MYOHEMOGLOBINURIA

A STUDY OF THE RENAL CLEARANCE OF MYOHEMOGLOBIN IN DOGS*

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The observation that hemoglobin extracted from muscle was excreted by the kidneys much more rapidly than blood hemoglobin was perhaps the first indication that these two substances are different (4, 7). Since the purification and crystallization of myohemoglobin by Theorell (13) in 1932 many of its chemical and physical properties have been elucidated. Also ideas concerning renal function have become more specific so that many of the features which were so puzzling to early investigators have now become fairly obvious.

Camus and Pagniez (4) in 1902 were the first to report the experimental production of muscle hemoglobinuria. Their papers contain little specific information regarding experimental procedures, but they conclude, significantly, that the amount of myohemoglobin recovered in the urine is proportional to the amount injected intravenously and that following such injections the urine becomes deeply colored while the plasma never exhibits more than a faint tinge. Similar results have been obtained in horses (3, 5) and the hemoglobinuria occurring in these animals in certain disease states has been attributed to the escape of myohemoglobin into the plasma and thence through the kidney. Whipple and his associates (1, 7, 10) also studied several aspects of the problem. Muscle hemoglobin was found to disappear rapidly from the plasma, and the renal threshold for this substance was estimated at about 15 mg. per kilo of body weight. Following repeated injections the iron content of the kidneys was somewhat increased although no iron-staining pigment could be demonstrated in the tubular epithelium, as in the case of blood hemoglobin.

The comparative physical and chemical characteristics of myohemoglobin and hemoglobin have been extensively dealt with in a recent review by Millikan (8). For the purposes of this study, however, the most significant findings are that the two substances are spectroscopically almost identical and that whereas hemoglobin has a molecular weight of 68,800 and contains four atoms of iron, a molecule of myohemoglobin weighs only 17,500 (12) and contains but one iron atom.

The experiments to be described are concerned with a study of the simultaneous renal clearances of myohemoglobin and creatinine in the dog. Since this method has previously been applied to hemoglobin (9), a quantitative

* We are indebted to Eli Lilly and Company for aid in conducting this work.
comparison of the renal excretion of the two substances can readily be made. The results show that myohemoglobin is cleared from the plasma about twenty-five times more rapidly than hemoglobin, but that the mode of excretion appears to be similar for the two substances.

Methods

All procedures employed for the determination of the renal clearances of myohemoglobin and creatinine were essentially similar to those described in a previous publication concerning the renal clearance of hemoglobin (9).

Female dogs were used to facilitate catheterization and tap water containing approximately 0.5 gm. of sodium bicarbonate per 100 cc. was given before and during each experiment in amounts sufficient to insure a moderate diuresis of alkaline urine.

A 10 per cent aqueous solution of creatinine was injected subcutaneously 1 hour before the commencement of each experiment in amounts approximating 1 cc. per kilo of body weight.

Urine was collected through a curved metal catheter, the bladder was washed with warm isotonic saline before and after each collection period, and the length of each period was approximately 20 to 30 minutes. The normal excretion rate of creatinine was determined during the first two periods and the myohemoglobin injections were made during the third. Following this, consecutive urine samples were collected until gross hemoglobinuria was no longer evident.

Blood samples of approximately 8 cc. were taken at about the mid-point of each period from the external jugular vein. In each instance, after puncturing the vein and withdrawing about 1 cc. of blood, a second syringe was inserted and the final sample collected. The blood was transferred with care to a tube containing 1.5 cc. of 1.4 per cent sodium oxalate and gently mixed. These precautions were necessary to prevent even very slight hemolysis which would interfere with the colorimetric determination of the relatively low plasma concentrations of myohemoglobin. Samples were centrifuged at high speed for 10 minutes and the plasma was transferred to another vessel immediately after noting the hematocrit. Appropriate factors were introduced into all final calculations to account for the anticoagulant.

Myohemoglobin was injected from a gravity bottle at a rate of from 20 to 30 cc. a minute. It was found desirable to inject as rapidly as possible, since myohemoglobin appeared in the bladder almost instantaneously, and the above mentioned speed was well tolerated.

In view of its extremely rapid elimination, the first urine period for myohemoglobin was measured from the time of completing the injection, and a blood sample was collected at the exact mid-point of this first period to avoid the necessity of interpolation.

Plasma and urine creatinine determinations were made according to the method described by Shannon, Jollife, and Smith (11). Myohemoglobin determinations were carried out according to the cyanmethemoglobin method of Evelyn and Salter (6). All colorimetric measurements were made on a Klett photoelectric colorimeter and appropriate blanks were carefully selected for each determination.

Preparation of Myohemoglobin.—In order to obtain concentrations of myohemoglobin high enough to permit the rapid injection of a moderately large quantity, the
quantitative extraction method of Whipple (14) and the crystallization method of Theorell (13) were slightly modified and combined. Blood-free muscles were obtained from dogs after viviperfusion, as described by Whipple (14). These were rendered reasonably free of fat and fascia, rinsed in saline, and minced in a meat grinder. Each kilo of ground muscle was mixed with about 1 liter of a 0.5 per cent solution of ammonia in water, shaken well, and stored overnight at 2-4°C. The mixture was then filtered through several thicknesses of cheesecloth. The turbid reddish grey supernatant fluid was shaken in a separatory funnel with about one-tenth of its volume of ether to remove any fat present and the relatively clear, red solution drained from the bottom was centrifuged. The pH was adjusted to 7.0 and basic lead acetate was added to precipitate a large portion of the protein impurities. The resulting heavy precipitate was removed by centrifugation, the pH again adjusted, and the excess lead removed by the addition of disodium phosphate. The final product, a clear, dark red fluid was dialyzed in cellophane sacs against distilled water at 2°C. Twelve hours were usually sufficient to remove all traces of phosphate. The solution was then rapidly frozen and lyophilized at reduced pressure and temperature. The brownish-red, flaky material so obtained was readily soluble in small volumes of distilled water (e.g. 300 mg per 10 cc. water). After filtration in a Büchner funnel it was ready for injection.

An early attempt was made to obtain a purer product by dialysis against saturated ammonium sulfate with subsequent removal of the sulfate by dialysis against water. However, after lyophilization a large proportion of the material was insoluble, indicating that denaturization had occurred. This procedure was abandoned because of the great loss of myohemoglobin, and because material obtained in this manner was found to be more toxic.

EXPERIMENTAL OBSERVATIONS

This report is concerned with the results of seven experiments performed on four separate animals. Varying amounts of myohemoglobin were injected intravenously, which caused initial plasma concentration ranging from 60 to 200 mg per 100 cc. Two animals received multiple injections but no appreciable difference was noted in the outcome of the various experiments. Small dogs were used exclusively in order to obtain maximum plasma concentrations with minimum amounts of myohemoglobin.

Slight reactions lasting \( \frac{1}{2} \) to \( \frac{3}{4} \) of an hour occurred following injection of the pigment in most experiments. The animals became listless and there was usually an associated diminution in the flow of urine and also in glomerular filtration, as estimated by creatinine clearance. These changes were attributed to the presence of some toxic impurities in the myohemoglobin preparations. In all other respects the clinical condition of the animals remained normal throughout.

Fig. 1 illustrates the rate at which myohemoglobin disappears from the plasma following intravenous injection. The composite graph was obtained by first plotting the disappearance curve for the experiment showing the highest
Initial plasma concentration of myohemoglobin. In each of the other experiments the initial plasma concentration was placed on this curve and subsequent concentrations were plotted in relation to it with regard to time. It will be noted that the rate of removal from the plasma is very rapid above a concentration of about 30 mg. per 100 cc., that it becomes much slower below this level, and that within any given range of plasma concentration the disappearance rate is independent of the initial level instituted. Below a concentration of about 40 mg. per 100 cc. the plasma has only a very slight reddish tinge and tends to become somewhat yellow in color.

The detailed data obtained in one characteristic experiment are recorded in Table I. Columns 3, 4, and 5, respectively, list the average plasma concentration, the milligrams excreted per minute, and the clearance rate of creatinine in each separate period. Similar data pertaining to myohemoglobin are found in
columns 6, 7, and 8. The creatinine clearance rates (column 5) also designate the volume of fluid filtering through the glomeruli per minute in any given period. A temporary diminution in filtration is seen in period 3 immediately following the injection of myohemoglobin. The myohemoglobin/creatinine clearance ratios listed in column 9 are obtained by dividing the figures in column 8 by those in column 5, resolving any effects due to fluctuations in glomerular filtration. The values in column 10 represent the amounts of myohemoglobin recovered in the bladder urine in each period, listed as a percentage of the total amount injected, and column 11 indicates the relative rate of urine flow from period to period.

**TABLE I**

*Summary of Experiment 1*

*Dog 40-65—Weight 10.5 Kilos*

<table>
<thead>
<tr>
<th>Period</th>
<th>Length of period</th>
<th>Creatinine</th>
<th>Myohemoglobin</th>
<th>Myohemoglobin/creatinine clearance ratio</th>
<th>Myohemoglobin in urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Plasma</td>
<td>Urine</td>
<td>Plasma</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mg./100 cc.</td>
<td>cc./min.</td>
<td>cc./100 cc.</td>
<td>cc./min.</td>
</tr>
<tr>
<td>1</td>
<td>2 min.</td>
<td>7.85</td>
<td>3.49</td>
<td>44.5</td>
<td>4.5</td>
</tr>
<tr>
<td>2</td>
<td>25.0</td>
<td>7.15</td>
<td>3.05</td>
<td>42.7</td>
<td>3.0</td>
</tr>
</tbody>
</table>

1.00 gm. myohemoglobin dissolved in 75 cc. water injected intravenously (95 mg./kg.)

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th></th>
<th></th>
<th></th>
<th>Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg.</td>
<td>mg./min.</td>
<td>cc./min.</td>
<td>cc./min.</td>
<td>flow</td>
</tr>
<tr>
<td>3</td>
<td>17.5</td>
<td>6.60</td>
<td>2.22</td>
<td>33.6</td>
<td>34.5</td>
</tr>
<tr>
<td>4</td>
<td>22.0</td>
<td>5.98</td>
<td>2.50</td>
<td>41.8</td>
<td>17.0</td>
</tr>
<tr>
<td>5</td>
<td>27.5</td>
<td>5.45</td>
<td>2.47</td>
<td>45.1</td>
<td>8.1</td>
</tr>
<tr>
<td>6</td>
<td>30.5</td>
<td>4.80</td>
<td>2.23</td>
<td>46.5</td>
<td>3.9</td>
</tr>
<tr>
<td>7</td>
<td>62.0</td>
<td>3.94</td>
<td>1.81</td>
<td>46.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

In Fig. 2 the excretion rates of myohemoglobin and creatinine in relation to their respective plasma concentrations are represented graphically. The creatinine curve shows a characteristic straight line originating at zero while a straight line relationship between the plasma concentration of myohemoglobin and the milligrams excreted per minute is seen to exist above a threshold plasma level of 17 mg. per 100 cc. In both curves the excretion during period 3 is lowered. The disappearance of these variations in the myohemoglobin/creatinine clearance ratio indicates that the same mechanism, presumably lowered filtration, is responsible for the observed changes in the excretion of both substances. When the myohemoglobin value in this period is corrected for the percentage drop in creatinine excretion, it is found to fall on the straight line joining the other points. A definite straight line relationship between the plasma concentration and the rate of excretion of myohemoglobin is found in
FIG. 2. Graphs A and B show the relationship between the rate of renal excretion and the plasma concentration for myohemoglobin and creatinine respectively, in experiment 1.

TABLE II
Summary of All Experiments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Dog No.</th>
<th>Weight (kg)</th>
<th>Myohemoglobin injected (mg.)</th>
<th>Renal threshold (mg./kg.)</th>
<th>Max. myohemoglobin clearance (cc./min.)</th>
<th>Average creatinine clearance (cc./min.)</th>
<th>Max. myohemoglobin/creatinine clearance ratio</th>
<th>Per cent of injected myohemoglobin in urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td></td>
<td></td>
<td></td>
<td>Actual</td>
<td>Corrected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>40-65</td>
<td>10.5</td>
<td>1.00</td>
<td>95</td>
<td>17</td>
<td>18.8</td>
<td>43.6</td>
<td>0.59</td>
</tr>
<tr>
<td>2</td>
<td>40-65</td>
<td>11.8</td>
<td>1.15</td>
<td>98</td>
<td>18</td>
<td>12.3</td>
<td>42.5</td>
<td>0.57</td>
</tr>
<tr>
<td>3</td>
<td>40-65</td>
<td>11.9</td>
<td>0.90</td>
<td>75</td>
<td>17</td>
<td>14.8</td>
<td>45.2</td>
<td>0.61</td>
</tr>
<tr>
<td>4</td>
<td>39-104</td>
<td>9.1</td>
<td>0.78</td>
<td>85</td>
<td>20</td>
<td>20.9</td>
<td>44.7</td>
<td>0.53</td>
</tr>
<tr>
<td>5</td>
<td>39-104</td>
<td>9.1</td>
<td>1.50</td>
<td>167</td>
<td>18</td>
<td>15.0</td>
<td>40.0</td>
<td>0.56</td>
</tr>
<tr>
<td>6</td>
<td>39-242</td>
<td>8.0</td>
<td>0.66</td>
<td>82</td>
<td>23</td>
<td>32.5</td>
<td>59.0</td>
<td>0.55</td>
</tr>
<tr>
<td>7</td>
<td>40-370**</td>
<td>10.7</td>
<td>0.99</td>
<td>92</td>
<td>—</td>
<td>16.3</td>
<td>62.0</td>
<td>0.57</td>
</tr>
</tbody>
</table>

* Figures in column 8 are corrected for the percentage drop in creatinine clearance during the initial myohemoglobin period.

** Dog 40-370—experiment discontinued due to trauma to urethra.
all experiments when similar corrections for variations in glomerular filtration are applied.

Table II summarizes the salient features of all the experiments. Columns 3, 4, and 5 list the amounts of myohemoglobin injected and their relationship to body weight. It will be seen that the renal threshold values in column 6 show a striking relative uniformity. However, it is felt that these values may be a little too high due to the slight effect of the yellow color in the plasma on the colorimetric readings at low plasma concentrations. The possibility of some contamination of the myohemoglobin solutions with blood hemoglobin was also considered. However, random histological sections of the muscle tissues used for extraction showed a complete absence of red blood cells after vivi-perfusion. It is also significant that on two occasions when samples of pigment, excreted by the kidneys below the renal threshold for blood hemoglobin, were reinjected, after dialysis against water, the threshold values observed were identical with those originally obtained.

The figures in column 7 are the actually determined clearance rates obtained in each experiment during the first period following the injection of myohemoglobin. In all but one instance there was a drop in creatinine clearance during this initial period below the average levels listed in column 9. The values in column 8 represent the maximum myohemoglobin clearance, under
ideal conditions, after a correction is made for the percentage drop in the simultaneous creatinine clearance.

The maximum myohemoglobin/creatinine clearance ratios in column 10 are those actually observed in each experiment.

Fig. 3, a composite of all the experimental data, shows the variation in the myohemoglobin/creatinine clearance ratio at different plasma levels. The ratio is seen to remain relatively constant above a plasma concentration of about 50 mg. per 100 cc. At this level it commences to diminish and falls sharply to zero as the threshold is approached.

DISCUSSION

The foregoing experimental data show clearly that there is a definite renal threshold for myohemoglobin at a plasma concentration slightly below 20 mg. per 100 cc. and that above this critical level the rate of renal excretion is directly proportional to the plasma concentration. The ratio between the simultaneous renal clearances of myohemoglobin and creatinine rises rather abruptly between the threshold level and a myohemoglobin plasma concentration of approximately 50 mg. per 100 cc. Beyond this point the clearance ratio tends to become relatively constant at a value between 0.55 and 0.60 indicating that under maximal conditions myohemoglobin is cleared from the plasma by excretion into the bladder urine at a rate which is 55 to 60 per cent of the creatinine clearance. Since creatinine clearance in the dog is a measure of glomerular filtration, the above findings imply that myohemoglobin is filtered through the glomerulus at a rate not less than 55 to 60 per cent of the maximum filtering capacity. The form of the excretion rate and clearance ratio curves, closely resembling those for such substances as glucose, strongly suggests, however, that tubular reabsorption of myohemoglobin is responsible for the observed threshold phenomenon. If this tubular reabsorption reaches a maximum at the threshold and remains constant at higher levels, the rate of glomerular filtration of myohemoglobin will be constant and somewhat higher than the observed excretion rate. Under these conditions the filtration rate may be graphically represented, in relation to the type of experimentally determined curve illustrated in Fig. 2, by a line parallel to it and originating at zero. By applying this type of analysis to each experiment an average rate of glomerular filtration is obtained for myohemoglobin which is approximately 75 per cent of creatinine filtration.

Iron-staining pigment was not observed by Whipple and his associates in the epithelium of the renal tubules following multiple small injections of myohemoglobin (10). However, since the total amounts injected were only about 3 gm., there would be little possibility under these conditions of demonstrating the minute quantities of iron that might have remained in the tubular epithelium.
It is also conceivable that, following reabsorption, myohemoglobin is fairly rapidly removed from the kidney.

A comparison of the findings on the excretion of myohemoglobin with those previously reported for a similar study of blood hemoglobin (9) reveals that the underlying mechanisms involved are similar for both substances and that such discrepancies as do exist are of a purely quantitative nature. The average renal threshold for myohemoglobin lies between 15 and 20 mg. per 100 cc. of plasma whereas that for blood hemoglobin is slightly over 100 mg. per 100 cc. Above these levels both substances exhibit a direct proportionality between the milligrams excreted per minute and the plasma concentration and the form of the curves relating the ratios of the respective clearances over creatinine clearance to plasma concentration is similar. The maximum myohemoglobin/creatinine clearance ratio averages 0.58 while the average hemoglobin/creatinine clearance ratio is 0.023. This indicates that under maximal conditions myohemoglobin is cleared from the plasma twenty-five times more rapidly than blood hemoglobin. On the basis of estimated values myohemoglobin is also found to filter through the glomerulus at twenty-five times the rate of blood hemoglobin.

Since the molecular weight of blood hemoglobin is four times that of muscle hemoglobin (12), the most reasonable explanation of the observed variations in excretion would seem to lie in differences in glomerular permeability for the two substances. Such differences in permeability are thought to be related to pore size although it is not definitely known whether actual anatomical openings exist in the membrane. In this connection it is interesting to note that the limit of complete glomerular permeability is thought to correspond to a pore size approximately that of an inulin molecule (molecular weight 15,000) (2). The permeability of the glomerular membrane for myohemoglobin, with a molecular weight of 17,500, however, appears to be only 75 per cent of the maximum.

It is possible that with this fuller understanding of the fundamental differences in the renal excretion of blood and muscle hemoglobin, the true nature of certain obscure clinical hemoglobinurias may become apparent.

SUMMARY AND CONCLUSIONS

When myohemoglobin is injected intravenously into dogs, in amounts ranging from 0.75 to 1.50 gm., it is rapidly eliminated from the plasma and approximately 65 per cent is excreted by the kidneys in from 1½ to 2½ hours.

Myohemoglobin does not appear in the urine below a threshold plasma concentration which is slightly under 20 mg. per 100 cc. but above this level the rate of renal excretion is directly proportional to the plasma concentration.

The maximum myohemoglobin/creatinine clearance ratio averages 0.58 contrasted with a value of 0.023 for blood hemoglobin. This indicates that the
rate of renal clearance of myohemoglobin is twenty-five times more rapid than that of blood hemoglobin. Evidence is presented that the excretory mechanism is essentially similar for the two substances but that differences in molecular weight account for different rates of glomerular filtration.

BIBLIOGRAPHY