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Techniques for the Demonstration of Myoglobin in Biological Materials

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Myoglobinemia and myoglobinuria accompanies many syndromes evoked by muscular damage due to an accident (crush syndrome, electrical current), or to other mechanisms as in cases of the "Haff disease", muscular dystrophy, or acute arterial occlusion [23].

Morphological diagnosis is based on the well known picture of the lower nephrone nephrosis [1]. It is very difficult, if possible at all, to distinguish the respective shares of myoglobin and hemoglobin in the causation of this condition [10]. The reports about direct observation of myoglobin crystals are very rare [5]. Recently myoglobin was demonstrated spectroscopically in the exisions of the kidney pyramids [22].

The detection of myoglobinuria in clinical conditions has not become a routine method [4], although this would be certainly highly desirable from the diagnostic point of view.

The molecular weight of myoglobin (Mb) is 17,000 with one heme in the molecule, while hemoglobin has four times this molecular weight (68,000) and four hemes per molecule. Myoglobin binds oxygen more firmly than Hb, is more resistant to alkaline denaturation and differs from it in salting out behaviour and amino acid composition. At concentrations over 20 mg per 100 ml of blood plasma, myoglobin appears in urine [2].

In our previous work we succeded in demonstration of Mb by means of paper electrophoresis and TLC in necrotic tissue [19, 20]. This paper sums up briefly these results and extends the possibility of detection of Mb to minute amounts in serum and urine by means of immunoelectrophoresis [26].

Material and Methods

Kidneys were obtained on autopsy of a patient, who died after surviving for 17 days after an accident which led to the crush syndrome. Anuria lasted for 7 days, death occured during the third dialysis with symptoms of hyperpotassemia and acute uremia (lower nephrone nephrosis was

established histologicaly). Second sample was from the kidney of a 36 years old woman after suicide by hanging.

Fat tissue and pelvis were removed, the kidney was cut to small pieces and ground with sea sand (25 g of sand per 100 g of kidney tissue) and 100 ml of 0.01 M acetate buffer pH 4.5. The ground tissue was left overnight in a refrigerator and afterwards centrifuged 30 min at 2000 r.p.m.

In the supernatant fluid the pH was made to pH 7.0 by adding 1N NaOH, the bulk of the proteins was precipitated by addition of lead acetate under stirring, in the proportion of 10 ml of 0.5 M lead acetate to 100 g of tissue. The pH was then made again to 7.0 and the precipitated proteins were centrifuged off. The excess of lead salts was removed by 30 min shaking of the supernatant in an electrical shaking machine with sodium phosphate and the precipitate was centrifuged off. A 20 µl sample of the clear supernatant, after making the pH to 7.0, was introduced on the origin of the electrophoretic paper.

Paper Electrophoresis

Conventional electrophoresis was carried out overnight (16 hours) at 200 V in a 0.15 M barbital buffer pH 8.6, paper Whatman 1.

Staining Procedure

The heme containing compounds were stained specifically with o-dianisidine [12] or benzidine-H_2O_2 reagent [7], proteins with bromphenol blue [9].

Thin-Layer Chromatography (TLC)

Descending TLC was performed on glass plates 18 cm × 4 cm, inclined at an angle of 12° accomodated in a glass tank. Suspension of Sephadex was prepared by shaking swollen Sephadex beads by means of an agitator in an 0.1 M phosphate buffer pH 7.4 or in a mixture of this buffer with the same volume of 0.9% NaCl. Sephadex G 50 Superfine, G 75 Superfine and G 200 Superfine were used. The volume ratio of gel solution was chosen according to the instructions of the producer [16]. Layers 0.9 mm thick were spread by means of a glass rod provided with cuffs of adhesive tape (Isolepa). The plates were connected with the solvent trough a bridge of Whatman 3 paper. Detection with benzidine-H_2O_2 was performed immediately after chromatography. Twenty µl of muscle extract was introduced on the chromatogram.

Immunoelectrophoresis

Electrophoresis was performed in 2% Difco Bacto agar in 0.1 M veronal buffer pH 8.6. The voltage of 200 V and a current of about 40 mA

was applied for 2 hours. The dimensions of the glass plates supporting agar jelly were 8.5 × 8.5 cm. 2 μl samples were used.

After the end of the electrophoresis rabbit antiserum was introduced into the troughs, which were cut in the agar gel, and left 48 hours to diffuse.

Preperation of Antiserum

Antiserum was prepared by immunization of rabbits with human Mb, isolated from human muscles by the method of Theorell [21, 22, 23] and later by the method of Luginbuhl [8]. The rabbits obtained 5 ml Mb with 5 ml Freund adjuvant i.m. on the 1st 20th, 35th day.

After the third injection the titres of the antibodies were evaluated. After the presence of the antibodies was established by the Ouchterlony precipitation method [11], the rabbits were killed, the blood collected and the plasma stored after the erythrocytes were centrifuged off.

The collected plasma was divided in 5 ml aliquots in penicillin bottles and freeze-dried. In this state the plasma was stored until use.

Results and Discussion

First we tried to distinguish Hb from Mb in the kidney by means of paper electrophoresis combined with a specific staining procedure. A sample of Hb and Mb prepared from human muscle [6, 17, 18, 21] was run in parallel with the kidney samples (Fig. 1). For the extraction of kidneys we used the procedure devised for the preparation of crystalline Mb and during this procedure we looked for the first step when we could establish the presence of Mb. This occurred after we precipitated the bulk of the proteins, besides Hb and Mb, with lead acetate. Further purification of Mb to remove Hb by salting out with ammonium sulfate is not necessary for the demonstration of Mb and is accompanied with a great loss of Mb. In this stage Mb can be easily distinguished from Hb. The method is reproducible and easy to perform.

The separation of Mb from Hb is a classical case for the use of molecular sieves (exclusion chromatography) since the molecular weight of Hb is about four times that of Mb.

Fig. 2 shows the separation of Hb from Mb on sephadex G 50 Superfine in phosphate buffer pH 7.4 after 40 min development. Detection with benzidine-H_2O_2. Similar results were obtained with Sephadex G 75 and with a mixture of the phosphate buffer and 0.9% NaCl (1:1). The minimum detectable amount of Hb by the benzidine-H_2O_2 reaction after chromatography was about 15 μg when a 2 cm long line had been spotted.

Because our future aim is to demonstrate Mb not only in necrotic kidneys, but also in vivo, we decided to work with a highly specific and sensitive method, i.e. with immunoelectrophoresis [13, 15, 26].

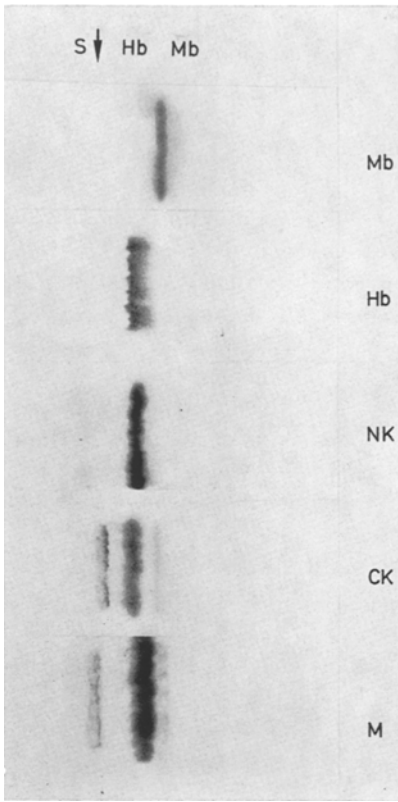

Fig. 1. Paper electrophoreogram of kidney extracts. *NK* extract of normal kidney, *CK* extract of crush syndrome kidney, *Mb* myoglobin, *Hb* hemoglobin, *M* muscle extract, *S* line of application. Barbital buffer pH 8.6. Stained with bromphenol blue.

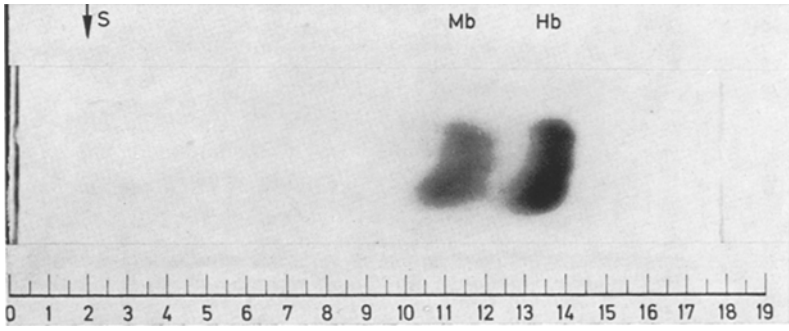

Fig. 2. Phosphate buffer (0.1 M, pH 8.6, 40 min. *Mb* myoglobin, *Hb* hemoglobin, *S* origin. Stained with the benzidine-H_2O_2 reagent

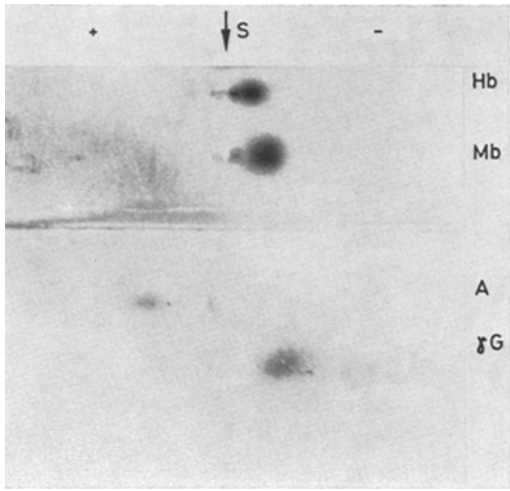

Fig. 3. Agarophoreogram. Stained with bromphenol blue

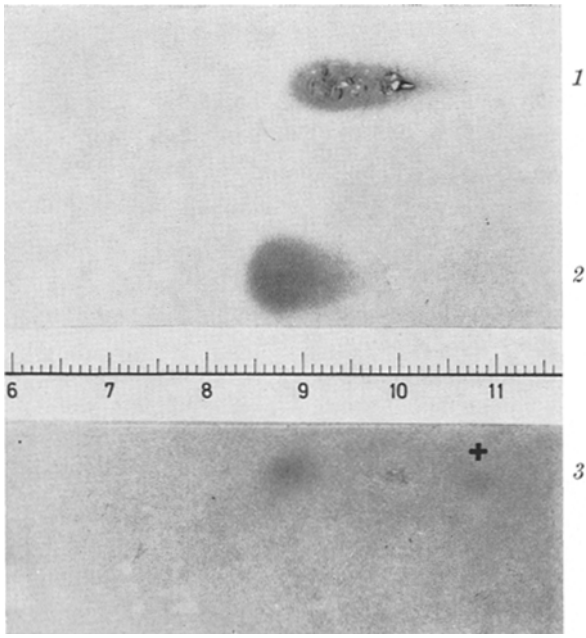

Fig. 4. Agarophoreogram of Hb (*1*), Mb (*2*), and a sample of urine (*3*). Mb-haptoglobin complex which is too weak to show up clearly on the reproduction, is marked with a + sign. Stained with a benzidine-H_2O_2 reagent. Origin at point 10

Agar electrophoreograms without immunodifusion were stained either for hemoproteins immediately after electrophoresis with the benzidine-H_2O_2 reagens, or after drying the next day with bromphenol blue (Fig. 3). Fig. 4 shows an agarphoreogram of urine from a case with myoglobinuria. We can see two benzidine-positive spots, one corresponding to myoglobin and the other to the myoglobin-haptoglobin complex. Both spots gave precipitate on immunophoreogram with the specific antiserum. The minimum amount of Mb which gave precipitation line was 0.5 μg (Fig. 5).

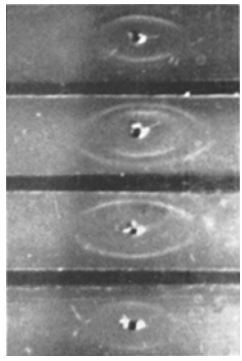

Fig. 5. Immunodiffusion of myoglobin samples. *1* 40 μg, *2* 10 μg, *3* 5 μg, *4* 0.5 μg

Summary

Paper or agar gel electrophoresis is a simple and convenient method for the demonstration of the presence of Mb in a kidney extract or biological fluids.

TLC on Sephadex may be used for the separation of Mb and Hb as well. Immunoelectrophoresis can serve for the detection of minute quantities of Mb in vivo.

Zusammenfassung

Papier- oder Agargel-Elektrophorese ist eine einfache und gebräuchliche Methode für den Nachweis von Myoglobin im Nierenextrakt oder in biologischen Flüssigkeiten.

TCL (Dünnschichtchromatographie) auf Sephadex kann ebenso gut für die Trennung von Myoglobin und Hämoglobin verwendet werden. Immunelektrophorese kann für die Entdeckung von Myoglobinspuren in vivo dienen.

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