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Immunological Studies of Duck Myoglobin* (33032)

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Myoglobin, the oxygen binding, heme protein of muscle, is widely found throughout the animal kingdom from molluscs (1) to man. Its concentration in muscle tissue of man is related to age, or maturity (2,3). It is present in both cardiac and skeletal muscle, but is deficient in smooth muscle (4,5). In some birds, certain skeletal muscles (e.g., the pectoral muscles of the chicken) may be pale in color, and deficient in myoglobin, while other muscles of the same bird may contain myoglobin. This report describes the production of a specific antiserum for an avian (duck) myoglobin, and its use in measuring age-related variations in muscle myoglobin content. Relationships between several bird myoglobins, and some physicochemical and immunologic characteristics of duck myoglobin are presented.

Materials and Methods. Myoglobin, concentrated from duck skeletal muscle by ammonium sulfate precipitation and gel filtration as previously described for that of man (3,6), was used as starting material for further chromatographic separation. Immunological precipitin reactions, and measurement of antigen content by radial diffusion in gels containing solutions of specific antibody have been described (3). Supernatant fluids obtained after centrifugation, at approximately 1500*g* for 30 min, of 30%, 0.15 *M* saline suspensions of muscle homogenates, were used as crude tissue extracts.

Results. 1. *Preparation of purified duck myoglobin by ion-exchange chromatography, for use as antigen.* Concentrated myoglobin, prepared from skeletal muscle of an adult muscovy drake by ammonium sulfate precipitation, was chromatographed on columns of DEAE-cellulose, at pH 8.0, with a variable ionic gradient (Fig. 1). Three components were resolved from the preparation. B, which eluted at low ionic strength, represented most of the heme protein content. The ratio of optical densities at 415 mμ and 280 mμ, for this component, was greater than 4. Two other heme-containing components were also

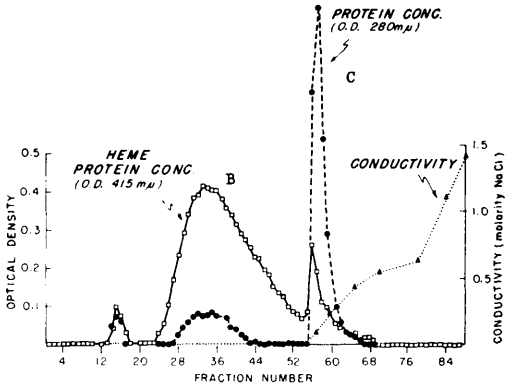

FIG. 1. DEAE-cellulose chromatographic elution diagram of partially purified duck myoglobin. Three heme protein containing components (A,B,C) are resolved. Starting buffer: 0.005 *M* phosphate, pH 7.5; final buffer: 0.005 *M* phosphate + 1 *M* NaCl, pH 7.5. Ionic gradient applied from a three-chambered Varigrad device. Percentage final buffer in each chamber: (1) 0, (2) 50, and (3) 33.3. Column 33 × 3 cm (18 gm of DEAE-cellulose, 0.89 meq/gm).

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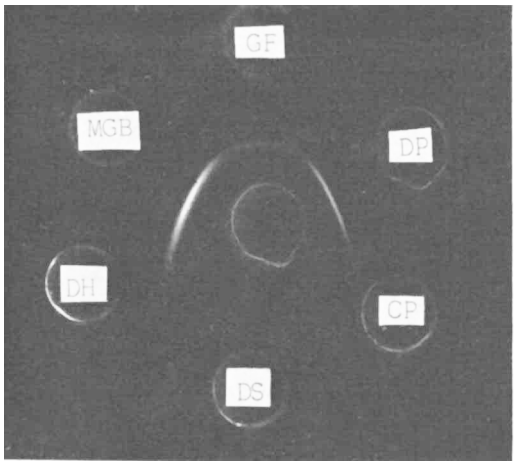

FIG. 2. Precipitin pattern of antimyoglobin serum in agar gel. Center well: undiluted antiserum; MGB, purified duck myoglobin, 0.08 mg/ml; GF, goose femorotibial muscle extract; DP, duck pectoral muscle extract; CP, chicken pectoral muscle extract; DS, undiluted duck serum; DH, duck hemoglobin.

present: A, contained a minor amount of protein; and C, which eluted when the ionic strength rose sharply and had a low 415/280 ratio suggesting the presence of nonheme compounds in this portion of the column's eluate.

Myoglobin, the heme protein of component B, was concentrated by lyophilization to contain 2 mg/ml, and subjected to analytic ultracentrifugation by the sedimentation velocity method (7). A single, slowly sedimenting component was observed, whose sedimentation constant was 1.75 S_{20w}.

2. *Preparation and properties of antimyoglobin serum.* Three of 4 rabbits after injection with a total of 0.5 mg of the purified duck myoglobin, emulsified in complete Freund's adjuvants (Difco Labs., Detroit, Mich.), developed precipitating antisera. Specificity was demonstrated in agar gel, double diffusion experiments (Fig. 2). A single precipitin line formed when the antiserum diffused against the purified duck myoglobin, as well as against a crude extract of duck pectoral muscle. These lines coalesced, at their junction, suggesting identity between myoglobin and the muscle antigen. There were no precipitation reactions between the antiserum and duck serum, hemoglobin preparations made from hemolyzates, or liver extract (not shown).

3. *Cross reactions of the antiserum.* The antiserum formed identical precipitin lines in agar gels with myoglobins of duck cardiac and skeletal muscle, goose cardiac and skeletal muscle, and chicken cardiac and thigh skeletal muscle. No precipitin reaction was detectable with chicken pectoral muscle (Fig. 2). However, addition of chicken pectoral muscle extracts to the antiserum did not inhibit its ability to precipitate duck myoglobin. The antiserum did not react with purified human myoglobin.

4. *Precipitin reaction.* Figure 3 demonstrates the results of addition of several concentrations of purified duck myoglobin to a standard amount of specific antiserum. Precipitates, which formed after incubation overnight at 4°C, were washed 3 times in 0.15 *M* saline, and analyzed for total protein content. Precipitation was maximal at 0.03 mg of myoglobin, and there was detectable inhibition of precipitation by 0.05 mg of the antigen. The graph of the ratio of antibody to antigen, at points of antibody excess, and at equivalence (8,9) is also shown. At infinitely low concentrations of myoglobin, the ordinate intercept, there was approximately 25 times as much antibody protein as antigen protein in the precipitate. Assuming a molecular weight of 18,000 for myoglobin and 150,000 for the average rabbit antibody molecule, there were 3.1 or approximately 3 molecules

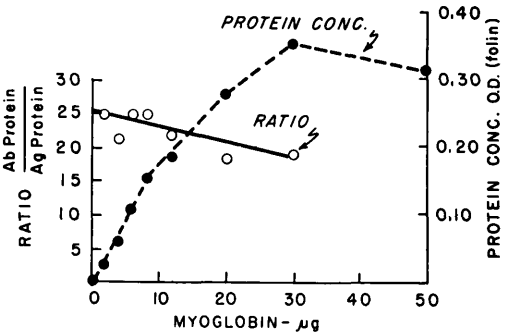

FIG. 3. Precipitin reaction of purified duck myoglobin and the specific antiserum. Varied amounts of myoglobin added to 0.1 ml of antiserum. Final total volume, 0.25 ml; buffer, 0.15 *M* NaCl; incubation, 30 min, 37°C, then 18 hours, 4°C.

TABLE I. Myoglobin Content of Avian Muscle (mg of myoglobin/gm of wet wt. of muscle tissue).

Muscle	Age		
	Adult	8 days	2 days
Duck (muscovy)			
Cardiac	4.6	4.2	2.1
Skeletal			
Femorotibial	3.5	0.9	0.7
Pectoral	2.2	0.2	0.3
Chicken (white leghorn)			
Cardiac	3.2		
Skeletal			
Femorotibial	3.0		
Pectoral	0.0		

of rabbit antibody associated with each molecule of duck myoglobin.

5. *Myoglobin content of avian muscle.* Since precipitating antibody was directed only against myoglobin, it was possible to measure the myoglobin content of crude saline muscle extracts by means of simple diffusion in agar gels premixed with a standard amount of specific antiserum. Duck skeletal muscle (Table I) contained approximately 2–4 mg of myoglobin/gm of wet weight. There were greater concentrations in cardiac and leg muscles than in pectoral muscle. The skeletal muscle of newly hatched ducklings was strikingly deficient in myoglobin, containing only 10–25% of the adult content. Cardiac muscle of ducklings, however, contained considerably more myoglobin than skeletal muscle. Almost one half the expected adult myoglobin content was present in the heart at 2 days after hatching. The content of myoglobin in adult chicken cardiac and femorotibial muscle was similar to that of the corresponding duck muscle tissue. However, no myoglobin was detected by the antiserum in chicken pectoral muscle.

Discussion. Ammonium sulfate precipitation and ion-exchange chromatography were used to purify duck myoglobin. Its sedimentation constant, 1.75 S_{20w}, represents an approximation since only one concentration of myoglobin was used for analysis. However, this value is sufficiently near to those reported for myoglobins of the horse, 2.08, and the sperm whale, 1.92, to imply a similarity of molecular size (10).

The specific antimyoglobin serum recognized no differences between cardiac or skeletal muscle myoglobins of the duck, goose, or chicken. This suggests a close similarity of structure between myoglobins of two genetic orders, *Anseriformes* (duck and goose) and *Galliformes* (chicken). The failure of human myoglobin to react with the antiserum indicates differences in molecular structure between it and the avian myoglobins tested.

Myoglobin was not detected immunologically in chicken pectoral muscle, however, there is spectrophotometric evidence of its presence there (11). Since there were no inhibitors to the antibody reaction in this tissue, it is assumed that the compounds assayed spectrophotometrically by Ashmore and co-workers are antigenically distinct from native myoglobin.

The striking increase in myoglobin content of skeletal muscle with maturity represents a phenomenon for which responsible factors are essentially unknown. Cardiac muscle contained more myoglobin after hatching and reached adult levels earlier in development than did the other muscle samples tested.

Summary. Purified duck myoglobin was prepared by ammonium sulfate precipitation and ion-exchange chromatography. Its sedimentation constant was estimated to be approximately 1.75. A specific precipitating antiserum to duck myoglobin was prepared in rabbits. This antiserum did not form precipitates with duck plasma, hemoglobin, liver extract or with components of crude muscle extracts other than myoglobin. No immunologic differences were detectable between duck cardiac or skeletal muscle myoglobin, and none were noted between myoglobins of goose, duck, or chicken present in muscle extracts. Chicken pectoral muscle lacked myoglobin. Precipitin data suggest that three regions on the duck myoglobin molecule act as combining sites or determinants for the rabbit serum. The myoglobin content of several avian muscles was measured immunologically. Skeletal muscle of newly hatched ducklings was deficient in myoglobin content,

while cardiac muscle of these immature birds contained almost one-half the adult amount 2 days after hatching, and reached nearly adult levels at 8 days after hatching.

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Antigenicity of Mycobacteriophages R1, D29, and Leo in Rabbits* (33033)

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Among the reports in the literature on mycobacteriophages, there are no systematic studies on the antigenicity of these phages. There are a few reports such as those of Bowman (1), Takeya *et al.* (2), and Mankiewicz (3, 4) which indicate that mycobacteriophages are relatively poor antigens as compared to the antigenicity of other bacteriophages (5). The data in the present paper on the serology of three mycobacteriophages support the concept that mycobacteriophages are poor antigens.

Materials and Methods. Media. Nutrient broth was used for growing the bacteria. Bottom-agar and soft-agar were the same as reported previously (6) and these were used in standard phage assay techniques.

Mycobacteriophages.[2] Lysates of mycobacteria infected with mycobacteriophages R1, D29, or Leo were prepared either on plates or in broth by the techniques of Bowman (6). The bacteria and bacterial debris were centrifuged out of the lysates at 5000*g* for 1 hour. The phages were then pelleted by high speed (25,000*g* for 6 hours or 100,000*g* for 30 min) centrifugation and resuspended in fresh broth. For further purification these suspensions were usually subjected to two or three cycles of high and low speed centrifugations. Such purified phage, suspended in broth, were used as the antigenic stimuli.

Bacteria.[2] *Mycobacterium tuberculosis.* ATCC No. 607 (henceforth referred to as *M.* 607) was used for growing and assaying the phages. *M. butyricum* (R1) was used as the initial source of mycobacteriophage R1.

Production of antiphage antibodies in rabbits. Each of the mycobacteriophages was injected, at weekly intervals, into rabbits for 12 weeks. Two rabbits each were injected with 1.0 or 0.5 ml of each phage by the following three routes: intravenous, subcutaneous in broth, and subcutaneous with incomplete Freund's adjuvant (7). The concentrations of phage used were between 10^{10} and 10^{11} plaque-forming units/ml (pfu). Blood samples were taken from each rabbit

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