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FAT BODY: A SITE OF HEMOGLOBIN SYNTHESIS IN *CHIRONOMUS THUMMI* (DIPTERA)

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ABSTRACT

Fourth instar larvae of *Chironomus thummi* were permitted to incorporate labeled amino acids and/or δ-aminolevulinic acid (δ-ALA) in vivo and in organ culture. The products secreted into the hemolymph or into the culture medium were examined by acrylamide gel electrophoresis. Nine electrophoretic bands can be resolved as hemoglobins without staining. When gels are sliced for scintillation counting, incorporated amino acids and δ-ALA are shown to be associated primarily with the same nine hemoglobin bands, suggesting that hemoglobins are assembled and secreted. Staining of gels with Coomassie brilliant blue reveals that there are several bands in addition to the visible hemoglobins. These bands incorporate amino acids, but not δ-ALA, suggesting that they are non-heme proteins. The results of culturing isolated salivary glands, gut, and fat body demonstrate that the fat body is the major site of hemoglobin synthesis and secretion. Labeled products of the gut represent about 5% of the total hemoglobins produced by the tissues, while no hemoglobins are produced by the salivary glands.

Although nine hemoglobins are visibly resolved on gels, labeling techniques reveal as many as 14 hemoglobins. This is the first demonstration of hemoglobin synthesis by specific tissues in culture in an invertebrate.

Unlike vertebrates in which the phenotypic expression of hemoglobins is limited, individual members of the genus *Chironomus* display a striking hemoglobin polymorphism. This hemoglobin multiplicity is both stage- and species-specific and can be effectively displayed by gel electrophoresis (Manwell, 1966; Thompson and English, 1966; Tichy, 1966, 1970; Braunitzer et al., 1968; Wülker et al., 1969; English, 1969; Laufer and Poluhowich, 1971; Schin et al., 1974). Hemoglobin is first seen in the second larval instar of *Chironomus* and may comprise up to 90% of the hemolymph proteins in the fourth instar (English, 1969). Specific hemoglobin patterns revealed by electrophoresis change during larval development (Wülker et al., 1969). At the conclusion of development to the adult, more than 95% of the hemoglobin has been degraded (Laufer and Poluhowich, 1971; Schin et al., 1974).

As many as 10 hemoglobins exist in *Chironomus thummi*. These were characterized in part by amino acid analyses including terminal amino acid determinations (Braun et al., 1968). English (1969) reported 12–14 hemoglobins in *Chironomus tentans*, while 8–10 were found in *Chironomus plumosus* and *Chironomus pallidivittattus* (Manwell, 1966; Tichy, 1968). The molecular weight of hemoglobin monomers determined by Sephadex

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gel filtration, sedimentation velocity, and analytical ultracentrifugation is estimated to be 15–16,000 (Braun et al., 1968; Thompson et al., 1968). Dimers having a mol wt of 31–32,000 (Svedberg and Eriksson-Quensel, 1934; Braun et al., 1968) may also occur. The presence of dimers is species-specific. They exist in a pH-dependent equilibrium with monomers in *C. thummi*, but are absent from *C. tentans* (Braun et al., 1968; English, 1969). *C. thummi* globin is similar in structure to sperm whale myoglobin (Amiconi et al., 1972) and shows stoichiometric binding of protoheme. Thus, there is one heme per globin.

The multiplicity of the hemoglobins and the temporal specificity with which they appear in development raise several interesting questions. In this report we identify the fat body as the organ which synthesizes both the heme and the globins of *Chironomus* hemoglobins. This report represents, to our knowledge, the first demonstration of hemoglobin synthesis in organ culture by an invertebrate.

MATERIALS AND METHODS

Experimental Organisms, Dissection, and Culture Conditions

Mid-fourth instar larvae of *C. thummi* taken from laboratory stocks, reared according to procedures of Laufer and Wilson (1970) and with an average wet weight of 4.0 mg, were used in these experiments. No distinction regarding sex was made.

The larvae were blotted and dissected in a drop of Cannon's medium containing penicillin and streptomycin (Ringborg and Rydlander, 1971). The entire gut (including malpighian tubules), the salivary glands, and/or the fat body loosely suspended in the hemolymph of the body cavity were removed and cultured. In some experiments, the fat body was cultured attached to the body wall. In others, the larva was subjected to a mock dissection and the entire larval contents were cultured, except for the head capsule and prolegs. A portion of the fat body is located between the intersegmental musculature and the body wall. Since no effort was made to isolate this fat body from the body wall, cultures of the body wall also included this fat body component.

The tissues were rinsed in Cannon's medium. Body walls (12 or 30) were cultured in 75 μl, guts (10–12) in 35 μl, salivary glands (35–40) in 50 μl, and fat body (12) in 40 or 50 μl, of fresh medium at room temperature (22–23°C) for 24 h.

After the incubation the medium was removed and processed. Thus, only hemoglobin synthesized and secreted into the culture medium was examined. In experiments in which tissues from the same larvae in culture were compared, the tissues were rinsed once with 50 μl of fresh medium and the rinse was pooled with the incubation medium. The samples were diluted 1:1 with dialysis buffer (0.009 M $Na\text{-}PO_4$, 0.04 M NaCl, 0.04% KCN, pH 8.1), and dialyzed against 500 ml of the above buffer overnight at 4°C with at least three changes of buffer to remove unincorporated isotope. Alternatively, G-25 Sephadex gel filtration was used to remove unincorporated isotope.

In another set of experiments, 28 mid-fourth instar larvae were placed in 0.75 ml of tap water containing ^{3}H-amino acids supplemented with 1–2 mg of food. After 24 h the larvae were bled. The hemolymph was dialyzed and treated like other samples before electrophoresis.

Isotopes

Isotopes were purchased from New England Nuclear, Boston, Mass. Tritiated L-amino acid mixtures [^{3}H(G_1)] were obtained with a spec act of 1.3 Ci/mmol. Carbon 14-labeled L-amino acids had a spec act of 49 mCi/mmol. [^{3}H]δ-aminolevulinic [3,5-H(N)] acid hydrochloride had a spec act of 5.050 Ci/mmol. The isotopes were dried down in the culture tubes under a stream of nitrogen gas or filtered air. Concentrations are indicated in the figure legends.

Incorporation of Isotopes

To determine if the isotopes were being incorporated into proteins and protoheme, cultures of 13 body walls including all of the fat body were incubated in the presence of either [^{3}H]δ-ALA or ^{3}H-amino acids. After 24 h the medium was collected and 50 μl of fresh fourth instar hemolymph were added to each sample. Unincorporated counts were removed from samples, and the heme and globin were separated with acid-acetone according to the procedure of Amiconi et al. (1972).

Of the total counts in the eluted samples treated with [^{3}H]δ-ALA, 87.7% were associated with the heme fraction. The protein fraction of the amino acid-labeled sample contained 86.5% of the total counts. Recovery of counts after acid-acetone treatment was 100% for both samples. These data suggest that amino acids are primarily incorporated into proteins including globin while most of the [^{3}H]δ-ALA is incorporated into protoheme.

Gel Electrophoresis and Scintillation Counting

Discontinuous acrylamide gels were cast in glass tubes (Davis, 1964; Ornstein, 1964). The 10% running-gel and the electrode buffers were prepared according to the procedure of Braun, Chrichton, and Braunitzer (1968), while the 3% stacking gel was prepared as adapted from Maizel (1969). (The concentration of ammonium persulfate in the 10% gel was reduced from 0.074% to 0.0093%, to prevent artifacts of rapid polymerization such as gel fracturing or bubbling.)

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A 5- to 10-μl aliquot of the dialyzed sample was removed for scintillation counting to determine the volume of the samples to be electrophoresed. 20 μl of clear fourth instar hemolymph, diluted 1:1 with dialysis buffer, were added to samples to provide visible hemoglobin (Hb) markers along with sucrose and bromophenol blue before electrophoresis. Marker Hbs were also electrophoresed on separate gels (Fig. 1). Electrophoresis was carried out at 200 V (<5 mA/gel) until the hemoglobin marker entered the 10% running gel. Separation was carried out at 100 V (<5 mA/gel) until the tracking dye was within 5 mm of the anodal end of the gel. During removal of the gels from the glass tubes, the stacking gel was occasionally lost. This is reflected in Figs. 2, 3, and 7.

Unlabeled gels were stained with Coomassie brilliant blue (0.6 g in 50% methanol/9.2% acetic acid) for 1 h and destained in 10% methanol/10% acetic acid overnight with continuous slow shaking. Radioactive gels were generally not stained, and in some cases they were scanned at 415 and 280 nm in a Gilford gel scanner (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) with a chart recorder attached to a Beckman DU spectrophotometer (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The positions of the red hemoglobin bands on all labeled gels were marked with 100-μm diameter tungsten wire pins. Careful note was taken of bands which were broad or which showed significant trailing. Unstained gels were fixed in 10% methanol/10% acetic acid for 3–24 h, frozen, cut into 1-mm slices, and digested in 30% hydrogen peroxide at 90°C.

Counting was done in 4.5 ml of Aquasol (New England Nuclear), or a fluor consisting of 2,500 ml toluene, 2,280 ml Triton X-100 (Research Products International Corp., Elk Grove Village, Ill.) and 160 ml Spectrafluor (Packard Instrument Co., Downers Grove, Ill.), on a Packard Tri-Carb or Nuclear Chicago Isocap/300 liquid scintillation counter (Nuclear Chicago Inc., Des Plaines, Ill.).

RESULTS

The general pattern of the multiple molecular forms of Hbs from fourth instar larvae obtained on gel electrophoresis in our laboratory is comparable to that of Hbs obtained by others (Braunitzer et al., 1968). The detection of these Hbs by several methods of examining gels (i.e., visual examination of stained and unstained gels, spectrophotometric scanning of unstained gels, scintillation counting of gel slices) and their correspondence to the Hbs identified by Braunitzer and his co-workers is shown in Table I.

Synthesis of Hemoglobin In Vivo

The same pattern of hemoglobins (Hbs) was obtained on acrylamide gel electrophoresis of fourth instar hemolymph in at least 60 experiments. Typically, nine Hbs were resolved in similar proportions and could be seen as red bands without staining (Fig. 1 and Table I). The Hb positions were numbered consecutively from the cathodal to the anodal ends of the gels. Band 1 migrates only slightly into the separating gel; bands 4 and 6 have broad trailing edges. When stained for protein with Coomassie brilliant blue, several faint additional bands were detectable, as well as material which did not enter the separating gel (Fig. 1). These are probably not hemoglobins (Fig. 1; and see below). In addition, after several weeks of destaining, bands 4 and 6 can sometimes be resolved into two bands each. Gels of labeled samples were not stained because of the lengthy destaining times required to develop these bands, but the trailing edges of the unstained bands were measured (see Materials and Methods).

We sought to determine whether all of the Hbs separated by electrophoresis were synthesized by

TABLE I

Correspondence of Hbs from Acrylamide Gels Detected by Different Techniques

Hb band nomenclature determined by:				
Visible red bands	Scan at 415 nm	Stained, visible blue bands	Peaks of [^{3}H]δ-ALA incorporation	Hb bands obtained by Braunitzer et al. (1968)
1	1	1	1	D1
2	2	2	2	D2
3	3	3	3	D3
4	4	4a	4x*	D4
		4b	4y	D5
			4z	
5	5	5	5	D6
6	6	6a	6x*	D7
		6b	6y	
			6z	
7	7	7	7	D8
8	8	8	8	D9
9	9a	9	9a	D10
	9b		9b	

The comparison of our work and that of Braunitzer et al. is an estimate made using a photograph of a gel of *C. thummi* Hbs in the reference cited.

* Bands designated x, y, and z were those resolved in the trailing edges of bands 4 and 6; which of these were derived from *a* and *b* (stained gels) was not determined.

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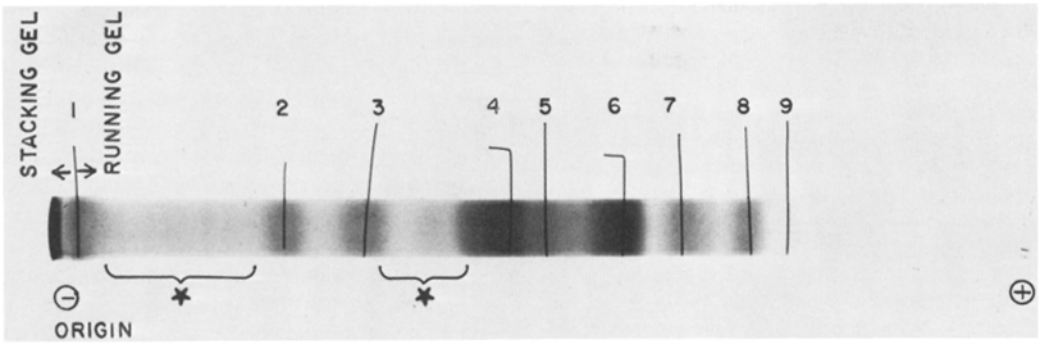

FIGURE 1 Acrylamide gel of mid-fourth instar *C. thummi* hemolymph. Electrophoresis was conducted as described in Materials and Methods. The nine Hbs were marked with tungsten wire pins, and the broad bands (4 and 6) were measured before staining with Coomassie brilliant blue. Asterisks indicate regions in which nonhemoglobin bands are found (see text).

larvae in vivo. Mid-fourth instar larvae fed on ^{3}H-amino acids for 24 h incorporated label into material which migrated with 9 Hbs (Fig. 2). Band positions are shown in the figures. Several less well resolved non-heme proteins were probably also labeled in slices 4–14. The most prominent labeling, however, coincides with the hemoglobins (see bands 4–6, especially). Apparently, different Hbs are labeled in vivo to varying degrees during the 24-h incorporation period. The possibility that several labeled Hb components contribute to the peaks of incorporation in bands 4–6 and 9 will be discussed later.

Hemoglobin Synthesis in Culture

We asked whether larval tissues would synthesize Hbs in culture in a pattern reflecting in vivo synthesis. More than 40 culture experiments were performed on fourth instar larval material. All results are internally consistent and demonstrated that Hbs are the major proteins synthesized and secreted by larval tissues. All of the experiments described were repeated at least once with similar results.

Larvae which had been cut antero-posteriorly and flattened to expose the contents of the body cavity were cultured with labeled amino acids for 24 h. Fig. 3 shows that the results are similar to in vivo incubations, though the resolution of the peaks is greater than in Fig. 2, the result of a longer 10% gel. Bands 4 and 6 and their trailing edges seem to be further resolved into 3 and 2 labeled components, respectively. While the resolution of the Hbs varies somewhat on gels in succeeding experiments, additional components in those bands can usually be demonstrated. Thus, larval tissues in culture are capable of incorporating labeled amino acids and secreting labeled proteins into the medium. The reason for differences in degree of labeling between in vivo and organ culture experiments are as yet unexplained. However, the same Hbs labeled in vivo are labeled in culture.

Various larval tissues were examined for their ability to synthesize and secrete Hb. Fat body attached to the body wall was cultured with amino acids. The gut, malpighian tubules and salivary glands were not present in these cultures. The results of electrophoresis were almost identical to those obtained in Fig. 3. Since the incorporation of ^{3}H-amino acids into material comigrating with Hb is not in itself conclusive evidence of Hb synthesis, fat body attached to the body wall was cultured in the presence of a tritium-labeled porphyrin precursor (δ-aminolevulinic acid, or δ-ALA) instead of amino acids (Fig. 4). Before fixation and slicing, the gel was scanned at 415 and 280 nm. The scan demonstrates that band 9 Hb may be composed of 2 Hbs. The fact that ^{3}H-amino acids (Fig. 3) and [^{3}H]δ-ALA are incorporated into the same Hb markers is indeed convincing evidence that the Hbs are being synthesized, assembled, and secreted by larval tissues in culture.

Compared to the profiles of amino acid incorporation, [^{3}H]δ-ALA labeling is localized to those regions of the gel with Hb. In addition to higher ratios of peak maxima to low level incorporation, the peaks are better resolved from each other. At least 80% of the [^{3}H]δ-ALA incorporation comigrates with visually distinguishable Hb bands.

It is evident that larval tissues other than salivary glands, gut, and malpighian tubules are a

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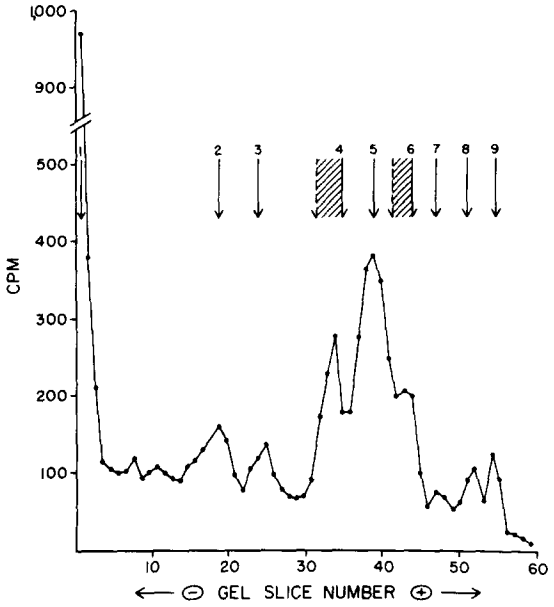

FIGURE 2 Electropherogram of labeled fourth instar hemoglobins after incubation of larvae in vivo for 24 h in the presence of ^{3}H-amino acids. The positions of the hemoglobins are indicated by the arrows over the incorporation profile. The bands are numbered from 1 to 9. Trailing edges of bands 4 and 6 are shown. 28 larvae were "fed" ^{3}H-amino acids (0.1 mCi/ml) for 24 h. The larvae were bled and treated as described in the Materials and Methods section, before electrophoresis. The gels were sliced into 1-mm sections for scintillation counting. The profile of incorporation into the stacking gel is not shown. Approximately 30% of the sample was electrophoresed.

major source of Hb. We suspected that the fat body, which is the source of many of the insect hemolymph proteins (Shigematsu, 1958; Laufer, 1960; Price, 1966; Price and Bosman, 1966; Rüegg, 1968), would also be the site of Hb synthesis.

The incorporation of both [^{3}H]δ-ALA and ^{14}C-amino acids into material secreted by fat body alone in culture for 24 h was examined. Double-label experiments were done to show whether both heme and globin are synthesized and then assembled by the fat body. Both isotopes are incorporated into all of the visible marker Hbs (Fig. 5). [^{3}H]δ-ALA is incorporated into material migrating with marker Hbs 1 and 3–9, with minimal label appearing in band 2. Bands 4 and 6 appear to have 2 and 3 labeled components, respectively, in both the ^{14}C- and ^{3}H-profiles. Amino acids are also incorporated into material which does not comigrate with either the visible Hb markers or tritium label. This material, primarily between Hb bands 1 and 2, and 3 and 4 (see Fig. 1), may be non-heme protein.

Since the amino acid compositions of *C. thummi* Hbs are not identical (Braun et al., 1968), and since the Hb was not purified before electrophoresis, the ratios of the [^{3}H]δ-ALA peaks to the ^{14}C-amino acid peaks are probably not indicative of a level of purity of the Hbs as a consequence of electrophoresis. However, very low ratios may be the result of contamination by non-heme proteins. In the figure, band 1 appears most heavily contaminated by non-heme proteins while bands 2, 3, and 5 may also contain comigrating, non-heme proteins.

The peak of [^{3}H]δ-ALA incorporation into band 5 Hb is represented by only one point while

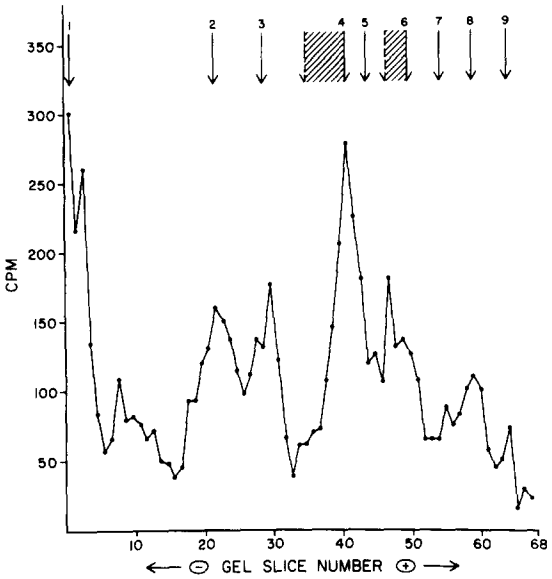

FIGURE 3 Electropherogram of labeled hemoglobins after in vitro incorporation of ^{3}H-amino acids by larvae dissected and cultured in toto. 30 larvae were dissected, and the head capsule and prolegs were removed. The body wall was cut lengthwise and the internal organs were displaced before culture in Cannon's medium containing ^{3}H-amino acids (0.5 mCi/ml). The medium was collected after a 24-h incubation, dialyzed, and electrophoresed as described. 2- μl of unlabeled fourth instar hemolymph diluted 1:1 with dialysis buffer was added to the sample as marker before electrophoresis. The positions of the marker hemoglobins are indicated by the arrows over the incorporation profile. The profile of incorporation into the stacking gel is not shown. Approximately 25% of the sample was electrophoresed.

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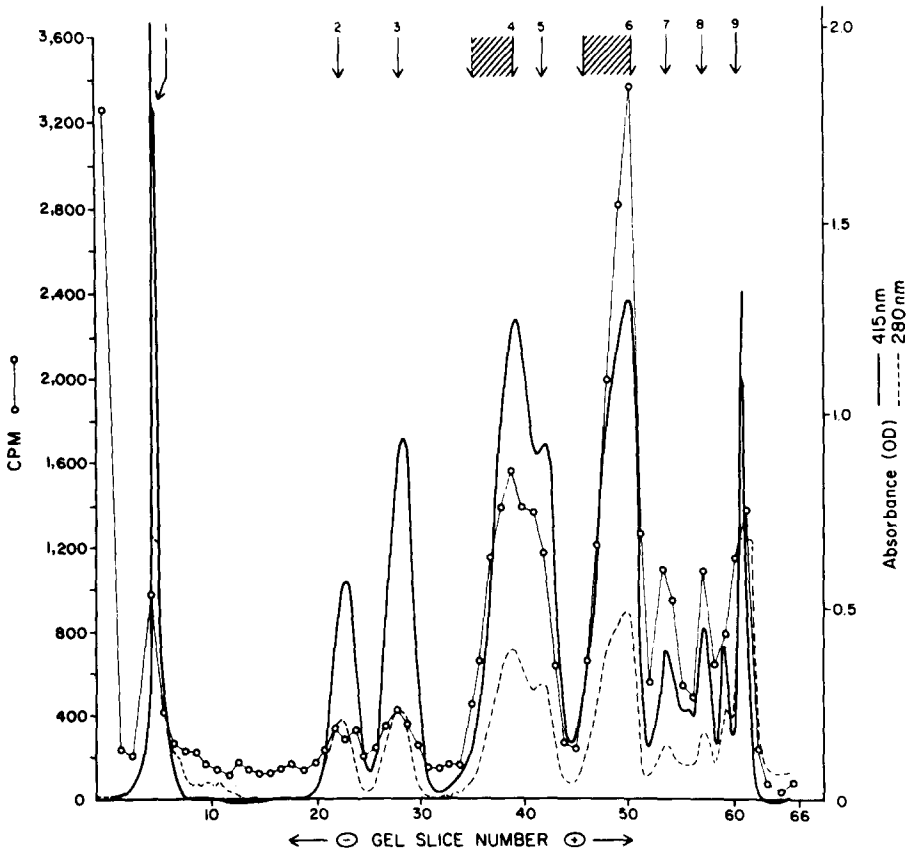

FIGURE 4 Electropherogram of labeled hemoglobins synthesized and secreted by body wall tissues in vitro in the absence of gut, malpighian tubules and salivary glands; incorporation of [^{3}H]δ-ALA. 12 larvae were dissected, the gut, malpighian tubules and salivary glands were removed, and the remaining body wall was cut lengthwise. The body walls were cultured for 24 h in the presence of [^{3}H]δ-ALA (1 mCi/ml). The medium was collected, dialyzed and electrophoresed with cold hemolymph hemoglobin markers as described. 33% of the sample was electrophoresed.

showing the most prominent ^{14}C-amino acid incorporation. To confirm that the Hb in band 5 was indeed being synthesized and secreted, Hbs in bands 4, 5, and 6 and the regions between these bands from a similar gel of a sample labeled with [^{3}H]δ-ALA alone were eluted overnight in 100 μl of dialysis buffer. The eluants were electrophoresed on gels identical to those used for the original sample. Band 5 Hb was the major labeled component on the electropherogram of the eluant from band 5. Some of band 5 Hb was also resolved in the eluant from the region between bands 4 and 5, but not in the other gels. Thus, [^{3}H]δ-ALA is incorporated into band 5 Hb. The failure of Fig. 5 to clearly show this is the result of "masking" of band 5 Hb by the leading and trailing edges, respectively, of Hb bands 4 and 6, which incorporate considerably more isotope.

Additional evidence that band 5 Hb is synthesized by the fat body is shown in the results of a similar double-label experiment using fat body (Fig. 6). Here, band 5 Hb is prominently labeled with [^{3}H]δ-ALA, and all of the Hbs show definitive evidence of being synthesized and secreted. Variation in the proportions of Hb synthesized in these experiments may be the result of injury to tissues during dissection.

Apparently, fat body in culture can synthesize, assemble, and secrete the same Hbs produced by intact larvae. To determine if Hb synthesis is

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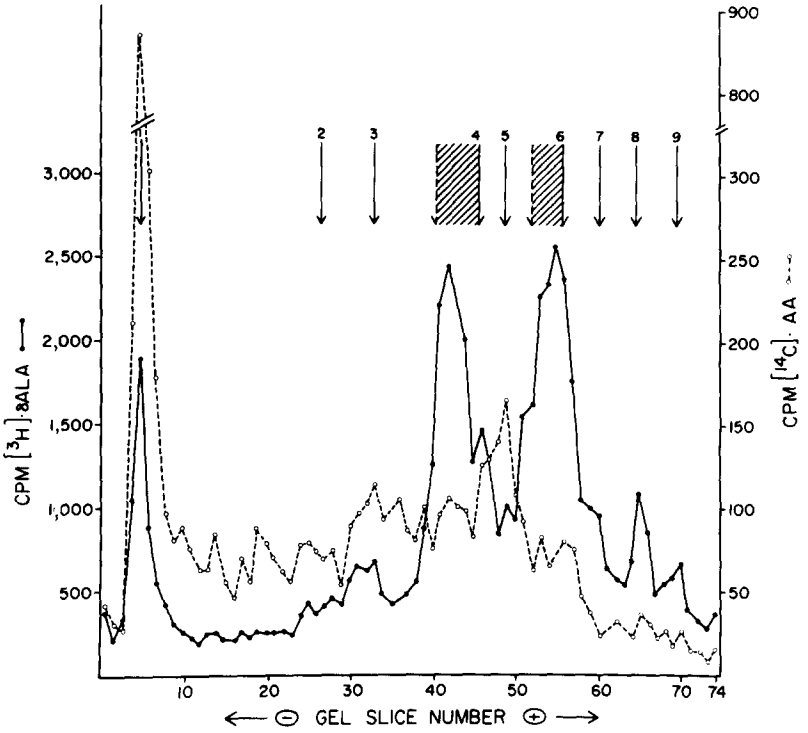

FIGURE 5 Incorporation of [^{3}H]δ-ALA and ^{14}C-amino acids into hemoglobins secreted by fat body in vitro for 24 h. Fat body from 12 fourth instar larvae were dissected and cultured in Cannon's medium containing [^{3}H]δ-ALA (1 mCi/ml) and ^{14}C-amino acids (0.5 mCi/ml). After the culture period, the medium was collected, dialyzed, and electrophoresed with cold hemolymph hemoglobin markers as described. 15% of the total sample was electrophoresed.

tissue-specific, organs other than the fat body were examined. Salivary gland and gut (including malpighian tubules) were cultured separately with either [^{3}H]δ-ALA or ^{3}H-amino acids for 24 h. Fig. 7 *a* reveals that some proteins incorporate ^{3}H-amino acids and are released into the culture medium by salivary glands. These proteins do not show [^{3}H]δ-ALA labeling (Fig. 7 *b*). Therefore, the salivary glands apparently do not synthesize and secrete hemoglobins. Gut tissue, on the other hand, seems to incorporate low levels of labeled δ-ALA and amino acids into several Hbs. Fig. 8 *a* shows significant ^{3}H-amino acid incorporation in the regions of Hb bands 3 and 5, while Fig. 8 *b* shows that Hb bands 1–5 are labeled with [^{3}H]δ-ALA. To estimate Hb contributed by both fat body and gut, experiments were done under conditions of identical time, isotope, and culture, comparing the incorporation of [^{3}H]δ-ALA into proteins secreted by either of these tissues from the same animals. The electrophoretic profiles from these experiments are similar to those from previous experiments in which the same tissues were used. The incorporation of isotope (cpm) into all materials secreted by the gut and displayed electrophoretically was 5.5% of the label secreted by both the gut and the fat bodies. In this calculation, no distinction was made regarding the specific incorporation of [^{3}H]δ-ALA into Hb. Thus, 5.5% represents the maximum amount of Hb synthesis, assembly, and secretion by the gut compared to the fat body on a per larva basis.

DISCUSSION

The results presented here confirm the Hb polymorphism in *C. thummi* described by Braunitzer et al. (1968). The only significant differences between our findings and those of Braunitzer and his co-workers are that bands 6 and 9 (their bands D7 and D10) are made up of at least two components, which can be resolved without the aid of isotope-

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labeling techniques (Table I). On the other hand, the incorporation profiles that we present indicate that bands 4 and 6 are each made up of 2–3 components. Therefore, our results indicate that there may be as many as 14 electrophoretically resolvable Hbs in *C. thummi*. According to Plagens (1971), only monomers exist for Hbs in the gel system used here. The number of monomeric globin polypeptides comprising each electrophoretic band is not known. N- and C-terminal analyses of Hbs from ion exchange columns show "microheterogeneities" which suggest that individual peaks may be made up of more than one Hb (Braunitzer et al., 1968). Thus, some of the Hb multiplicity revealed by labeling techniques may be due to the synthesis of several different globin chains. Alternatively, this result could be caused by posttranslational modifications of existing globin chains or by the partial hydrolysis of the globin polypeptides (which would also explain the terminal amino acid microheterogeneities).

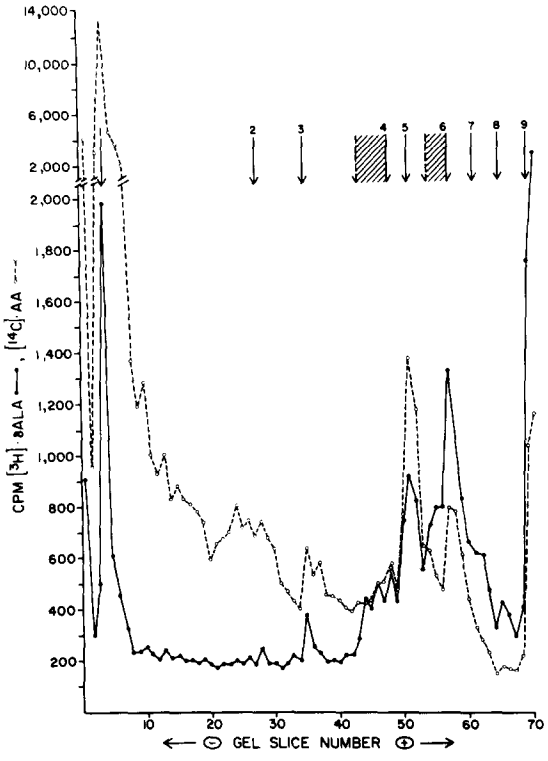

FIGURE 6 Incorporation of [^{3}H]δ-ALA and ^{14}C-amino acids into hemoglobins secreted by fat body in vitro for 24 h. Repeat of experiment in Fig. 4.

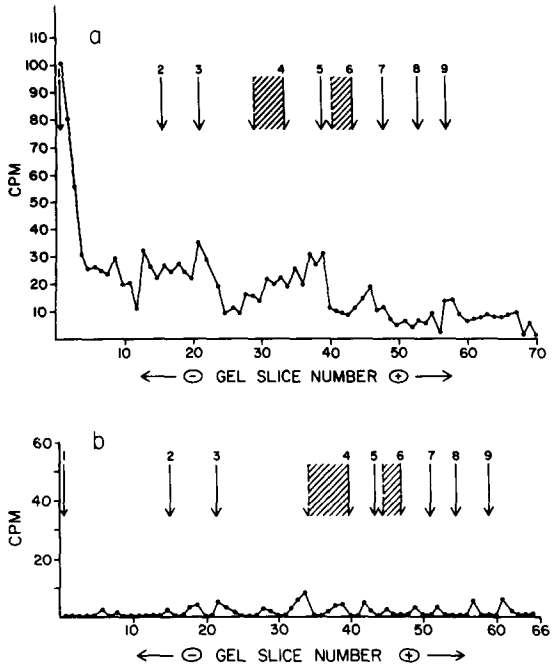

FIGURE 7 Incorporation of ^{3}H-amino acids and [^{3}H]δ-ALA into products secreted by salivary glands in vitro. (*a*) 24-h incubation in presence of ^{3}H-amino acids (1.5 mCi/ml). (*b*) 24-h incubation in presence of [^{3}H]δ-ALA (1.0 mCi/ml). 35–40 salivary glands were present in each culture. Culture and electrophoretic conditions are as described for fat body. The entire sample was electrophoresed in each case.

The results of isotope incorporation experiments with *C. thummi* indicate that fat body is the major site of Hb synthesis in the fourth instar. Fat body synthesizes both the heme and globin moieties of all nine of the electrophoretically distinct Hbs (and their components) present in this stage. This is the first demonstration of hemoglobin synthesis, assembly and secretion by an invertebrate organ in culture.

It appears that more isotope is present in certain of the Hbs than others at the end of the 24-h incubation period. These data may reflect differences in the net rates of the various Hbs synthesized by the species. It is also possible that some differences in total counts occur as a consequence of variation in the specific activities of the amino acids in the incubation mixture. Furthermore, the amino acid analyses of Braunitzer et al. (1968) show that in all of the globins of *C. thummi* analyzed, the ratios of the amino acids are not identical.

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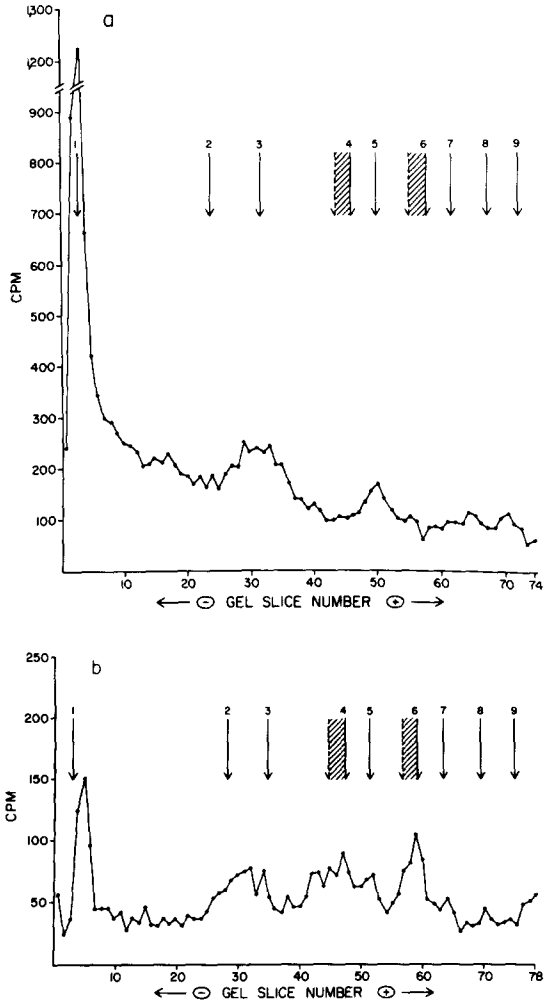

FIGURE 8 Incorporation of ^{3}H-amino acids and [^{3}H]δ-ALA into products secreted by entire gut (including malpighian tubules) in vitro. (*a*) 24-h incubation in the presence of ^{3}H-amino acids (1.5 mCi/ml). (*b*) 24-h incubation in the presence of [^{3}H]δ-ALA (1 mCi/ml). 10 guts were present in each culture. Culture and electrophoresis conditions are as described for fat body. The entire sample of [^{3}H]δ-ALA-labeled material was electrophoresed while 50% of the ^{3}H-amino acid-labeled material was electrophoresed.

The differences observed in the proportions of the Hbs synthesized by two cultures of fat bodies from the same stage animals but taken on different days and treated identically may be due to injury of the tissues during dissection. The fat body, in particular, is very fragile and may respond to injury by alterations in Hb synthesizing patterns.

The gut tissue (including the malpighian tubules) also synthesizes and secretes a small quantity of Hb (about 5% of the total secreted by both gut and fat body from the same animals in vitro). Since the fat body used in organ culture experiments does not include that found between the body wall musculature and the epidermis, the amount of Hb synthesized by fourth instar gut compared to Hb synthesized elsewhere in the insect may be considerably less than this figure. We have found that the body wall of *C. thummi*, in the absence of gut, malpighian tubules, salivary glands, and the fat body customarily used in our cultures is still capable of Hb synthesis and secretion. It is reasonable to assume that the source of this synthesis is the remaining fat body.

The cells of the fat body that are responsible for Hb synthesis are not known, although all fat body cells appear the same by phase-contrast microscopy. Cells originating from fat body in the first larval instar of the dipteran parasite *Gastrophilus* have been implicated in the synthesis of Hb (Dinulescu, 1932; Keilin and Wang, 1947). These "tracheal cells" which are permeated by tracheoles in *Gastrophilus*, may have their counterpart in the *Chironomus* fat body.

There are at least two possible explanations for the production of Hb by the gut. It may represent residual Hb synthesis from an earlier larval stage when the gut is a major site of Hb production. On the other hand, there may be a population of cells common to both gut and fat body, which are the actual sites of Hb synthesis. Such a cell type might be the hemocytes, which like the fat body are of mesodermal origin and adhere to organs in the body cavity of the larva (cf. Wigglesworth, 1965; Landureau and Grellet, 1975).

Manwell (1966), Wülker et al. (1966), Laufer and Poluhowich (1971), and others have demonstrated that the electrophoretic patterns of larval hemolymph Hbs change with development. These changes are temporally related to molting and metamorphosis and may therefore be under the regulatory control of molting hormones (e.g., ecdysones). We now have evidence that changes in Hb synthetic patterns are also correlated with molting (reported elsewhere). Ecydsone control of fat body synthesis of vitellogenin in the adults of *Aedes aegypti*, the yellow fever mosquito, has already been established (Fallon and Hagedorn, 1972; Hagedorn et al., 1973; Fallon et al., 1974; Hagedorn, 1974). Although the fat body of higher Diptera (e.g., *Drosophila*) undergoes histolysis at metamorphosis and is replaced by a new organ in

the adult, this may not be the case in lower dipterans such as *Chironomus* and *Aedes* (Wigglesworth, 1949; Trager, 1937). If larval and adult *Chironomus* fat body are one and the same organ with a homogeneous cell population, it must function to secrete two major protein products, Hbs and vitellogenins, each at a different stage in the life cycle of the insect. Since a major secretory function is usually taken as a sign of terminal cell differentiation, the prospect of two "terminally differentiated states" of one tissue is an especially intriguing one.

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REFERENCES

AMICONI, G., E. ANTONINI, M. BRUNORI, H. FORMANECK, and R. HUBER. 1972. Functional properties of native and reconstituted hemoglobins from *Chironomus thummi thummi*. *Eur. J. Biochem.* **31**:52–58.

BRUAN, V., R. R. CRICHTON, and G. BRAUNITZER. 1968. Über monomere und dimere Insektenhämoglobine aus *Chironomus thummi*. *Hoppe-Seyler's Z. Physiol. Chem.* **349**:197–210.

BRAUNITZER, G., H. GLOSSMAN, and J. HORST. 1968. Zur Frage der nativen Hämoglobine der Larven von *Chironomus thummi thummi*. *Hoppe-Seyler's Z. Physiol. Chem.* **349**:1789–1791.

DAVIS, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**: 404–427.

DINULESCU, G. 1932. Recherches sur la biologie des gastrophiles: anatomie, physiologie, cycle evolutif. *Ann. Sci. Nat. Zool.* **15**:1–183.

ENGLISH, D. S. 1969. Ontogenetic changes in hemoglobin synthesis of two strains of *Chironomus tentans*. *J. Embryol. Exp. Morphol.* **22**:465–476.

FALLON, A. M., and H. H. HAGEDORN. 1972. Synthesis of vitellogenin by the fat body in *Aedes aegypti:* the effect of injected ecdysone. *Am. Zool.* **12**:697a. (Abstr.).

FALLON, A. M., H. H. HAGEDORN, G. R. WYATT, and H. LAUFER. 1974. Activation of vitellogenin synthesis in the mosquito *Aedes aegypti* by ecdysone. *J. Insect Physiol.* **20**:1815–1823.

HAGEDORN, H. H. 1974. The control of vitellogenesis in the mosquito *Aedes aegypti*. *Am. Zool.* **14**:1207–1217.

HAGEDORN, H. H., A. M. FALLON, and H. LAUFER. 1973. Vitellogenin synthesis by the fat body of *Aedes aegypti*. I. Evidence for transcriptional control. *Dev. Biol.* **31**:285–294.

KEILIN, D., and Y. L. WANG. 1947. Hemoglobin of Gastrophilus larvae. Purification and properties. *Biochem. J.* **40**:855–866.

LANDUREAU, J. C., and P. GRELLET. 1975. Obtention de lignées permanentes d'hemocytes de blatte. Caractéristiques physiologiques et ultrastructurales. *J. Insect Physiol.* **21**:137–151.

LAUFER, H. 1960. Blood proteins in insect development. *Ann. N. Y. Acad. Sci.* **89**:490–515.

LAUFER, H., and J. POLUHOWICH. 1971. A factor controlling the concentration of hemoglobin in *Chironomus* during metamorphosis. *Limnologica.* **8**:125–126.

LAUFER, H., and M. WILSON. 1970. Hormonal control of gene activity as revealed by puffing of salivary gland chromosomes in dipteran larvae, in laboratory experiments. *In* General and Comparative Endocrinology. R. E. Peter and A. Gorbman, editors. Prentice-Hall Inc., Englewood Cliffs. N. J. 135–200.

MAIZEL, J. V., JR. 1969. Acrylamide gel electrophoresis of proteins and nucleic acids. *In* Fundamental Techniques in Virology. K. Habel and N. P. Salzman editors. Academic Press, Inc., New York. 334–362.

MANWELL, C. 1966. Starch gel electrophoresis of the multiple haemoglobins of small and large larval *Chironomus*—a developmental haemoglobin sequence in an invertebrate. *J. Embryol. Exp. Morphol.* **16**:259–270.

ORNSTEIN, L. 1964. Disc electrophoresis. I. Background and theory. *Ann. N. Y. Acad. Sci.* **121**:321–349.

PLAGENS, U. 1971. Vergleichende Untersuchung der Hämoglobine verschiedener Chironomiden. Ph.D. Dissertation, Ludwig-Maximilians Univ., Munich.

PRICE, G. M. 1966. The *in vitro* incorporation of (U-^{14}C) valine into fat body protein of the larva of the blowfly, *Calliphora erythrocephala*. *J. Insect Physiol.* **12**:731–740.

PRICE, G. M., and T. BOSMAN. 1966. The electrophoretic separation of proteins isolated from the larva of the blowfly, *Calliphora erythrocephala*. *J. Insect Physiol.* **12**:741–745.

RINGBORG, U., and L. RYDLANDER. 1971. Nucleolar-derived ribonucleic acid in chromosomes, nuclear sap and cytoplasm of *Chironomus tentans* salivary gland cells. *J. Cell Biol.* **51**: 355–368.

RÜEGG, M. K. 1968. Untersuchungen zum Proteinstoffwechsel des Wildtyps und der Letalmutante (ltr) von *Drosophila melanogaster*. *Z. vgl. Physiol.* **60**:275–307.

SCHIN, K. S., J. POLUHOWICH, T. GAMO, and H.

LAUFER. 1974. Degradation of hemoglobin in *Chironomus* during metamorphosis. *J. Insect Physiol.* **20**:561–571.

SHIGEMATSU, H. 1958. Synthesis of blood protein by the fat body in the silkworm *Bombyx mori* L. *Nature* (*Lond.*). **182**:880–882.

SVEDBERG, T., and I.-B. ERIKSSON-QUENSEL. 1934. The molecular weight of erythrocruorin. *J. Am. Chem. Soc.* **56**:1700–1706.

THOMPSON, P. E., W. BLEECKER, and D. S. ENGLISH. 1968. Molecular size and subunit structure of the hemoglobins of *Chironomus tentans*. *J. Biol. Chem.* **243**:463–467.

THOMPSON, P. E., and D. S. ENGLISH. 1966. Multiplicity of hemoglobins in the genus *Chironomus* (Tendipes). *Science* (*Wash. D. C.*). **152**:75–76

TICHY, H. 1966. A multisample electrophoresis apparatus using vertical polyacrylamide gel slabs. *Anal. Biochem.* **17**:320–326.

TICHY, H. 1968. Hemoglobins of *Chironomus tentans* and *pallidivittatus*. Biochemical and cytological studies. 4 Wiss. Konf. Ges. Dtscher Naturforsch. u. Arzte, Springer-Verlag, Berlin 248–252.

TICHY, H. 1970. Biochemische und cytogenetische Untersuchungen zur Natur des Hämoglobin-Polymorphismus bei *Chironomus tentans* und *Chironomus pallidivittatus*. *Chromosoma* (*Berl.*). **29**:131–138.

TRAGER, W. 1937. Cell size in relation to the growth and metamorphosis of the mosquito, *Aedes aegypti*. *J. Exp. Zool.* **76**:467–491.

WIGGLESWORTH, V. B. 1949. The utilization of reserve substances in *Drosophila* during flight. *J. Exp. Biol.* **26**:150–163.

WIGGLESWORTH, V. B. 1965. The Principles of Insect Physiology. Methuen & Co. Ltd., London.

WÜLKER, W., W. MAIER, and P. BERTAU. 1969. Untersuchungen über die Hämolymphproteine der Chironomiden (Dipt.) *Z. Naturforsch. Teil B. Anorg. Chem. Org. Chem. Biochem. Biophys. Biol.* **24b**:110–116.