Determination of the Molecular Weight of Proteins, Esterases, and Basic Phospho-Monoesterases in the Hemolymph of *Galleria mellonella* L. Prepupa and Pupa Using a Thin-Layer Chromatography

The study of the molecular weight of insect proteins by means of thin-layer gel chromatography follows very closely similar studies in human serum[1-3]. LOUGHTON[4] reports that the first determination of molecular weight of insect hemolymph proteins by means of ultracentrifuga was carried out by SVEDBERG and ERIKSON-QUENSEL[5], who calculated the molecular weight 31,400 for the hemoglobin of *Chironomus*. GLASER and STANLEY[6] and LAUFFER[7] estimated, also by means of ultracentrifuga, the high-molecular protein with sedimentation constant 17S in the hemolymph of *Bombyx mori*. BUCK[8] quoted the unpublished results of OUCLEY, LEVENBOOK and WILLIAMS 1951 who distinguished 3 proteins in the hemolymph of *Hyalophora cecropia*. The highest molecular weight of one of these proteins was approximately 450,000. The most extensive study of the hemolymph proteins in *B. mori* was carried out by ODA[9]. Using ultracentrifuga they determined 3 proteins in the hemolymph. The molecular weight of the protein with the fastest sedimentation was 630,000 and that of the protein with the slowest sedimentation was 160,000. LOUGHTON[4] using ultracentrifugation of the hemolymph of the sixth larval instar of *Malacosoma americanum*, previously separated in DEAE-cellulose column, determined 6 proteins with different sedimentation constants by ultracentrifugation of the individual fractions. In his recent study, AGRELL[10] using thin layer of acrylamide determined the molecular weights of basic proteins in the homogenate of head, thorax and abdomen of *Calliphora erythrocephala* ranging from 9,000–100,000.

Materials and methods. The thin-layer chromatography proved to be a fairly accurate method of estimating the molecular weight of proteins[11]. Sephadex G-200, Superfine, allowed to swell in water for 72 h and then washed in 0.05 *M* veronal buffer, pH = 8.4, for additional 8 h[12], was used for the experiment. A 200 × 200 mm glass plate was used as a base for the thin-layer coating. Gel layer in thickness of 0.5 mm was spread with a 10–15° slope of the plate. Whatman 3[13] was used as a wick for connecting the thin layer of gel with the buffer reservoir. Before spreading the sample on the thin layer, veronal buffer was allowed to flow through Sephadex G-200 for 120 min. The following substances were used as reference samples: cytochrom C, mol. wt. 13,000, product of Reanal, Hungary; hemoglobin, mol. wt. 68,000, product of Reanal, Hungary; γ-globulin (2× recrystallized), mol. wt. 180,000, Calbiochem, USA; thyreoglobulin (2× recrystallized), mol. wt. 680,000, Koch-Light, Great Britain. 5 µl-aliquots of the above reference samples of 3% protein solutions were transferred with a pipette on the layer of Sephadex G-200.

The hemolymph was obtained by cutting the leg of prepupa (1–2 days old), and by piercing the wing region of the lateral side of pupa (1–7 days old), previously centrifuged (1000 r.p.m.) in a water medium. Phenylthiourea was used as antimelanizant. The hemolymph was centrifuged at 20,000 r.p.m. and the supernatant was diluted or condensed to 3% protein solution in hemolymph.

After finishing the gel filtration (3 h), the surface of the thin layer of Sephadex was covered with Whatman 3 filter paper for the time of 20 min, then the filter paper was taken off and stained in amido black 10B (120 min) for identification of protein. The excess of stain was removed by 2% acetic acid.

2-Naphthyl acetate or 1-naphthyl butyrate (produced by Prof. HUNTER) were used as a substrate for the identification of esterase with diazotation agent Fast Blue BB salt[14,15].

2-Naphthyl sodium phosphate was used as a substrate for the identification of basic phospho-monoesterase with diazotation agent Fast Red TR salt[16].

The calibration curve was constructed on the basis of the distances of the spots of reference protein samples from the starting point, determined in millimetres, and the known values of their molecular weights. The corresponding values of the molecular weights of the filtrated proteins and enzymes were subtracted from this curve.

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Table I. Estimation of the molecular weight of proteins, esterases and basic phosphomonoesterase in hemolymph of prepupa and pupa *G. mellonella* L.

Prepupa of *G. mellonella*						Pupa of *G. mellonella*					
Molecular weights in hemolymph						Molecular weights in hemolymph					
Proteins	%	Esterases	%	Basic phospho-monoesterases	%	Proteins	%	Esterases	%	Basic phospho-monoesterases	%
12,700	23.9	40,000	90	–	–	12,600	30	65,000	93	–	–
120,000	5.0	–	–	–	–	170,000	17.5	–	–	–	–
475,000	71.1	530,000	10	475,000	100	490,000	52.5	570,000	7	550,000	100

Results. The results are summarized in Table I and Table II.

Table II. Estimation of the molecular weight of proteins in the fat body and midgut of prepupa *G. mellonella* L.

Prepupa of *G. mellonella*			
Molecular weight of proteins			
Midgut	Fat body	Midgut	Fat body
70,000	110,000	520,000	475,000

Zusammenfassung. Mittels dünnschichtiger Gel-Filtration Sephadex G-200 (Superfine) verteilten sich die Hämolymphproteine von Vorpuppen und Puppen der *Galleria mellonella* in 2 Hauptfraktionen. Für die erste Fraktion wurde das Molekulargewicht um 13000, für die zweite Fraktion um 500000 (im Mittel 75% des Gesamteiweisses der Hämolymphe) bestimmt.

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Heterogeneity and Globin Composition of Adult Chicken Hemoglobin

The heterogeneity of hemoglobin (Hb) in an individual of a certain animal species is likely to be a general rule, though the molecular basis and physiological significance of this finding are not yet clearly understood.

We have been interested in the study of hemoglobin composition in amphibia[1], fishes[2], and chickens[3], during both embryonic development and adult life.

The present study reports our findings on the heterogeneity and globin composition of the hemoglobin of adult chickens of both sexes and of various breeds (White Leghorn, Arbor Acres, etc.).

The number and composition of chicken Hb have been the subject of many investigations[4–9]; the discrepancies emerging from different results might be due to the various techniques used, though a strain-dependent heterogeneity cannot be ruled out completely.

Materials and method. Blood samples were drawn by heart- or veni-puncture in heparinized syringes. Red blood cells were then treated as previously reported for human red blood cells[10]. Clear Hb solutions were transformed in the cyano-derivative[11] for prolonged storage and for all the chromatographic and electrophoretic separations. Electrophoresis of Hb was carried out in starch gel at pH 8.6 as previously described[2], or in cellulose acetate at pH 8.5 using the buffer system suggested by HUEHNS[12]. Separations were performed at 5 °C with a current of 400 Vs for 1–2 h. The fractions were stained with Amido Black 10 B, or benzidine, or Ponceau Red (only for cellulose acetate). Chromatography of hemoglobin was carried out by Amberlite CG-50 type II. The original procedure worked out by ALLEN et al.[11] for human hemoglobin has been of great help. A buffer consisting of Na_2HPO_4 (molarity of $Na^+ = 0.092$ + KCN $0.005M$, brought with $5M$ phosphoric acid to pH 7.45) was found to be satisfactory. The Amberlite columns (1×35 cm for analytical, and 2×45 cm for preparative work) were operated at 5 °C, at a flow-rate of 2 ml/h; the elution of Hb types was completed by heating the columns at 28 °C and raising the flow-rate to 3.5–4 ml/h.

Globin chains composition and properties were investigated through starch gel electrophoresis in $6M$ urea, with and without $0.1M$ mercaptoethanol. The following buffer systems were used for preparing the gels: (1) Glycil-glycine ($0.01M$) brought with $5M$ NaOH to pH 9.3; (2) *Tris*-EDTA-borate buffer[12], pH 8.55; (3) *Tris*-EDTA-borate ($0.0187M$ *Tris*, $0.047M$ boric acid, $0.001M$ EDTA), pH 7.3; (4) phosphate buffer (KH_2PO_4 $0.006M$), brought with $1M$ KOH to pH 6.2; (5) acetate buffer (Na acetate $0.005M$), brought with $5M$ acetic acid to pH 5.4; (6) acetate buffer (Na acetate $0.005M$), brought with $5M$ acetic acid to pH 4.7; (7) citric acid-*Tris* (citric acid $0.004M$ + *Tris* $0.009M$), brought with $5M$ KOH to pH 3.5; (8) citric acid-*Tris* (citric acid $0.004M$ + *Tris* $0.009M$), brought with $5M$ KOH to pH 2.8; (9) formic acid buffer, according to MULLER[9], pH 1.8. This buffer was also used without urea. The buffer reservoirs had the same pH and salt composition, but with a buffer concentration about 8 times higher than the corresponding gels; urea and mercaptoethanol were likewise omitted.

Results and discussion. The electrophoresis of chicken hemoglobin, in agreement with other authors[8,9], showed 2 fractions; we have called Hb 1 the minor more anodic component (25–30% of the total Hb), and Hb 2 the major component (70–75% of the total Hb).

The chromatography on Amberlite columns separated 3 hemoglobin peaks (Figure 1). The first peak is eluted together with some non-heme proteins in the break-through-volume (as shown by the ratio O.D. 541/280), and represents about the 9% of total Hb. The second and the third peak are eluted when the column is heated at 28 °C, and represent respectively the 20 and the 70% of the total Hb. When submitted to starch gel electro-

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