The Metachromatic Activity of Urine Following the Injection of Heparin


Heparin was injected in man, dog, rabbit and sheep and the metachromatic activity (MA) of the urine measured directly or after precipitation with benzidine. The presence and amount of MA in the urine depended on route, size and time relations of the injection, but generally was much less than the MA of the heparin injected. An excretion product, uroheparin, has been described. It is concluded that following injection heparin may be (a) destroyed by heparinase, (b) degraded to uroheparin or (c) excreted unchanged. Following (b) or (c) metachromatic activity is found in the urine.

Urineary excretion of heparin and its metabolic products has been investigated by a number of authors with conflicting results. Howell and Maedonald originally claimed to have demonstrated excretion of heparin in urine on the basis of recovery of slight anticoagulant activity from urine after heparin injections, but as no control urine was tested there was no evidence that the injected heparin was responsible for the activity, which was no greater than that due to sodium chloride. Jaques precipitated urine with acid alcohol and while obtaining satisfactory recoveries of added heparin failed to find any increased anticoagulant in the urine of dogs after injection. Wilander, on the other hand, demonstrated anticoagulant activity in the urine of rabbits after injection of heparin, equivalent to 20 to 25 per cent of the amount injected. Copley and Schnedorf found that the urine of rats and rabbits gave a metachromatic reaction with toluidine blue after the injection of heparin, and they assumed that this showed heparin itself to be excreted in the urine. Reiner and Winterstein reported that after previous injection of heparin in rabbits, a substance appeared in urine which had an inhibitory action on coagulation. The quantity corresponded to almost 20 per cent of the heparin administered, but varying amounts of a substance inhibiting coagulation also appeared in normal rabbit urine, and these authors found no evidence of excretion in man even following large doses. P. Astrup developed a method for separating heparin from urine, using benzidine. This gave quantitative recovery of added heparin even in low concentrations, but when applied to urine after injection of heparin, only 1.0 to 12 per cent of the injected heparin could be found as judged by the inhibitory activity on the clotting of citrated human plasma. By metachromasia, Piper concluded that in rabbits 25 to 50 per cent of injected heparin was excreted.

Several recent reports, beginning with P. Astrup have demonstrated that different products appear in the urine after the injection of heparin. Marbet and Winterstein found that in man, while as much as 50 per cent of the injected heparin could be recovered as estimated by a nephelometric test, this showed only 7 to 20 per cent of the antithrombic activity. A preliminary communication of work in Toronto on this subject by Best and Jaques in 1946 reported the isolation, following the injection of heparin, of a urinary substance similar to heparin but with low antithrombic activity and suggested the term uroheparin for this. However, the complexity of the problem as demonstrated in these early experiments delayed publication. The present report does not propose a complete solution to the problem. It does, however, demonstrate that elimination of heparin involves several mechanisms. Further, while the study has been...
conducted chiefly on the dog and man, experiments with other species indicate that the confusion in the literature cited above is due to the fact that the mechanism of excretion observed varies with the dose, species and the type of test used to measure the excreted material.

Methods and Materials

Antithrombic Assay. This was conducted on the principles of the Jaques and Charles' assay. A dilution of 1:1,000 (0.1 units heparin per milliliter) was used for the standard solution and the thrombin solution was adjusted to give an end point at 0.02 to 0.03 units of heparin. The quantities of standard solution and unknown taken for comparison (after diluting the unknown to approximately 0.1 units per milliliter) were 0.1, 0.2, 0.3, 0.4 ml. This gave an approximate antithrombic titer (±30%).

Table 1.—Recovery of Added Heparin from Urine by Astrup's Method

<table>
<thead>
<tr>
<th>Heparin Added Units</th>
<th>Metachromatic</th>
<th>Antithrombic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Units</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>1.4</td>
<td>1.25</td>
</tr>
<tr>
<td>50</td>
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<td>200</td>
<td>175</td>
<td>87</td>
</tr>
<tr>
<td>400</td>
<td>371</td>
<td>93</td>
</tr>
</tbody>
</table>

The heparin was added to 50 cc. aliquots of urine.

Metachromatic Assay. As discussed by Bank and Bungenberg de Jong, Jaques, Mitford and MacDonald, and Jaques, this property of heparin refers to the fact that on addition of heparin to certain basic dyes a new absorption band is produced at a shorter wave length. This new absorption band for the dyes azure A and toluidine blue, appears at 500 m\(\mu\) compared to 620 m\(\mu\) for the dye alone. Crude extracts of heparin and heparin in urine precipitate the heparin-dye compound in such a way as to make most photoelectric instruments useless. Reasonable color matches can be obtained by the Lovibond tintometer, however, and the metachromatic determinations were conducted with this instrument. The actual assay was conducted as described by Jaques, Mitford and MacDonald using the narrow cell described by Jaques, Monkhouse and Stewart. For rapid qualitative testing for metachromasia in urine, a spot test was found very useful. One drop of dye was added to 3 drops of sample on a glass plate over a white background. The dye turned pink at low concentrations of heparin and gave a precipitate at high concentrations. The test was positive for concentrations of heparin as low as 0.1 units per milliliter.

Units. All assays were conducted by direct comparison of activity of the sample with that of a standard solution of Connaught Laboratories heparin. In the later work, the standard solution was assayed against a solution of the International Standard of heparin. Much of the data was obtained in 1945-1946 before the International Standard was available, but the unit used differed from the present International unit by less than 10 per cent. All assay values are expressed in equivalent units of the International Standard of heparin, the equivalent metachromatic activity being expressed as MA units, the equivalent antithrombic activity as AT units.

Separation of Heparin from Urine. The metachromatic assay has been applied to urine directly and also after separation of the heparin by one of two methods. The first method was based on earlier work of Miss Ann Macdonald and is similar in principle to that of Marbet and Winterstein. The urine was concentrated, the pH adjusted to 5.5 and two volumes of 5 per cent brucine phosphate at the same pH added. The precipitate was hydrolyzed in alkaline ammonia water in a water bath at 65°C. for 10 minutes, and after centrifuging and washing the residue, the combined supernatant was acidified with acetic acid and precipitated with two volumes of alcohol.

The second method for isolation of heparin from urine, has been that of Poul Astrup. To 50 ml. of urine was added 25 ml. of benzidine hydrochloride and 2 ml. of 5 N hydrochloric acid. The benzidine-heparin precipitate was shaken in a separatory funnel with 10 ml. of 10 per cent ammonium hydroxide and 50 ml. ether, and after filtering the lower aqueous phase, 0.5 per cent sodium chloride and four volumes of ethyl alcohol were added. The precipitate of crude heparin was dried. Weights given for the benzidine stage are for this precipitate. The method was tested by adding varying quantities of commercial heparin to 50 ml. aliquots of urine. Results are shown in table 1. Recoveries were close to 90 per cent. This method was shown by Astrup to be satisfactory for the determination of heparin in concentrations as low as 20 mg. per 50 ml. urine. Although Astrup was unable to ensure that losses would never exceed 20 per cent, little difficulty has been encountered by us in obtaining an average recovery of 90 per cent with heparin added to urine.

Heparin used in all experiments was commercial beef heparin supplied by the Connaught Medical Research Laboratories, University of Toronto. We are indebted to Dr. R. D. Defries of the Laboratories for a gift of the heparin used.

Experimental Results

The Metachromatic Enhancing Activity of Urine. Copley and co-workers devised a
semiquantitative test for heparin in urine based on a direct test of urine for metachromasia. In a number of the early experiments we used this principle but modified it by using the solutions and Lovibond Tintometer as described by Jaques, Mitford and Ricker. This is illustrated in table 2. Varying amounts of heparin and normal dog urine were mixed and assayed. It is evident that heparin gives a higher red reading with the dye when mixed with urine. This is in contrast to all previous substances studied. These without exception interfered with or inhibited the metachromatic reaction. For convenience we refer to this property of urine as its metachromatic-enhancing (ME) activity and the substance responsible for it as the ME factor. Heparin in urine could be freed from this factor by precipitation, as with brucine phosphate.

Experiments on the properties of ME factor demonstrated that the activity was usually soluble in 60 per cent alcohol, dialyzed, was insoluble in organic solvents and could not be distilled from either acid or alkaline pH. On evaporation of urine, the ME factor was found in the residue. Complete ashing destroyed the ME factor but heating of urine residues to definite charring did not destroy it, suggesting that it was an organic compound of high melting point. Piperidine when tested was found to have metachromatic activity but when diluted to the concentration found in normal urine (10–30 mg. per liter) it showed neither metachromatic activity nor metachromatic-enhancing activity.

**Metachromatic Activity of Urine following the Injection of Heparin.** While metachromatic activity has previously been observed in urine following the injection of heparin, there have been no quantitative studies of this phenomenon. As shown in figure 1, 5,000 units of heparin were injected intravenously in a dog and the urine collected by catheter at approximately 15-minute intervals. Metachromatic activity appeared in the urine within five minutes of the injection and most of the metachromatic activity was excreted in the first 40 minutes. The total net activity in the urine was equivalent to about 15 per cent of that injected. While early workers reported crude heparin caused polyuria, it can be seen that increase in urine flow occurred after the metachromatic activity had largely disappeared from the urine.

A series of such experiments was conducted in which varying amounts of heparin were given as single intravenous injections, the urine collected and the metachromatic activity of the urine measured. Doses of heparin varied from 50 to 2,000 units per kilogram. Metachromatic values were expressed as equivalent units of heparin, after correction for the metachromatic enhancing activity of the urine. The results are shown in figure 2. The metachromatic activity appeared in the urine within five minutes of the injection in all cases where activity was observed. No metachromatic activity was observed in the urine after an injection of 50 units per kilogram. Above this value, the metachromatic activity in the urine (solid line in fig. 2) increased with size of dose in a progressively increasing fashion. With doses of 50 to 350 units of heparin per kilogram, relatively little metachromatic material was excreted. Assuming the blood volume to be 100 ml. per kilogram, the latter value will correspond to an initial blood level of 3.5 units per milliliter. Above this value, excretion increased rapidly with increase in dosage. Of
Fig. 1. Excretion of metachromatic activity after intravenous injection of heparin in the dog. Three thousand Connaught Laboratory units of heparin injected intravenously at zero time. Urine collected by catheter under Amytal.

Fig. 2. Total metachromatic activity of urine after single intravenous injections of heparin in the dog. Twelve animals were used, weighing from 8 to 23 Kg, and the urine was collected by catheter under Amytal. Solid points are actual found values and solid line is drawn for the equation, \( y = 1.8071x - 2.7098 \) where \( y = \log \) of metachromatic activity in urine, \( x = \log \) of metachromatic activity injected. Broken line is for difference between injected and excreted metachromatic activity.

greater interest was the difference between injected metachromatic activity and that excreted (dotted line of fig. 2). This will represent the portion of heparin injected which did not appear as metachromatic activity in the urine. With doses up to 350 units per kilogram, most of the heparin appeared to follow this route. At 600 units per kilogram (blood level about 6 units per milliliter), this reached a constant value, suggesting that this system was saturated. Injection of amounts of heparin greater than this resulted in the excretion of a corresponding amount of metachromatic activity.

Metachromatic Activity of Urine after the Continuous Intravenous Injection of Heparin. Previous work showed that rates of injection of 1 unit per kilogram per minute in dogs gave a constant raised clotting time, presumably due to a constant level of heparin in the circulation, and led to the clinical use of this method of administration of heparin. If this constant level of heparin were due to a constant rate of excretion, it should be recoverable from the urine. A series of experiments were, therefore, conducted in which heparin was given as a continuous intravenous injection for varying periods of time and the urine collected by catheter and assayed. The urine was also checked by the spot test and the time noted when it no longer showed metachromatic activity. The results are shown in table 3.
These experiments yielded a completely unexpected result. In four experiments with varying rates of constant infusion only traces of metachromatic activity were detectable in the urine (<1 unit per milliliter). However, in further experiments, a single injection or "primer" of 50 units of heparin per kilogram was given at the start of the experiment and 25 to 50 per cent of the metachromatic activity injected was recovered in the urine. As the "primer" did not amount to more than 10 per cent of the metachromatic activity injected, the excreted activity could not be considered as simply the transfer of this metachromatic activity but the material which can then be assayed for both metachromatic and antithrombic activity. This method has, therefore, been applied to the urine from rabbits, dogs, men and sheep which have received heparin by intravenous muscular, or subcutaneous routes. The results of a typical experiment are shown in figure 3. A man of 60 Kg. received approximately 400 mg. (44,360 MA units by assay) subcutaneously. The urine was collected by voiding, the bladder being emptied immediately before the injection. The total metachromatic activity found in the urine was equivalent to 5 per cent of that injected, of which half was excreted in 8 hours. Control values for urine without injection of heparin were 0.02 MA, 0.004 AT units per milliliter of urine, so that the results reported were related to the injection of heparin. The antithrombic activity of the benzidine precipitates from the urine samples was in all cases much lower than the metachromatic activity of the same samples. The ratio AT:MA showed a constant value of approximately 0.15. As commercial beef heparin standardized against the International Standard was used as the standard in these assays, both by definition and actual assay, the ratio AT:MA for the heparin injected was 1.0. This manifestly

<table>
<thead>
<tr>
<th>Kg.</th>
<th>Units/Kg.</th>
<th>Total</th>
<th>Recovery</th>
<th>Urine showed MA</th>
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<td>77</td>
<td>1.58</td>
<td>7.2</td>
<td>6</td>
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</table>

* Single intravenous injection at beginning of experiment.
† Not assayable but positive on spot test.

**Table 3.** Urinary Excretion of Metachromatic Activity with Constant Intravenous Injection of Heparin
differently material separated from urine after injection of heparin and showing very low antithrombic activity compared with metachromatic activity, we have named uroheparin.

A number of such experiments have been conducted with varying doses in different species with the results shown in table 4. It will be observed that in all cases the total excretion of uroheparin as well. The same was true of the intravenous injection in the dog, the ratio being 1.2 for the first 24-hour sample, and 0.4 for the second. The value after subcutaneous injection in men was 0.1, indicating excretion chiefly of uroheparin. The value of the ratio for the urine samples following subcutaneous injection in a sheep was

![Graph](chart.png)

**Fig. 3.** Activity of benznidide precipitate from urine after heparin injection. Man, 60 Kg.; 400 mg. (44,360 MA units) injected subcutaneously. Heparin in urine precipitated by the method of P. Astrup, in blood by the method of Monkhouse and Jaques, with the metachromatic activity measured in a Beckman spectrophotometer at 500 m. MA = metachromatic activity; AT = Anti-thrombic activity.

excretion of metachromatic activity was only 2 to 15 per cent of the activity injected as heparin. However, as judged by the ratio AT:MA, the nature of the product excreted varied with the dose and species. In the case of the first rabbit, the ratio AT:MA was 1, indicating excretion of the heparin injected. However, when the heparin was given subcutaneously, the ratio fell to 0.5, indicating about 0.6, or in between the other two values. Whether this represents a mixture of injected heparin and uroheparin or less extensive degradation of the heparin, we do not know.

**Properties of Uroheparin.** A number of attempts have been made to obtain this material from urine in purified form. It has been found that uroheparin is strongly absorbed by barium sulfate, brucine sulfate and
other precipitates formed with agents used to purify heparin and that some loss occurs on dialysis. These properties make difficult its separation from sulfates. Its metachromatic properties are destroyed by precipitation with glacial acetic acid, which is a useful method for freeing heparin of inorganic contaminants. The material also appears to be more sensitive to heat than heparin itself. It gives the same metachromatic band with azure A as heparin.

A partially purified product has been obtained for preliminary comparison of its activity in assay systems. Subject SH received 440 mg. of heparin subcutaneously. The total 48-hour urine was collected and precipitated with benzidine. The precipitate was hydrolyzed with ammonia, extracted with ether, and the aqueous phase precipitated with four volumes of ethyl alcohol to give 5.145 Gm. (1.43 units per milligram, 0.17 AT units per milligram); 3.9 Gm. were dissolved in water, filtered, dialyzed, and taken to dryness to give 92.6 mg. (28.6 MA units per milligram, 1.2 AT units per milligram), a yield of 47 per cent. Three subjects received a total of 1287 mg. heparin subcutaneously. The urine was collected for 10 hours after the injections in one fraction and for the next 50 hours as a second fraction. The urine from the three subjects was pooled to give two samples: pre-10, 2450 cc; and post-10, 7550 cc. Both urines were precipitated with benzidine and purified as above to give 63.3 mg. for pre-10 and 336 mg. for post-10. The three samples were assayed for metachromatic activity, antithrombic activity and for anticoagulant activity on a cat. The results are shown in table 5. The specific metachromatic activity of 25 to 30 units per milligram indicates that the material is sufficiently purified that impurities will not interfere with the assays to any degree. The data is valuable in giving necessary preliminary information about uroheparin, but the values are to be considered approximate figures pending accurate assays on more purified material. For comparison are shown the values calculated from data reported by Jaques, Charles and Waters,18 Jaques and Charles12 for dog and sheep heparin. In the case of these heparins, the AT:MA ratios were markedly different from those for beef heparin (2.6 and 0.24), while the AT:MA ratios (1.5 and 0.42) did not show as marked a difference. In contrast with the uroheparin samples, the AT:MA ratio showed the greatest difference: that is, these samples possessed very low antithrombic potency together with a significant anticoagulant potency. We can correlate these results with the original urine. The yield of metachromatic activity in the purified material compared to the initial benzidine precipitate was 30 per cent for pre-10, 37 per cent for post-10, and the AT:MA ratios of the crude benzidine precipitates were: SH, 0.12; pre-10, 0.14; post-10, <0.1, in general agreement with the ratios obtained on the purified material.

**Discussion**

The metachromatic activity of the urine following heparin injections may represent several substances. Until uroheparin is isolated in sufficient purity and quantity for extensive studies, it cannot be said whether this is one substance or a mixture of partially degraded heparins. However, the results reported in
tables 4 and 5 together with the yields on purification indicate that uroheparin may cover more than one chemical species. Therefore, purification of uroheparin requires quantitative recovery of the metachromate activity of urine. It is evident from the results in table 5, and the description of properties that whereas these show greatest differences from beef heparin in their anticoagulant activity (AC:MA → 1), and much less differences in antithrombic and similar activities (AT:MA → 1), uroheparin shows greatest difference from beef heparin in the latter assay.

As seen above, Astrup's benzidine precipitation method serves to recover metachromate material from urine for assay and as shown in table 4 will give either or both of two products, the commercial beef heparin injected and uroheparin, with the AT:MA ratio distinguishing between them. Until uroheparin has been obtained demonstrably free of heparin, so that its AT:MA ratio can be determined accurately, the ratio can be used only in a qualitative manner. It must be pointed out that as the ratio AT:MA depends on two assays each with a 5 per cent error, differences in the ratio must be at least 10 per cent to be significant. In the case of assays on the benzidine precipitates from urine, there is inter-
ference in the assays from impurities, and as the assay method used was only an approximate estimate of antithrombic activity with a 30 per cent error, not an actual titration as described by Jaques and Charles, much wider limits must be set.

The small amounts of metachromatic material obtained from normal urine by benzidine precipitation are not sufficient to affect the values reported following injection. However, it is of interest in itself as to its possible relation to naturally occurring heparin.

Following heparin injections, the metachromatic activity excreted in the urine represents only a small proportion of the heparin injected except after very large doses. The recent enzyme studies of Jaques and Keeri-Szanto appear to provide an explanation of this. They find that heparinase causes a parallel loss of metachromatic and antithrombic activity. It is evident then from the results reported here together with this finding, that uroheparin is not the product of heparinase action. Presumably it is the product of another enzyme, requiring future identification. Also, the chief metabolic products of heparin resulting from the action of heparinase are not metachromatic and other methods must be developed for their determination as excretion products.

CONCLUSIONS

1. After injection of heparin in man, dogs, rabbits and sheep, metachromatic activity (MA) was observed in the urine.

2. A factor was present in dog and human urine which enhanced the metachromatic activity of heparin.

3. The amount of metachromatic activity in the urine following single intravenous injections in dogs depended on size of dose but was much less than that of the heparin injected. The unrecovered metachromatic activity reached a limit with a dose of 800 units per kilogram. Continuous intravenous injections of 1 unit per kilogram per minute of heparin were not accompanied by significant excretion of metachromatic activity in the urine, but the additional injection of 50 units per kilogram initially resulted in the excretion of 25 to 50 per cent of the total metachromatic activity injected.

4. On quantitative precipitation of urine with benzidine, metachromatic activity equivalent to 2 to 13 per cent of the heparin injected was recovered with all species.

5. An excretion product of heparin with metachromatic activity, uroheparin, was obtained and its properties described.

6. It is concluded that following injection, heparin may be (a) destroyed by heparinase, (b) degraded to uroheparin, (c) excreted unchanged. In the case of b and c metachromatic activity is found in the urine.

REFERENCES


URINARY EXCRETION OF HEPARIN

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