Isolation and Preservation of Human Myoglobin for Use in Immunologic Detection of Myoglobinuria. (31208)

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(Introduced by Ralph P. Reece)
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Detection of human urinary myoglobin has been hampered by the difficulty of readily separating hemoglobin and myoglobin when both appear in the urine. This report is concerned with methods of extracting stable antigenic myoglobin from muscle tissue, separating hemoglobin and myoglobin in urine and the application of immunologic techniques to detect myoglobin in urine. At the suggestion of one of us (E.L.R.) these methods were applied in a preliminary clinical study to determine if myoglobinuria occurs among patients who have suffered recent myocardial infarcts.

Materials and methods. Extremely low yields of human myoglobin were obtained from cardiac or skeletal muscle by salting out of hemoglobin at pH 8 and crystallization of myoglobin at pH 7. The protein was labile to temperature and storage and very weakly antigenic despite the use of Freund's adjuvant, and antiserum prepared against the extract consistently gave slight cross-reactions with hemoglobin. Since the addition of 40% formaldehyde for preservative purposes markedly improved the stability and antigenicity of the extracts, the following procedure adapted from that described by Luginbuhl (1) was used for myoglobin extraction: Muscle tissue, obtained from autopsy performed within 12 hours after death, was minced, rinsed, and homogenized with cold distilled water (2 ml/g of tissue). The pH was adjusted to 8 with 2 N NaOH and this pH was maintained throughout the extraction by periodic addition of the base. All procedures were performed at 4°C. The extract was stirred for 1 hour, centrifuged for 30 minutes at 16,000 $\times$ *g* and the supernatant filtered. Fifty-six and five-tenths grams of ammonium sulfate were added for each 100 ml of filtrate and, after stirring, the mixture was again centrifuged for 30 minutes at the same speed. Ammonium sulfate was now added to the supernatant to produce 100% saturation, the suspension stirred for 4 hours and then centrifuged at 39,000 $\times$ *g* for 30 minutes. To this final supernatant, sufficient formaldehyde was added to lower the pH to at least 4.5. It was then filtered prior to storage at —4°C. Protein determination performed after a few weeks of storage indicated the yield to be about 600 mg/100 g of wet tissue compared to less than 200 mg for the unpreserved extract.

The choice of a maximum pH of 4.5 was based on the fact that at times our final extracts showed reddish clumps of suspended hemoglobin. The amount of formaldehyde necessary to precipitate these suspended particles usually lowered the pH to 4.5 or less. In each instance the amount of formaldehyde needed to precipitate contaminating hemoglobin varied with the amount of the pigment in solution, the ionic strength of the extract and the time and temperature of exposure to the preservative.

Six rabbits were used. Each was immunized with an intramuscular injection of 0.5 ml of the formaldehyde-treated extract, containing approximately 1 mg of the protein, plus 0.5 ml of complete Freund's adjuvant. Ten days later a second injection of 0.5 ml of the extract alone was given, and the rabbits bled one week after this. The rabbits did not show adverse reactions to the injections.

The pooled antisera were reacted by immunoelectrophoresis in Veronal buffer at pH 8.6 and by double diffusion plate tests against its homologous antigen and hemoglobin.

The following procedures were used to detect myoglobin in human urine. Urines of 35 subjects were studied. The specimens were obtained at random from 10 normal control subjects (laboratory personnel) and from 25 hospitalized patients. Ten of these patients were admitted with suspected myocardial in-

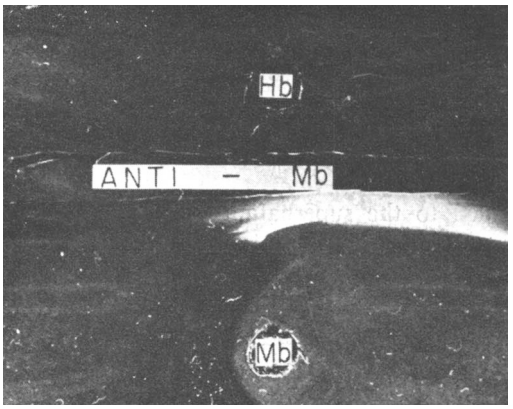

FIG. 1. Immunoelectrophoretic study of anti-formaldehyde-treated myoglobin against the homologous antigen and hemoglobin.

farctions, and 15 with other non-specific ailments. Urine specimens were obtained immediately after admission and at specific intervals thereafter. For example, as a rule, all urines excreted during the first 3 days were tested, and after that about one sample a day was tested until the patient left the hospital. The freshly passed urines were added to the 10 ml mark of vials containing 0.5 ml of formaldehyde. Each vial was refrigerated for periods up to 4 weeks and just prior to testing the specific gravity was determined and the urine filtered. The suspected diagnosis, whether myocardial infarct or other, was withheld from the laboratory staff.

Hemagglutination tests were performed using fresh or preserved human O type red blood cells coated with 0.2 mg myoglobin per ml of 2.5% red blood cell suspension. Double diffusion tube tests(2) were performed in 2.5 i.d. × 40 mm pieces of glass tubing, lined with 0.3% agar. A plug of 0.65% agar was placed in the center of the tube occupying a space of about 7 mm. The bottom of the tube was filled with 0.05 ml antiserum and the top with 0.05 ml urine. Both sides were then sealed with paraffin and incubated at 20°C overnight. Two-fold serial dilutions of the urines, up to 1:32, were tested against the undiluted antiserum in the same manner.

Results. Fig. 1 and 2 show that the anti-myoglobin did not react with hemoglobin (fresh or formaldehyde-treated) and the homologous reactions indicate that 4 or more antigenic components have resulted from the formaldehyde-treatment of myoglobin. Fig. 2 also shows the similarity between the 2 pigments in that antiserum prepared to fresh hemoglobin reacted against the formaldehyde-treated myoglobin, giving 3 bands of precipitate.

All urines with a specific gravity of 1.010 or over reacted with the undiluted antiserum. The number of precipitate bands depended on the amount of reactive substance present in the urine. Undiluted preserved urines of normal subjects and hospitalized patients usually showed one band of precipitate. On dilution of the urine with an equal quantity of saline, the precipitate was not seen. Undiluted and 1:1 dilution of urines collected from patients within 24 hours after infarction usually showed 2 bands of precipitate. On further dilution one band disappeared and this remaining band could be dectected in urines diluted 4 or 8 times. This type of "positive" result was obtained for periods up to 10 days following hospitalization. Pigmented urines containing bile (obtained from

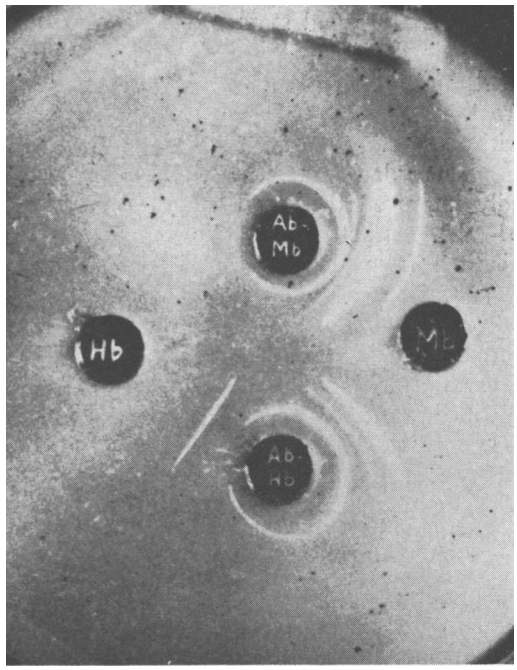

FIG. 2. Double diffusion plate reaction of anti-formaldehyde-treated myoglobin and anti-hemoglobin against formaldehyde-treated myoglobin and hemoglobin.

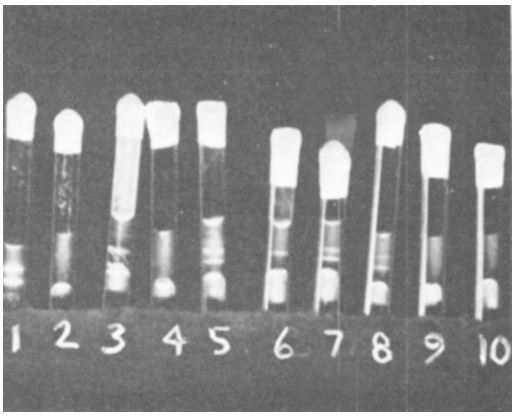

FIG. 3. Double diffusion tube tests of anti-formaldehyde-treated myoglobin (bottom) against formaldehyde-treated urines. (Urines diluted 2-fold.)

patients with cancer of the colon, liver cirrhosis and hepatitis) gave similar reactions. Fig. 3 shows the results of double diffusion tube tests of the urines of control subjects as well as those with recent myocardial infarcts, reacted against the anti-myoglobin. A random distribution of negatives and positives was used to demonstrate the ease by which a precipitate band can be seen. No. 2, 4, 9, and 10 are the urines of normal control subjects. No. 7 shows the reaction of the antiserum against the bile contaminated urine of a patient with liver cirrhosis. No. 1, 3, 5, 6, and 8 were obtained from patients with suspected myocardial infarcts, within the first 24 hours of admission to the hospital. Results of hemagglutination inhibition tests indicated that antisera titers of 1:5000 or more could be completely inhibited by 30 minutes incubation with urine from a patient with recent myocardial infarct or with bile. Control urines caused only partial inhibition.

Discussion. Human myoglobin has been reported to be a heterogeneous molecule with at least 3 electrophoretically-distinct components (3 and 4). Formaldehyde-treated myoglobin is shown in Fig. 1 and 2 to consist of 4 or more antigenic components. The exact biochemical nature of the reaction between formaldehyde and myoglobin is, not to our knowledge, known. It is possible that the results obtained may be caused solely by the low pH of the reaction. Rumen and Appella(5) have noted that at acid pH, seal myoglobin globin forms at least 2 components with sedimentation constants of about 4 and 8 S. It has been shown(6) that hemoglobin globin can dissociate or aggregate into large molecular weight substances depending on the specific pH in the acid or alkaline range. We are currently continuing this aspect of the investigation, comparing the immunologic results obtained with acid in place of formaldehyde treatment of myoglobin.

The clinical application of this study has indicated that the immunologic procedures may be a useful presumptive test for myoglobin or myoglobin-like substances in urine. It has long been known that several diseases, as well as excess physical exercise or muscle crushing, can cause myoglobinuria. In this preliminary study, no attempt was made to evaluate the amount of exercise undertaken by the control subjects or to obtain information on possible muscle dyscrasias among the hospitalized patients. It is significant that anti-myoglobin serum reacted with all urines tested and that larger amounts of myoglobin or a myoglobin-like substance were found in the urine after suspected myocardial infarction or on excretion of bile.

Summary. Addition of formaldehyde to ammonium sulfate extracts of myoglobin from human muscle increased the stability and the antigenicity of the myoglobin. Antiserum to this preserved extract does not react with hemoglobin. Immunologic tests of formaldehyde-preserved urine indicated that myoglobin or a myoglobin-like substance was present in all urines tested. Urines containing bile and urines from patients with recent myocardial infarctions contained larger amounts of the immunologically reactive substance.

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In vitro Distribution of Inulin and PAH in Dog and Sheep Kidney Cortex and Medullary Slices.* (31209)

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Measurements of the renal blood flow using PAH have generally ignored the fate of PAH in the medullary blood flow. It has been suggested that the medullary segments of the renal tubules do not extract PAH, and therefore the measurements of the renal blood flow as determined by the clearance of PAH reflect only the renal cortical flow(1,2). The experiments reported here are in accord with this concept, for they have demonstrated that the medullary cells *in vitro* did not extract and concentrate PAH in a system in which cortical cells effectively concentrated the hippurate. Concurrent estimates of the inulin space were performed in order to determine the extent of intracellular concentration of PAH by kidney cortex and medullary slices *in vitro*.

Methods. All experiments were performed on hydropenic dogs or sheep. Each animal was anesthetized with pentobarbital sodium and exsanguination was accomplished by cutting through the jugular veins and carotid arteries. The kidneys were quickly removed, cut in half along the longitudinal axis and placed into iced beakers. Slices of cortex and medulla were prepared with the Stadie-Riggs tissue slicer. Approximately 250 mg of cortex or medullary slices (239-271 mg) were incubated in 6 ml of Krebs-Henseleit solution for 2 hours following equilibration with 95% O_2-5% CO_2. The medium contained sodium acetate 10 mM, PAH 48 μg/ml and inulin-carboxyl-C^{14}, 0.5 microcurie per 6 ml of medium. At the conclusion of the incubation period the slices were quickly removed from the incubation medium, dipped twice into 30 ml of distilled water, blotted lightly on Whatman #2 filter paper, reweighed and transferred into centrifuge tubes containing 2 ml of water. The incubation medium also was transferred to centrifuge tubes and the centrifuge tubes were immediately placed into a boiling water bath for 3 to 4 minutes.

PAH in the tissue extract and in the medium was determined according to the method described by Smith and colleagues(3). PAH was determined with and without hydrolysis with 2 N HCl in tissue extracts and medium from 3 dog experiments; in the remaining 3 experiments no hydrolysis was performed. PAH was determined on both hydrolyzed and non-hydrolyzed tissue extracts and medium from all samples in the sheep experiments.

The inulin space (ECF) was determined according to methods outlined by Rosenberg, Downing and Segal(4). For determination of total tissue water, approximately 250 mg cortex and medullary slices were incubated in Krebs-Henseleit solution containing sodium acetate 10 mM and PAH 48 μg/ml. These slices were washed in distilled water, at the conclusion of the 2-hour incubation period, blotted on Whatman #2 filter paper, reweighed and placed in weighed beakers. The beakers were placed in an oven at 100°C for 15 to 18 hours and then reweighed.

The intracellular fluid of cortex and medullary slices and the concentration of PAH in the intracellular fluid were determined according to the following formulae:

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