. Each globin possessed a distinct set of electrophoretic characteristics, suggesting a series of gene loci which code for each molecular type.

4. The first intraspecific differences were observed in Chironomus hemoglobin electropherograms. Starch gels of the Wisconsin strain showed a definite band five and a much reduced production of band three, while the Iowa strain exhibited a prominent band three but completely lacked band five. Disc acrylamide electropherograms of the Iowa strain exhibited prominent bands two and four while the Wisconsin strain lacked band two and showed a less intense band four.

5. Hybrids between the two strains were completely viable and exhibited electrophoretic separations which showed a codominant pattern of hemoglobins. Preliminary examinations of electrophoretic differences between the two strains suggest that there may be at least two linkage groups.

6. Further studies of Chironomus hemoglobins offer a potentially valuable tool for the study of gene action at the polytene chromosome level as it relates to protein synthesis.

RÉSUMÉ

Changements ontogéniques de la synthèse de l’hémoglobine dans deux souches de Chironomus tentans

1. L’étude de l’hémoglobine de Chironomus tentans par électrophorèse sur acétate de cellulose, gel d’amidon et gel de polyacrylamide, illustre la multiplicité des formes de l’hémoglobine. On observe jusqu’à 12–14 formes distinctes.

2. Au cours de l’ontogénèse des changements quantitatifs affectent chaque forme d’hémoglobine, mais toutes sont généralement présentes. Une exception à ces observations se produit dans la très jeune larve de deuxième stade quand la synthèse débute, et en fin de quatrième stade larvaire quand l’hémoglobine se dégrade. La succession ontogénique de production de l’hémoglobine chez Chironomus se compare au relais foetal-adulte qui se produit chez l’homme.

3. L’analyse sur gel de la globine des hémoglobines principales, en présence d’urée à concentration 8 M, confirme l’hypothèse que, contrairement aux hémoglobines des vertébrés supérieurs celles de Chironomus sont constituées d’une seule chaîne polypeptidique. Chaque globine a ses caractéristiques électrophorétiques propres suggérant l’existence d’une série de Loci qui codent pour chaque type de molécule.
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4. Les premières différences intraspecifiques ont été observées sur les électrophorégrammes d'hémoglobine de Chironomus. Les électrophorégrammes de la souche Wisconsin montrent une bande cinq et une bande trois beaucoup plus réduite, tandis que dans la souche Iowa la bande 3 est prédominante et la bande cinq fait complètement défaut.

Les électrophorégrammes sur disque de polyacrylamide de la souche Iowa montrent des bandes deux et quatre prédominantes, tandis que la souche Wisconsin a une bande quatre beaucoup moins intense et pas de bande deux.

5. Les hybrides entre les deux souches sont normalement viables, et les séparations électrophorétiques montrent une codominance de la distribution des hémoglobines. L'observation préliminaire des différences électrophorétiques entre les deux souches suggère qu'il peut y avoir au moins deux groupes de Linkage.

6. De nouveaux travaux sur les hémoglobines de Chironomus pourraient offrir un moyen d'étudier, au niveau des chromosomes polytènes, le mécanisme qui relie l'action des gènes et la synthèse protétique.

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