"Myoglobinolytic" Activity of Human Skeletal Muscle and Other Tissues* (33688)

LAWRENCE J. KAGEN AND RAYA GUREVICH
(Introduced by C. L. Christian)

Department of Medicine, Columbia University College of Physicians and Surgeons, and the Edward Daniels Faulkner Arthritis Clinic of the Presbyterian Hospital, New York, New York 10032

Skeletal muscle extracts of several species possess proteolytic, catheptic activity (1–3), as well as the ability to break down myoglobin into trichloroacetic acid-soluble components (4). We wish to present further evidence for a "myoglobinolytic" activity of human skeletal muscle extracts, as well as for other human tissue extracts, and to indicate that the myoglobin substrate for this reaction is altered from native myoglobin, and that the products produced no longer precipitate with an antiserum specific for myoglobin. An attempt was made to purify the active component of skeletal muscle.

Materials and Methods. 1. Human myoglobin and specific rabbit antimyoglobin serum were prepared, as described (5). The concentration of myoglobin in crude muscle extracts was measured by the method of radial diffusion into agar premixed with a standard amount of antimyoglobin serum (5, 6). The pH of each sample was corrected to 7.7 (by the addition of a few drops of 0.1 *N* HCl or NaOH) after incubation, before antibody assay.

2. Acrylamide gel electrophoresis (Canal Indust. Corp., Bethesda, Md.) was performed in 7% gel. Stacking gel was at pH 8.9, separating gel at pH 9.5.

3. DEAE-cellulose ion-exchange chromatography with an ionic strength gradient, and analytical ultracentrifugation were performed as described (5).

4. Supernatant fluids obtained after centrifugation of 30% homogenate suspensions were used as crude tissue extracts. Human pectoral muscle from surgical specimens was used as the source of skeletal muscle.

* Supported in part by grants from the USPHS (AM-11659-01) and the John A. Hartford Foundation, New York.

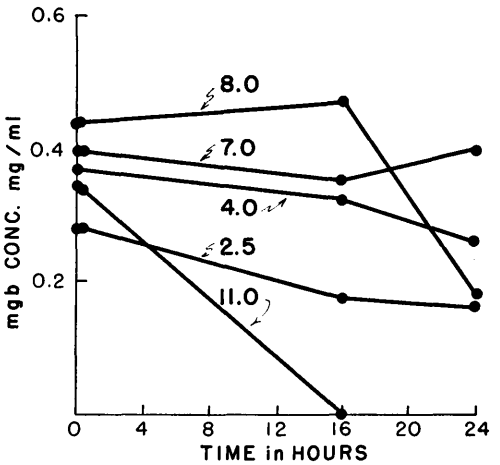

FIG. 1. Concentration of purified human myoglobin immunologically detectable after incubation at several pH values. Buffer: barbital–acetate, incubation: 37°, pH: indicated by arrows.

5. Incubations were carried out in barbital, sodium acetate buffer (7).

Results. 1. Loss of antigenic activity of myoglobin after incubation in acid muscle extracts. The antigenic content of known concentrations of purified human myoglobin, incubated at 37°, was fairly stable over a wide pH range (Fig. 1). As shown, myoglobin's antigenic content was lost after incubation for 16 hr at pH 11.0, and diminished at pH 2.5. Little loss of content occurred at pH 4.

In the presence of crude human skeletal muscle extracts, however (Fig. 2), myoglobin's antigenic content completely disappeared after 1 hr at pH 3.4 and 4.0, and 50% of the immunologically detectable myoglobin was lost at pH 5.0. Little or no loss occurred on incubation at pH 7.4.

2. Presence of "myoglobinolytic" activity in other human tissues and fluids. Purified human myoglobin was incubated with a variety of extracts of human tissues and fluids at

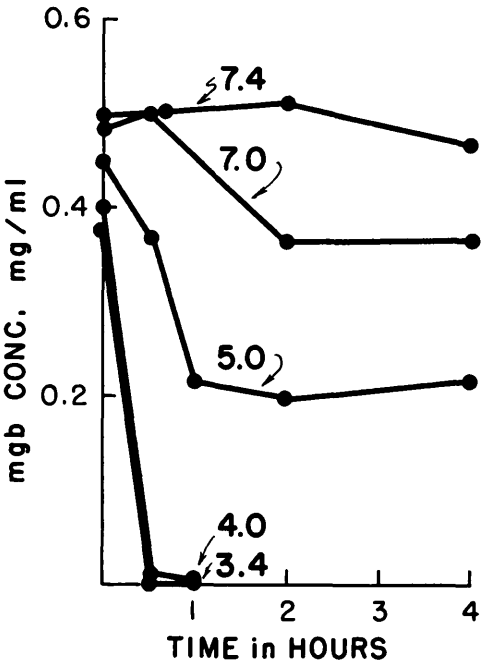

FIG. 2. Concentration of myoglobin immunologically detectable in human skeletal muscle extracts after incubation at several pH values. Buffer: barbital–acetate, incubation: 37°, pH: indicated by arrows.

pH 3.4, at 37°, for 2 hr. Extracts of skeletal muscle, cardiac muscle, uterine smooth muscle, liver, spleen, and unconcentrated urine caused the loss of antigenic activity of myoglobin at acid pH. Unconcentrated serum had no effect however.

3. Character of the active portion of skeletal muscle extract. An attempt was made to purify and study the properties of the portion of skeletal muscle extract which, on incubation, caused the loss of immunologically-detectable myoglobin. The optimum pH for activity was in the acid range between 3 and 4; the activity was present in the soluble tissue fraction (i.e., present in supernatant fluid after centrifugation at 30,000*g* for 1 hr), was nondialyzable, stable to heating at 56° for 1 hr, and precipitable by ammonium sulfate (Table I).

The activity of the muscle extract fractions was determined by their incubation with standard solutions of purified human myoglobin, at 37°, pH 3.4. The percentage loss of myoglobin content, compared to the zero time incubation sample was used as a measure of "myoglobinolytic" activity.

Table I indicates results obtained after such incubation of dialyzed supernatant fluids, and redissolved, dialyzed precipitate fractions obtained after addition of varied concentrations of ammonium sulfate. As shown, essentially all "myoglobinolytic" activity of skeletal muscle extracts was present in the supernatant fluids, at 5, 10, and 25% saturation of ammonium sulfate. At 50% saturation, activity was present in both the precipitate and supernatant fluid, and at 100% saturation all "myoglobinolytic" activity was precipitated.

4. Partial purification of the active portion of skeletal muscle extract by ion-exchange chromatography. Supernatant fluids at 20% ammonium sulfate saturation were adjusted to 100% saturation, and the resulting precipitates were dissolved in, and dialyzed to equilibrium with 0.0025 *M*, pH 7.8 sodium phosphate buffer. Ion-exchange chromatography using DEAE-cellulose was then performed on this soluble extract fraction containing 103 mg of protein (Fig. 3). The column's eluate fractions were pooled into 5 portions (A, B, C, D, E) and each of these was tested for "myoglobinolytic" activity. A semiquantitative scale indicates the approximate relative activities in each based upon the dilution which still retained "myoglobinolytic" activity. As shown, it was not possible

TABLE I. "Myoglobinolytic" Activity of Human Skeletal Muscle Extract after Precipitation with Ammonium Sulfate.

% Saturation of ammonium sulfate	% Loss of myoglobin content after incubation (2 hr, 37°, pH 3.4) with	
	Dialyzed supernatant fluid	Redissolved dialyzed precipitate fraction
0	57	—
5	74	14
10	69	16
25	57	0
50	77	89
100	0	85

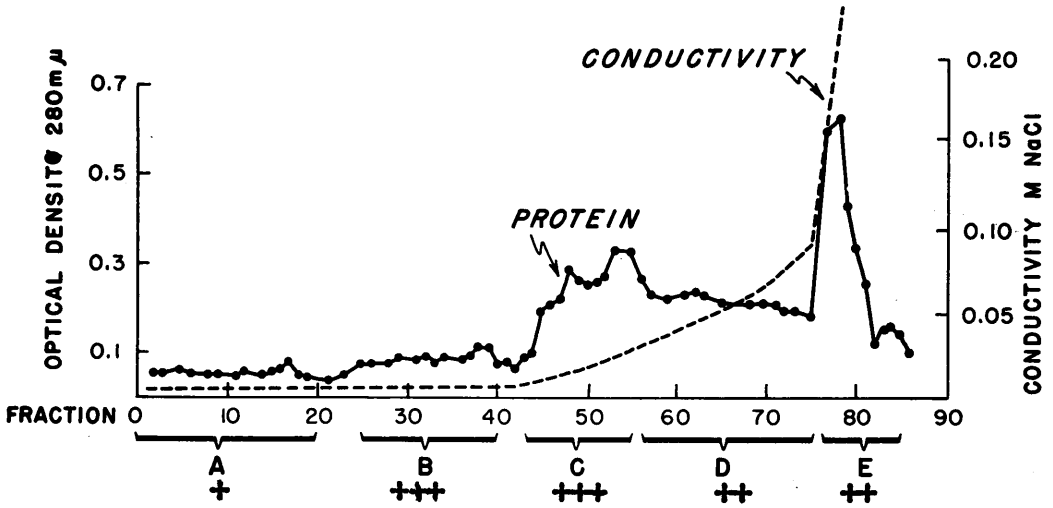

FIG. 3. DEAE-cellulose chromatographic elution diagram of the fraction of human skeletal muscle extract precipitated by 100%, but not by 20% saturated solution of ammonium sulfate. Five pooled fractions (A–E) tested for myoglobinolytic activity and scored 0–4+, based upon their relative dilution which still retained activity. Column: 30 × 3 cm (approx 20 g of DEAE-cellulose, 0.89 meq/g). Gradient applied from a Varigrad device, chamber 1) 200 ml of 0.0025 *M* phosphate, pH 7.8; and 2) 180 ml of 0.0025 *M* phosphate, pH 7.8 + 20 ml of 1 *M* NaCl.

to obtain separation of this activity into one discrete zone, however fraction B, which was active while having a low protein concentration was used for further studies. Less than 10 μg/ml of this preparation was able to completely destroy the antigenic content of 3 mg of myoglobin within 2 hr at pH 3.4.

5. Character of the reaction. Myoglobin, at acid pH, underwent changes in spectrophotometric and sedimentation behavior. Figure 4 demonstrates the shift in spectral absorption maximum from 410 mμ at pH 7.0 to 370 mμ at pH 3.4. Addition of fraction B (less than 10 μg of the partially purified fraction from DEAE-cellulose chromatography) did not change this property, although with time a loss of optical density at 370 mμ occurred. Figure 5 indicates the results of analytical ultracentrifugation of myoglobin at pH 7.0 and 3.4. Under acid conditions, in the barbital–acetate buffer, myoglobin sedimented more rapidly. Addition of fraction B (not shown) diminished the amount of rapidly sedimenting material without the formation of other detectable peaks. Figure 6 shows the patterns obtained after electrophoresis in acrylamide gels. At pH 7.0 most of the myoglobin was detectable as a single major band with 3 fainter bands moving more anodally. This pattern of apparent electrophoretic heterogeneity (or subfractions) of purified human myoglobin has been noted previously (8). At pH 3.4, however, the major band's electrophoretic mobility had increased and it now appeared more anodally, with two faintly discernible slower bands also present. After

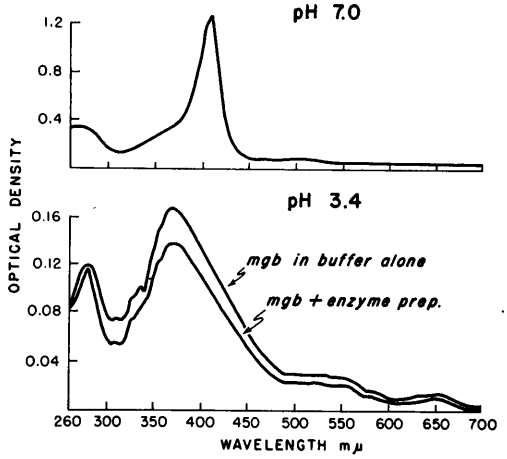

FIG. 4. Optical density spectral patterns of human myoglobin at pH 7.0, and 3.4 enzyme prep: fraction B less than 10 μg/ml, incubation: 37°, 2 hr.

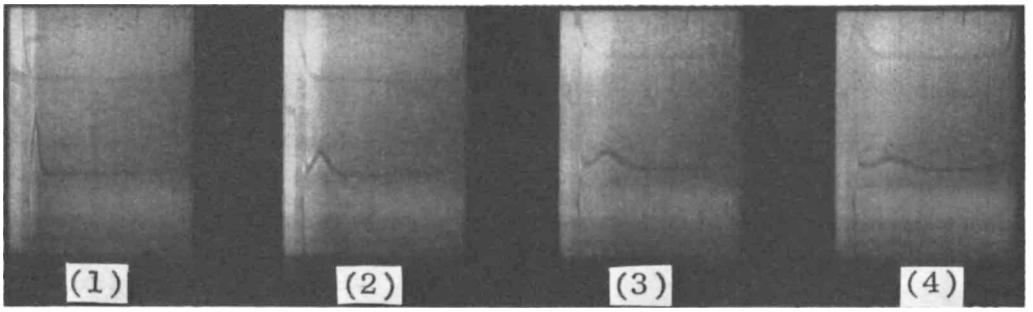

FIG. 5. *Schlieren* patterns during ultracentrifugation of purified human myoglobin: (upper cell) at pH 7.0; (lower cell) at pH 3.4; mgb conc 3.6 mg/ml, speed 47,660 rpm; photo interval, 8 min between frames. Direction of centrifugal force and bottom of the cell: to the right.

incubation with less than 10 μg of fraction B, at pH 3.4, there was a loss of this major band, and the appearance of a diffuse zone of several slowly moving bands. Fraction B alone, in the concentration used, was not detectable in the gel.

6. Addition of the partially purified extract (fraction B) to human serum albumin. Addition of fraction B to human serum albumin (Fraction V, Pentex, Inc., Kankakee, Ill.) failed to cause any loss of its antigenic content, when tested by the radial diffusion method in agar containing rabbit antiserum to human serum albumin.

Discussion. The ability of human myoglobin to form a precipitin pattern in agar with its specific antibody was destroyed after its incubation at acid pH, with extracts of several human tissues (skeletal, cardiac, uterine smooth muscle, spleen, liver) as well as with urine. This "myoglobinolytic" property was not found in human serum. Incubation of

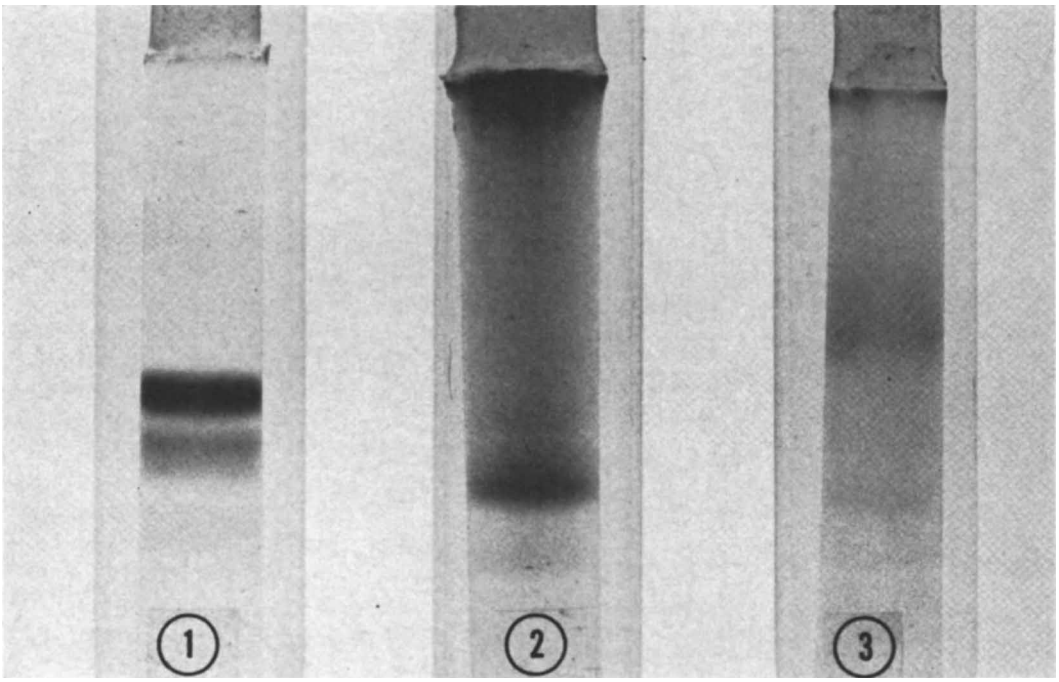

FIG. 6. Acrylamide gel disc electrophoresis patterns of human myoglobin after incubation: 1) at pH 7.0; 2) at pH 3.4; and 3) at pH 3.4 with fraction B; anode is below.

myoglobin alone, under these conditions failed to cause a loss of antigen content.

An attempt was made to partially purify the active "myoglobinolytic" portion of the skeletal muscle extract. It was found to be in the soluble cell fraction, nondialyzable, stable at 56° for 0.5 hr, and most active between pH 3 to 4. These characteristics suggest that it may have been a catheptic type of proteolytic enzyme. It was not possible, however, to obtain a discrete separation of this activity by ion-exchange chromatography, so that there may have been several different enzymes with similar activity. The range of specificity of the "myoglobinolytic" fraction is not known, although it did not seem to destroy the antigenic properties of human serum albumin, tested in a similar manner. It is of interest that "myoglobinolytic" activity existed in tissues (liver, spleen, uterus) which do not normally contain myoglobin. This may suggest a wider substrate specificity.

The electrophoretic heterogeneity of purified myoglobin in acyrlamide gel electrophoresis was previously noted and was thought to be due to the presence of subfractions of myoglobin since all four fractions were benzidine positive, reactive with antimyoglobin serum, and a partial interconvertibility between them in repetitive electrophoresis could be demonstrated (8).

At acid pH, both the spectral and sedimentation properties of the purified human myoglobin changed. The increase in the apparent sedimentation constant of myoglobin implies the possibility of molecular aggregation. This altered, acid myoglobin could then have reacted with the "myoglobinolytic" fraction. As a result, as shown by acrylamide electrophoresis, the concentration of acid myoglobin was reduced, and a diffuse zone of slowly moving electrophoretic bands appeared. This suggests an alteration of the acid myoglobin complex to form a new series of compounds, perhaps proteolytic breakdown products.

Summary. Human skeletal muscle extracts at acid pH brought about a loss of myoglobin's antigenic precipitating activity. This property was also present in cardiac muscle, uterine smooth muscle, spleen, liver, and urine, but was absent in serum. The "myoglobinolytic" activity of human skeletal muscle was soluble, nondialyzable, inactive near neutral pH, most active at pH 3.4, stable at 56° for 0.5 hr, and precipitable by ammonium sulfate. It was not possible to obtain adequate separation of this activity using DEAE-cellulose chromatography. Partially purified preparations of this "myoglobinolytic" activity caused the loss of the precipitating ability of myoglobin with antiserum, as well as the loss of its electrophoretic character, and the appearance of new electrophoretic species. The substrate for this reaction appeared to be a complex molecular form of acid myoglobin. The "myoglobinolytic" activity of tissue extracts is presumed to be due to the presence of one or more acid activated proteolytic enzymes. The substrate specificity is not known. However, active fractions did not seem to attack human serum albumin.

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Received Sept. 20, 1968. P.S.E.B.M., 1969, Vol. 130.