THE CLEARANCE OF INULIN AND SODIUM P-AMINOHIPPURATE IN THE RAT

SYDNEY M. FRIEDMAN, JOHN R. POLLEY* AND CONSTANCE L. FRIEDMAN

From the Department of Anatomy, McGill University, Montreal, Canada

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The natural advantages of the rat as an experimental animal have made it worth while to establish reliable methods for the estimation of renal function in this species. In 1942, two of the present authors presented a method using inulin and diodrast which gave reproducible results and was of value in comparative experiments, although admittedly cumbersome (1). Dicker and Heller, in 1945, criticised the absolute value of the method since ether had been used and renal function probably considerably depressed (2). They presented a modified procedure which was, however, still too cumbersome to be of value for large groups of animals. In both these methods, the emptying of the bladder at the start of the procedure and again ten to thirty minutes later entailed a double hazard, since incomplete drainage at the start or at the end of the clearance period would give abnormally high or low clearance values respectively. Further, no attempt had been made to determine whether plasma concentrations of the test substances measured at the end of the clearance period were valid for the whole of that period.

More recently, Meyer Friedman has estimated the clearance of sodium p-aminohippurate (PAH) in the rat (3), but his methods are open to certain criticisms. It seemed to us that a useful clearance procedure should be established on a firm basis with some absolute validity and adapted for large series of animals. P-aminohippurate, whose advantages are well known (4), was used throughout instead of diodrast.

EXPERIMENTAL. The relation of dose to plasma level. Preliminary to arranging a satisfactory clearance procedure, the response of the plasma level to administration of the test substance (inulin or PAH) was investigated. This was considered necessary if the test substance were to be given as a single dose without sustaining infusions and if a single blood sample were to serve as a guide to the plasma level during the test period.

a. The PAH plasma level after injection. It was hoped that a quantitative picture of the plasma level could be obtained by following a single subcutaneous administration of PAH. Since clearance is expressed in terms of plasma, while such curves could only be studied using small samples of whole blood from the tail, preliminary experiments were performed to determine whether the whole blood analysis could yield the plasma concentration by calculation. The method of Bratton and Marshall for the analysis of PAH was used (5). Male albino rats were injected subcutaneously with small quantities of PAH and samples of

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blood taken by heart puncture. The hematocrit was determined and a sample of blood analysed directly. The remaining blood was then centrifuged and the supernatant plasma analysed. The precipitated red cells were washed with isotonic saline until the washings were free of PAH. The cells were then laked and analysed for their PAH content. Table 1 shows that the plasma level of PAH can be calculated accurately from the whole blood analysis if the hematocrit value is known.

PAH as the aqueous solution of the sodium salt was then injected into normal rats. The desired doses, 30 to 100 mgm., were prepared by diluting the clinical 20 per cent solution in 2 per cent sodium sulfate so that the required amount was contained in 4 cc. This quantity was injected subcutaneously and successive small samples of tail blood were analysed, 0.1 cc. of blood being used for the determination. Hematocrit values were determined at varying times in each experiment. As a final precaution, the PAH content of the final tail blood sam-

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td><strong>BLOOD PAH A</strong></td>
</tr>
<tr>
<td>mgm. %</td>
</tr>
<tr>
<td>2.0</td>
</tr>
<tr>
<td>1.5</td>
</tr>
<tr>
<td>2.0</td>
</tr>
<tr>
<td>2.4</td>
</tr>
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</table>

ple was compared with that of heart blood obtained at the same time. Curves of this type and in this range were constructed for 14 adult male rats. As shown in figure 1, the 40 mgm. dose yields a steady plasma level. In this case, the value at 52 minutes was 5.2 mgm. per cent, while the calculated average for that period was 5.3 mgm. per cent. Doses above 40 mgm. cause the plasma level to rise to a maximum within approximately 30 minutes and then to fall. With smaller doses the peak level is reached more rapidly. Apparently, with doses above 40 mgm. the hippurate enters the blood faster than it can be cleared by the kidneys. Thus, where the dose of PAH administered is of a size to produce a pronounced maximum in the curve, the end point plasma value may not represent the mean. Although the plasma concentration falls less rapidly after 60 minutes, clearance determinations based on end point values obtained even beyond this time still deal with a rapidly falling curve. On the other hand, repeated curves obtained from animals given a 40 to 50 mgm. dose showed that a plasma sample taken 50 minutes after injection was representative of conditions existing over the entire period.

These data indicate that the renal saturation level for PAH lies in the neighbourhood of 6 mgm. per cent. No variation in hematocrit was noted despite the repeated small bleedings and there was close agreement between the last tail
Fig. 1. The relation of the plasma concentration of PAH to the administered dose

Fig. 2. A type of plasma concentration response to administered PAH
blood analysis and the analysis of the simultaneous heart blood sample. The curve shown in figure 2 indicates that even with low doses, end point plasma values may be misleading. In this animal, the plasma level rose slowly to 6.3 mgm. per cent, then fell rapidly to 3.6 mgm. per cent at 54 minutes, a value not truly representative of the mean. Numerous experiments have shown that when the 50 minute plasma value lies below 4.5 mgm. per cent, the curve may be of this type.

![Figure 3](http://ajplegacy.physiology.org/)

**Fig. 3.** The relation of plasma concentration 50 minutes after subcutaneous administration of various doses of PAH.

Two conclusions with regard to clearance procedures for PAH using the single dose technique follow from this analysis. 1. If the clearance period begins immediately after injection and lasts 50 minutes, a steady plasma level can be maintained by a suitable dose. 2. If the clearance period starts 30 or 60 minutes after injection, requiring a larger initial dose of PAH, a variably falling plasma level during the clearance period results.

To ascertain the dose of PAH necessary to yield correct plasma levels 50 minutes after injection in different size animals, a large series of determinations was carried out. The data are summarised in figure 3. The plasma concentration 50 minutes after injection is almost linearly proportional to the dose adminis-
tered. The dose is not sharply critical, a difference of 10 mgm. producing a
change in plasma level of approximately 2 mgm. per cent. In this composite
curve a slight break occurs in the 40–50 mgm. dose range, corresponding to
5–7 mgm. per cent in the plasma. This break, not very apparent in the over all
grouping of animals weighing 100 to 200 grams, was very obvious with a more
restricted weight grouping. Thus, for animals weighing 120 to 140 grams, and
140 to 160, the curve breaks sharply between 5 and 7 mgm. per cent, a further
indication that this is the level of renal saturation.

b. The inulin plasma level after injection. As in the case of PAH it was of
importance to investigate the variation of the plasma concentration with time
after the administration of inulin. This problem was not studied previously
because of the lack of suitable chemical procedures. With the publication by
Harrison (6) of a method which could measure as little as 2γ of inulin it became
feasible to investigate this problem provided that 1, the plasma value could be

| TABLE 2 |
|---|---|---|
| **BLOOD INULIN** | **PLASMA INULIN** |  |
| | **Calculated** | **Analysed** |
| mgm. % | mgm. % | mgm. % |
| 6.5 | 13.0 | 14.0 |
| 10.0 | 20.0 | 20.8 |
| 8.0 | 16.0 | 15.6 |
| 13.5 | 27.0 | 26.5 |

obtained from the whole blood value by calculation, and that 2, in view of the
small size of the sample, the necessity for yeast treatment to remove fermentable
sugars could be avoided.

It was expected that no inulin would enter the red cell. Thus, assuming a
hematocrit of 50 per cent (normal for the rat) the plasma concentration should
be twice that of the blood. A series of animals was studied to satisfy this assump-
tion. Inulin was injected, a blood sample taken, and the inulin content of both
blood and plasma determined. Table 2 presents typical data, and shows that
the whole blood concentration obtained by analysis is directly convertible to
the plasma value providing the hematocrit remains unchanged.

In the analysis of whole blood it was found that the inulin content could be
estimated without yeast treatment by subtracting a blank blood value obtained
from the same animal before the administration of inulin. Indeed, a calibration
curve for inulin in blood differed from the aqueous curve only by the blank value
of the particular blood used to establish the curve (fig. 4). Investigation of a
series of normal animals showed that this blank value was fairly uniform.

A 2 per cent solution of inulin in saline was then prepared, adsorbed on char-
coal while hot, and filtered hot through a Seitz EK disc. Three cubic centi-
meters (60 mgm.) of this inulin solution were injected intraperitoneally to insure
uniform and rapid absorption into the blood stream. In our experience inulin
solutions are poorly absorbed from the subcutaneous tissues. The typical plasma concentration curves for a series of animals are shown in figure 5.

From a study of such curves it appeared that the intraperitoneal injection of this amount of inulin yielded a plasma concentration which increased smoothly for at least 50 minutes. The mean value of the inulin concentration in plasma for that period corresponded closely to two-thirds of the value obtained by analysis of a sample taken at 50 minutes.

![Diagram](http://ajplegacy.physiology.org/)

**Fig. 4.** The colorimetric reading of inulin in blood is the sum of its inulin content plus the blank value for whole blood.

**The clearance of inulin and PAH.** It became increasingly clear that for the determination of both inulin and PAH clearances, an experimental period extending for 50 minutes from the time of injection was an eminently suitable arrangement. Not only are the plasma concentrations reasonably steady, but in addition, the necessity of draining the bladder at the start of the period is eliminated. This diminishes the hazard of incomplete urine collection. Based on this, a series of clearance determinations were carried out in normal male rats. Although the clearance of inulin and PAH were determined simultaneously, for convenience in discussion they will be separately presented.

a. **The clearance of inulin.** The clearance of inulin, $C_{\text{IN}}$, was investigated in 60 adult rats. The average values obtained were plotted against the plasma
concentration, which ranged from 30 to 60 mgm. per cent. The inulin clearance was independent of plasma level in the range investigated; the average value for $C_{IN}$ when the plasma concentration was 31–40 mgm. per cent was 0.72 cc./100 grams/min., when 41–50 mgm. per cent, 0.75 cc., and when 51–60 mgm. per cent, 0.70 cc. In these experiments urine flow was not excessive, averaging 0.5 to 0.7 cc./hour, and inulin clearance was independent of the rate of urine flow.

![Fig. 5.](image)

Fig. 5. The plasma concentration of inulin after intraperitoneal administration of an aqueous solution in three animals.

b. The clearance of PAH. According to the classical work of Smith and his co-workers (7, 8) the clearance of a substance which is wholly removed from the plasma during one passage through the kidney measures the renal plasma flow. The clearance of this substance will be independent of its concentration in the plasma providing this does not exceed the capacity of the tubules. When the plasma concentration exceeds the saturation level of the tubules, further excretion becomes referable only to filtration, and the clearance is depressed.

In figure 6 the clearance of PAH, $C_{PAH}$, is plotted against the 50 minute plasma concentration. The data were obtained from the same 60 animals discussed above. It is evident that the clearance is independent of plasma level where this is not less than 4.5 mgm. per cent nor more than 7 mgm. per cent.
Above 7 mgm. per cent the clearance is progressively depressed while, on the other hand, abnormally high clearance values are obtained when the concentration falls below 4.5 mgm. per cent. This latter finding is interpreted as a confirmation of the caution stated previously, that a 50 minute plasma level below tubular saturation probably reflects a falling curve during the clearance period. Such end point values are lower than the true mean and hence yield falsely high clearance values.

![Figure 6](https://example.com/figure6.png)

**Fig. 6.** The clearance of PAH at various plasma concentrations. Each point is the average of determinations in several animals weighing 120 to 160 grams.

c. *The measurement of tubular mass.* The tubular excretion, T, of a test substance is the minute urinary output of that substance less the amount excreted by filtration. In calculating T, since the water content of plasma, W, is relatively constant at about 0.9, it is taken as unity. Similarly, for those plasma concentrations under discussion a negligible error is introduced by considering the freely filterable fraction of PAH, F, to be constant at 0.8 (1).

Once the tubules are saturated, T becomes a constant, Tm, which reflects the amount of functional excretory tubular tissue. As shown in figure 7 the saturation level, where T becomes a constant, is again 5 to 7 mgm. per cent. Beyond 7 mgm. per cent a secondary rise in T is observed. This is expected, since no correction was made for the change in F as the plasma concentration...
increased. Moreover, above tubular saturation the curve of plasma concentration for the period is humped and the 50 minute plasma level used in the calculation of $T$ is lower than the actual mean plasma concentration for the period.

It is important, therefore, in the study of clearance values in normal animals that the terminal plasma level of PAH lie within a restricted range, if values derived are to have absolute as well as relative meaning.

Procedure for the simultaneous determination of inulin and PAH clearances in large series. In designing a clearance procedure applicable to the study of large series of animals the method should be as simple as possible. To effect this the inulin and PAH analyses were modified so that they could be carried out simultaneously as far as possible. The animal and analytical procedures were as follows.

Animal methods. Four cubic centimeters of PAH solution (12.5 mgm./cc. in 2 per cent sodium sulfate) are injected subcutaneously in the lumbar region. The 50 mgm. dose will yield the correct plasma level in almost all animals of 160 to 210 grams. If the weight is 120 to 160, a 45 mgm. dose suffices, while for animals of 220 to 260 grams the dose may be raised to 55 mgm.

Immediately following this, 3 cc. of warm inulin solution (2 per cent inulin in saline) is injected intraperitoneally. Although the inulin solution in the earlier experiments was treated to remove pyrogen, we have latterly found this unnecessary. The collection period

Fig. 7. Tubular excretion of PAH at various plasma concentrations. Each point is the average of determinations in several animals weighing 120 to 160 grams.
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is timed from the completion of the second injection. The rat is placed directly into a metabolism funnel. Fifty minutes after injection the rat is picked up over the funnel and the bladder drained by suprapubic pressure. Micturition is usually quite free and spontaneous, however. Immediately following urine collection 0.75 cc. of blood is obtained by heart puncture, using a 24 or 25 gauge needle. With practice, the blood is taken during the 51st minute. The animal is then returned to its cage unharmed and the blood centrifuged for 15 minutes at high speed.

The funnel is rinsed thoroughly and urine and funnel washings made up to a total volume of 100 cc., 0.2 cc. of which is used for the analytical procedure. If the diluted urine is heavily contaminated with feces it may be cleared by filtration; under ordinary circumstances, however, it remains reasonably clear. Attempts to filter the urine directly by devices within the funnel are not advisable since there may be the loss of a drop of urine containing a high concentration of the test substances. Urine made up as above yields a negligible blank value for both PAH and inulin despite feces contamination.

Chemical methods. 1. Add 0.2 cc. plasma to 6 cc. distilled water, rinsing the pipette thoroughly.
2. Add 2 cc. of 18 per cent trichloracetic acid. Shake and centrifuge at moderate speed for 5 minutes.

PAH

3a. To 3 cc. of supernatant add 0.3 cc. of 0.1 per cent sodium nitrite. Shake and allow to stand for 3 minutes.
4a. Add 0.3 cc. of 0.5 per cent ammonium sulphamate. Shake and allow to stand for 3 minutes.
5a. Add 1.5 cc. of 0.1 per cent N-(naphthyl) ethylene diamine dihydrochloride. The color develops rapidly. Allow to stand 10 minutes, then transfer to a micro colorimeter tube for reading in a photoelectric colorimeter using a 525 μ filter. The colorimeter is set to zero with a colorless reagent blank.

Inulin

3b. Place 2 cc. of supernatant in a boiling tube.
4b. Add 4 cc. of Harrison's reagent.
5b. Place the tube in boiling water for 30 minutes, cool at room temperature for 30 minutes, transfer to a micro colorimeter tube and read in photoelectric colorimeter with a 625 μ filter. The colorimeter is set to zero with a reagent blank.

Experiments with rat plasma show a negligible blank for PAH and a small relatively constant blank for inulin where the solutions are free of turbidity. At inulin plasma levels above 30 mgm. per cent the blank value may be ignored.

Urine diluted as stated in the section dealing with animal methods is handled in the same way as plasma, 0.2 cc. being further diluted in 6 cc. of water and exposed to the precipitating agent.

Using this technique, the animals are conveniently handled at the rate of 16 a day by one operator and one analyst.

In calculating the normal clearance values obtained where the terminal PAH level is between 5 and 7 mgm. per cent it was observed that renal function did not increase in direct proportion to body weight, so that expressing results in terms of 100 grams yielded lower values for larger animals, as seen in table 3. This difference disappeared if the results were corrected according to kidney weight using the formula of Braun Menendez (9). The formula, \( y \) (kidney weight) = 2.06 \( x \) (body weight) + 277, is in reality only a special case of the general power formula \( y = ax^b \), where \( a \) and \( b \) are constants; kidney weight is
actually a function of surface area. Meeh's application of the power formula to surface area in the rat, \( y(\text{cm}^2) = 11.23 \text{ weight}^{2/3} \), was applied to the data and table 3 shows the identity of values obtained for the two series of animals when the data were recalculated for \( 100 \text{ cm}^2 \).

<table>
<thead>
<tr>
<th>GROUP</th>
<th>NUMBER OF ANIMALS</th>
<th>WEIGHT (gms.)</th>
<th>( \text{CIN cc./100 g.} )</th>
<th>( \text{CPAH cc./100 g.} )</th>
<th>( \text{TmPAH mg./100 g.} )</th>
<th>( \text{CIN cc./100 cm}^2 )</th>
<th>( \text{CPAH cc./100 cm}^2 )</th>
<th>( \text{TmPAH mg./100 cm}^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>142</td>
<td>0.84</td>
<td>5.02</td>
<td>0.23</td>
<td>0.39</td>
<td>2.32</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( \pm 0.06 )</td>
<td>( \pm 0.36 )</td>
<td>( \pm 0.01 )</td>
<td>( \pm 0.02 )</td>
<td>( \pm 0.17 )</td>
<td>( \pm 0.01 )</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>177</td>
<td>0.69</td>
<td>4.40</td>
<td>0.19</td>
<td>0.36</td>
<td>2.31</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( \pm 0.10 )</td>
<td>( \pm 0.34 )</td>
<td>( \pm 0.01 )</td>
<td>( \pm 0.04 )</td>
<td>( \pm 0.18 )</td>
<td>( \pm 0.01 )</td>
</tr>
</tbody>
</table>

In table 4 normal clearance values obtained from 14 animals selected at random from our large series are shown. The results group closely as shown by the low standard deviation in each case.

**DISCUSSION.** In the original attempt to apply the inulin and diodrast clearance methods to the rat the importance of a representative plasma sample was recognized. A clearance period of one hour was used and a mid point blood sample obtained. Since one hour had been allowed for equilibration of the test substance before beginning the clearance period it was felt that the slope of fall of the plasma level during the second hour would be uniform and a mid point sample would accurately represent the mean. While consistent results were
obtained with the method it was admittedly laborious. Dicker and Heller (2) modified the technique to use a short clearance period of 10 to 30 minutes and introduced the device of a terminal blood sample as an estimate of the mean. It was not practical to test the validity of this end point determination since the diodrast analysis required sizable amounts of plasma and precluded the possibility of studying a plasma curve.

More recently Meyer Friedman (3) has studied the clearance of PAH in the rat and arrived at certain interesting conclusions. His method is open to considerable criticism, however, in the light of the present findings. For example, he studied the clearance of 100 mgm. of PAH in the hour following subcutaneous injection. This amount gives a markedly humped plasma curve and is well above the saturation limit. We cannot but feel that the ensuing end point plasma levels, as published, are essentially meaningless.

Dicker and Heller reported no variation in the inulin clearance with urine flow in the range studied. The present observations confirm these findings for low rates of urine formation (up to 0.75 cc./hr.). On the other hand, both Friedman (3) and Braun Menendez and Chiodi (10) have shown an increase in the inulin clearance with higher flows. Estimates derived from Friedman's data indicate that C_PAH is actually increased by the excessive hydration, and this increase in renal plasma flow would seem the obvious explanation for the increased glomerular filtration rate observed. It does not seem over-cautious to assume that urine flow rates exceeding 1 cc. per hour involve far reaching changes in renal hemodynamics which invalidate any attempt to interpret the clearance values obtained.

The clearance data of Braun Menendez and Chiodi are especially interesting. Their data are complicated by the presence of hydration since they were interested in this particular feature, but, in general, using a technique similar to that of Dicker and Heller they obtained values for C_IN and TmD of the same order as those here presented. Similarly, C_D was comparable to the normal value here given for C_PAH when urine flow was of similar magnitude and plasma diodrast did not exceed 3.5 mgm. per cent iodine.

In the present series the normal renal plasma flow, C_PAH, is higher than that previously reported and the C_PAH/TmPAH ratio higher than in any other species yet studied. It is not unexpected that this should be so when, for example, the normal pulse rate in the rat is approximately 300 per minute (11) when compared with 70 for man.

**SUMMARY**

1. Renal function has been investigated in the rat using inulin and sodium p-aminohippurate.

2. The calculation of results is most suitably based on surface area. The normal values obtained, expressed per 100 cm² of body surface are as follows: C_IN 0.36 ± 0.04 cc./min.; C_PAH 2.31 ± 0.18 cc./min.; TmPAH 0.10 ± 0.01 mgm./min.; F. F. 15.7 per cent ± 2.3; and C_PAH/TmPAH 22.1 ± 2.0.

3. Renal plasma flow per unit of tubular excretory tissue is greater than in other animals investigated and may be related to hemodynamic differences.
Acknowledgment. The PAH used in these experiments was supplied through the courtesy of the Research Division of Sharp and Dohme, Ltd., Glenolden, Pa.

REFERENCES
(1) Friedman, S. M. and C. A. Livingstone. This Journal 137: 564, 1942.