Vasodilator Effects of a Substance Present in Normal Human Urine in Comparison with the Effect of Methacholine and Sodium Nitrite

A Statistical Analysis of Reliability of the Method of Assay

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This paper presents a method for bioassay of vasotropic substances. Data on the accuracy of the method are presented in terms of the response to repeated injections of methacholine. Some of the factors affecting the response to methacholine are analyzed. The peripheral vasodilator effect of a nondialyzable substance present in human urine is demonstrated and the effectiveness of this substance is related to that of methacholine.

A PREVIOUS paper3 from this laboratory described the temporary decline in arterial pressure produced by the intravenous injection of a substance present in normal urine. It was suggested that this substance probably acts by causing peripheral vasodilation.4* In this paper we describe a method for assaying vasotropic substances by injecting them intra-arterially while recording the venous outflow from the isolated hind limb of the dog. This paper demonstrates by means of this assay technic that the vasodepressor substance in urine has a direct vasodilator effect. The suitability of this method of assay of vasotropic substances is discussed and certain of the factors which lead to variability in the responses to intra-arterial injections are analyzed. This method of assay has the advantage that repeated injections may be made in the same animal and only 0.2 to 0.8 ml. of urine is required whereas 2 to 10 ml. are required when the assay is made by intravenous injections.3

METHODS

a. Operative Preparation and Flowmeter Equipment. The general arrangement of the animal and equipment are shown in figure 1. Twenty-five dogs weighing 7 to 22 Kg. (average 13.6) were anesthetized with 30 mg. per kilogram of sodium pentobarbital.* The femoral artery and vein of one hind leg, usually the left, were dissected free. The vein was prepared for peripheral cannulation while a small superficial arterial branch was prepared for central cannulation, the former to be used for venous outflow and the latter for the intra-arterial injections. Dissection was carried through from the femoral triangle to the sciatic sheath. Tourniquets were passed through and around the medial and lateral muscle masses and tightened so as to occlude all collateral venous and arterial channels,6,7 while excluding the femoral artery and vein and the femoral and sciatic nerves. Cessation of venous outflow following occlusion of the femoral artery was used as evidence of effective occlusion of collateral circulation. In the opposite leg, the femoral artery was cannulated for registration of mean arterial pressure and the femoral vein for return of the blood obtained from the perfused leg. In many experiments, a pressure regulator* was connected to a cannula inserted into a carotid artery to assist in stabilizing the arterial pressure. Coagulation of the blood was prevented by heparin; the initial dose of 5 mg. per

* Kindly furnished by Premo Pharmaceutical Laboratories, New York, N. Y.

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Preliminary reports of this work were presented at the 1948 and 1949 meetings of the American Physiological Society.1,2 During the study, L. T. F. was a fellow of the Life Insurance Medical Research Fund.

* For additional literature on the urinary depressor substance the reader may refer to references 3 and 14 to 16.
kilogram was followed by 1.6 mg. per kilogram every half hour thereafter.

The rate of outflow was measured by the piston-type of volume recorder shown in figure 2. This meter operates on the Gaddum principle of a constant cycle length in which the height of each stroke indicates the rate of flow. The cycle length of 10 seconds used approximately two and a half seconds for filling and seven and a half seconds for draining. This timing allowed completion of both the filling and emptying phases during each 10 second cycle. The meter contained an L stopcock rotated by a solenoid which in turn was actuated from a relay connected with the laboratory timing circuit. When rotated to the A position the L stopcock connected the reservoir which collected blood from the femoral vein with the measuring tube in which was a piston recorder. When rotated to the B position the stopcock connected the measuring tube with a constant level outflow device which caused the piston recorder to return to the same point at the end of each stroke. With this device, there was never any outflow blood which was not measured. The recorder used had a range of 0 to 20 ml. per 10 seconds. This was quite adequate since, under control conditions, most of the hind legs studied had control flows of 2 to 6 ml. per cycle. Thus the meter had ample capacity to handle the increased flow seen during the experimental period. The over-all apparatus was calibrated by running fluid through it at various constant rates and measuring the amplitude of deflection. The calibration was linear. The apparatus is described more fully elsewhere.9, pp. 71–73; 10

The blood leaving the flowmeter was collected and reinfused into the dog every four minutes; the volume reinfused each time was approximately 100 ml. Blood for the initial filling of the pressure regulator and flowmeter and for providing a reserve of blood was obtained from a separate dog.

b. Control Experiments. In six control experiments methacholine, dissolved in pyrogen-free physiologic saline (0.2 µg. per ml.), was given in amounts of 0.2 to 0.8 ml. over a period of 20 seconds; control injections of the saline were given in like volume. In these control experiments flows and responses to the drug injections were recorded for one to two and three-quarters hours in the intact preparation; following this the vagi were severed and the carotid arteries ligated and flow responses recorded for two to six additional hours; the femoral and sciatic nerves were then cut as high as possible, and the flow responses were followed for an additional three and one-half hours. In experiment 2 the dog died at the time of nerve section; in experiment 3 the dog survived one-half hour and in experiment 4, one and one-quarter hours. In experiments 3 and 4 only one injection of methacholine was made after the nerve section. In experiments 5 and 6, flow responses were recorded for two and three and one-half hours in the intact preparation; after this the femoral and sciatic nerves were sectioned as high as possible and flow responses followed for three and three and one-half hours respectively. The total hours of perfusion were: experiment 1, nine and one-half hours; experiment 2, four and one-half hours; experiment 3, six and three-quarters hours; experiment 4, five and three-quarters hours; experiment 5, seven hours; and experiment 6, five hours.
"Method of Preparation of Urines. All urine specimens were collected in autoclaved flasks from male subjects after careful cleansing of the penis and after discarding the initial voided urine. Part of the urine, indicated as P, was stored unaltered; another portion, F, was filtered through a Seitz filter; a third portion, D, was dialyzed in sterile viscose cellophane bags through frequent changes of distilled water at 4 to 9 C. for 24 hours; a fourth portion, FD, was both filtered and dialyzed. All samples were either used immediately, or were stored at −20 C. until used. Representative samples were found pyrogen-free using the rabbit as test animal. All equipment used in the preparation and storage of the urine was thoroughly washed and then rinsed with pyrogen-free saline before autoclaving. All specimens of urine were examined for clarity and frequent checks were made of the urine in various stages of preparation by inoculating the urine on tryptose phosphate broth and thioglycolate media. Cultures were read after 48 hours. Sterile, pyrogen-free saline, handled in the same manner as the urines, served as controls. Urines prepared in the above manner were studied for their vasomotor activity by intra-arterial injection into dogs as described above for methacholine. The potency of the urine was expressed in terms of the amount of methacholine required to give a similar vasodilator response.

Results
A. Method for Measuring Flow Curve

Figure 3 shows a typical flow curve in which is recorded the response to two alternate intra-arterial injections of 0.4 ml. of urine and to two injections of 0.4 ml. of methacholine chloride (0.2 μg. per ml.) The flowmeter had a cycle length of 10 seconds (6 cycles per minute). The calibration on the left gives the flow per cycle. The calibration on the right gives the flow per minute represented by the amplitude per stroke of the flowmeter (that is, the flow per cycle × 60/cycle length in seconds). During the control period the flow was 2.4 ml. per cycle, that is, 14.4 ml. per minute. Within 2 to 3 cycles after the intra-arterial injection was started, the venous outflow had begun to increase. It reached maximum of 5.5 to 6.4 ml. per cycle (33 to 38 ml. per minute) in five cycles after the start of the injections and returned approximately to control values in seven cycles with the urine and in 10 cycles with the methacholine.

The responses to injections were analyzed as follows:

1. \( \Delta F \) (increment in flow) = \( MF \) (maximum flow) − \( CF \) (control flow).

2. Per cent \( \Delta F \) (percentile increment in flow) = \( \Delta F \times \frac{100}{CF} \).

Selection of the highest flow cycle during the response to the drug may lead to some variability, since the cycle length of the flowmeter
usually used was 10 seconds. However, this effect was minimized by making each of the injections at approximately the same moment in the flowmeter cycle. The method of calculation was checked also by integrating the flow throughout the vasodilator effect. This method did not give any more constant response than the use of the single greatest cycle. Since use of the highest cycle is much quicker and appeared to be equally as accurate we have expressed all the results in this paper in this manner. It was found by experience that a response equal to a 100 to 200 per cent increase in flow (per cent $\Delta F$) was most convenient for use as the change was of sufficient magnitude to be measured easily, yet not so great that the effect lasted an excessive length of time.

B. Responses to Intra-arterial Injection of Saline

Control injections of the saline used in preparing the methacholine solution were given in volumes equivalent to those of the urine and reference solutions in most of the experiments. In most cases 0.4 ml. of the solution caused either no increase in flow, or a minimal, brief increment amounting to a percentile increment in flow (per cent $\Delta F$) of 15 to 60. In one experiment saline caused percentile increments of flow of 90 to 147, comparable with those produced by the urine; this experiment was discarded.

C. Reference Vasotropic Substances

It was assumed that the response to a given amount of vasotrophic substance would vary from animal to animal, but that the ratio of the response to substance A and the response to substance B might be somewhat more constant from animal to animal. If this were shown to be true then the potency of substance B could be expressed in terms of the amount of substance B required to give the same vasodilator effect as a standard amount of reference substance A. Two reference substances were examined, sodium nitrite and methacholine. It was found that 0.2 to 0.4 ml. of a 10 per cent solution of sodium nitrite gave percentile increases in flow ranging from 34 to 584 (average 138) in the nine experiments in which the substance was used; and, that 0.4 ml. of a solution containing 0.2 $\mu$g. per ml. of methacholine gave percentile increases in flow of 26 to 688 (average 162) in the 10 experiments in which it was used. The flow curves for sodium nitrite and urine were similar but sodium nitrite, upon repeated injection, tended to cause methemoglobinemia. Methacholine induced responses the duration and contour of which were similar to those caused by the vasodilator substances in urine. (See fig. 3.) Furthermore, the time interval between the drug administration and the beginning of response, the magnitude of response and the duration of response did not change markedly as the experiment progressed and no tachyphylaxis was noted to repeated injections of the methacholine.

D. Control Experiments using Methacholine

(1) Magnitude of Control Flows. As shown in table 1, the control flows ranged from 13 to 39 ml. per minute in the six intact preparations. The animal weights varied from 10 to 13 Kg. Since the perfused mass of the legs was not determined, no attempt was made to relate the flows to the mass of the extremity.

(2) Variability of Control Flows in the Control Experiments. In the six intact preparations (table 1) control flows decreased steadily in three dogs, falling 25 to 67 per cent; in one the flow rose 100 per cent; and in the remaining two control flows did not change significantly. The coefficient of variation for the control flows in these experiments ranged from 9 to 24 per cent (average 9 per cent). Mean arterial pressure in the various experiments between 80 and 116 (average 101 mm. Hg); the coefficient of variation for mean arterial pressure in these six control experiments ranged between 1 and 11 per cent (average 6 per cent). Since, in five of the six experiments, mean arterial pressure fluctuated only $\pm$ 5 mm. Hg, the changes in control flow could not be correlated with the fluctuations in mean arterial pressure. Additional injections of anesthetic had to be given at irregular intervals. These injections (2 ml. of 3 per cent sodium pentobarbital) increased the control flow 30 per cent to 400 per cent and the increase in flow persisted for 5 to 10 minutes.

(3) Effects of Vagal Section, Carotid Artery
Ligation and Femoral and Sciatic Nerve Section on Control Flow in the Control Experiments. After a series of injections either the vagus nerves were sectioned and the carotid arteries ligated to minimize pressoreceptor influences, or the femoral and sciatic nerves were cut to eliminate nerve impulses going to the perfused leg. In one instance section of the vagus nerves and ligation of the carotid arteries caused the control flow to decrease almost immediately. In the three other experiments leg flow decreased after section of the vagus nerves and ligation of the carotid arteries, but the results were less clear cut because the control flow was declining at the time of the section. However, these procedures usually increased the variability of the perfusion pressures, the control flows, and maximum flows and the changes in flow. Following section of the femoral and sciatic nerves the control flows increased by 150 to 430 per cent in three of the four experiments in which prior section of the vagus nerves and ligation of the carotid arteries had been performed. The remaining dog of this group died at the time of nerve section. Section of the femoral nerves in the two intact animals caused 32 per cent and 100 per cent increases respectively in control flow.

(4) Variation of Response to Repeated Injections of Methacholine in the Control Experiments. In order to determine the reliability of the assay method, we made repeated injections of the same quantity of methacholine in each of the six control experiments. The results are shown in table 1, part I. Column A identifies the experiment; B gives the animal weight, C the number of similar injections, D the perfusion pressure (mean arterial pressure), E the control flow (CF), F the maximum flow (MF), G the maximum increment in flow (ΔF), and H the percentile increment in flow (ΔF/CF × 100 = per cent ΔF) in the intact preparations.

In the six intact animals (table 1), the maximum flows tended to vary slightly less than the control flows (coefficients of variation 4 to 16 per cent), but the increment in flow (ΔF) varied more than either the control flow or the maximal flow, that is, the coefficients of variability for ΔF were 4 to 41 per cent. When the results were expressed as per cent ΔF rather than as ΔF, the coefficients of variability were increased in three of the six experiments on the intact dogs, and were essentially unchanged in three (range 4 to 48 per cent).

(5) Effect of Severing the Vagus Nerves, Ligation of the Carotid Arteries, and Section of the Femoral and Sciatic Nerves on the Variability of the Response to Methacholine. The possibility was considered that changes in control flow due to variations in the activity of the reflex homeostatic mechanisms might be playing a part in causing the variability in response to successive injections. Therefore, as noted above, in several of the experiments (a) the vagi were severed and the carotid arteries ligated (column I, table 1); (b) the femoral and sciatic nerves were severed (experiments 5 and 6, column J, table 1), or (c) both procedures were carried out successively (experiment 1, column J, table 1). As shown in table 1, these procedures decreased the variability in two experiments and increased the variability of the changes in flow in two experiments.

(6) Relationship of Response to Vasomotor Tone in the Extremity in the Control Experiments. In attempting to determine some of the causes of variability of response to the methacholine, we analyzed the effects of changes of vasomotor tone upon the responses. Comparisons of pairs of injections, in the intact dogs, in which the perfusion pressure was constant but the control flow had varied, fairly consistently indicated that the response was greater the lower the control flow, that is, the higher the control vasomotor tone. During the increase of vasomotor tone that followed vagal and carotid section the responses tended to be decreased, and during the decrease in vasomotor tone that followed sciatic or femoral nerve section the responses tended to be increased. However, the change was not consistent enough to be taken as indicating a definite relationship. During the periods of increased flow which followed injections of additional amounts of anesthetic, the increments in flow in response to methacholine usually were less than those recorded just prior to the injection of anesthetic.
### Table 1.—Control Experiments. Variability of Responses to Repeated Injections of Methacholine and Urine

<table>
<thead>
<tr>
<th></th>
<th>Intact Animal</th>
<th>After Vagi Cut and Carotid Arteries Ligated</th>
<th>After Cutting Femoral and Sciatic Nerves</th>
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<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Exp. No.</td>
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<tr>
<td>Wt. Kg.</td>
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<tr>
<td>No. of Injs.</td>
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<tr>
<td>Mean Art. Press.</td>
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<td></td>
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<tr>
<td>mm. Hg</td>
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<tr>
<td>Control Flow</td>
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<td></td>
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<tr>
<td>ml./min.</td>
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<tr>
<td>Control Flow</td>
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<tr>
<td>ml./min.</td>
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<td></td>
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<tr>
<td>Maximum Flow</td>
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<tr>
<td>ml./min.</td>
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<td></td>
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<tr>
<td>ΔF (MF – CF)</td>
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<td></td>
<td></td>
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<tr>
<td>ml./min.</td>
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<td></td>
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<tr>
<td>Percent ΔF (ΔF</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CF × 100)</td>
<td></td>
<td></td>
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<tr>
<td>Range Av.</td>
<td></td>
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<td>Range Av.</td>
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<tr>
<td>C. of V.</td>
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<tr>
<td>Range Av.</td>
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<tr>
<td>C. of V.</td>
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<tr>
<td>Range Av.</td>
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<tr>
<td>C. of V.</td>
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#### I. Responses to Intra-Arterial Injections of Methacholine—0.4 ml.

(0.2 μg./ml.)

|                  |    |    |    |    |    |    |    |    |    |    |    |    |
| Exp. No.         | 1  | 2  | 3  | 4  | 5  | 6  | 1  | 2  | 3  | 4  | 5  | 6  |
| Wt. Kg.          |    |    |    |    |    |    |    |    |    |    |    |    |
| No. of Injs.     |    |    |    |    |    |    |    |    |    |    |    |    |
| ΔF (MF – CF)     |    |    |    |    |    |    |    |    |    |    |    |    |
| ml./min.         |    |    |    |    |    |    |    |    |    |    |    |    |
| Percent ΔF (ΔF  |    |    |    |    |    |    |    |    |    |    |    |    |
| CF × 100)        |    |    |    |    |    |    |    |    |    |    |    |    |
| Range Av.        |    |    |    |    |    |    |    |    |    |    |    |    |
| Range Av.        |    |    |    |    |    |    |    |    |    |    |    |    |
| C. of V.         |    |    |    |    |    |    |    |    |    |    |    |    |
| Range Av.        |    |    |    |    |    |    |    |    |    |    |    |    |
| C. of V.         |    |    |    |    |    |    |    |    |    |    |    |    |

#### II. Responses to Intra-Arterial Injections of Dialyzed Urine—0.4 ml. (60D)

|                  |    |    |    |    |    |    |    |    |    |    |    |    |
| Exp. No.         | 5  | 6  | 7  | 5  | 6  | 7  |    |    |    |    |    |    |
| Wt. Kg.          |    |    |    |    |    |    |    |    |    |    |    |    |
| No. of Injs.     |    |    |    |    |    |    |    |    |    |    |    |    |
| ΔF (MF – CF)     |    |    |    |    |    |    |    |    |    |    |    |    |
| ml./min.         |    |    |    |    |    |    |    |    |    |    |    |    |
| Percent ΔF (ΔF  |    |    |    |    |    |    |    |    |    |    |    |    |
| CF × 100)        |    |    |    |    |    |    |    |    |    |    |    |    |
| Range Av.        |    |    |    |    |    |    |    |    |    |    |    |    |
| Range Av.        |    |    |    |    |    |    |    |    |    |    |    |    |
| C. of V.         |    |    |    |    |    |    |    |    |    |    |    |    |
| Range Av.        |    |    |    |    |    |    |    |    |    |    |    |    |
| C. of V.         |    |    |    |    |    |    |    |    |    |    |    |    |

* Urine and methacholine injections given alternately.
† C. of V. equals standard deviation over mean × 100.
E. Vasodilator Effects of Urine and Various Urinary Extracts

(1) Typical Flow Response to Urine. Figure 3 reproduces two flow responses to the intrarterial injection of 0.4 ml. of urine. As may be seen by comparison with the other two responses, the amplitude of the flow increment and the duration of the effect in this experiment is about the same as that noted with 0.08 μg. of methacholine.

(2) Variability of Response to Repeated Injections of Urine into the Same Animal. The effects of repeated injections of the same quantity (0.4 ml.) of the same dialyzed urine preparation (60D) were studied in three intact animals, (table 1, part II, columns A–H), and again in the same animals after cutting the femoral and sciatic nerves (columns K and L). Two of these animals (5 and 6) were the same as those used for testing methacholine; in these the injections of the urine preparation and methacholine were given alternately. There was no systematic variation in response which would suggest tachyphylaxis. The magnitude and the variability of the responses to the urine preparation were of the same order as those noted with methacholine. Section of the sciatic and femoral nerves increased slightly the variability of the response to the urine preparation in all three experiments.

(3) Variation in Vasodilator Effect of the Urine in Different Experiments. Plain sterile urine was injected intra-arterially in doses of 0.1 to 0.4 ml. in 16 intact animals. The percentile increment in flow (per cent ΔF) in the various experiments in response to 0.4 ml. of urine varied from 6 to 410 (average 180). When different urines were tested in the same animal they varied considerably in their potency. Urines from some human subjects possessed no vasodilator effects. Such urines were eliminated from the study. Injection of the same urine preparation into each of several legs gave responses which varied quite widely; the variation between leg preparations was, however, of the same order as that noted for methacholine.

(4) Relationship of Vasodilator Effect of Urine to That of the Reference Substances. Because of the wide variability of the response of both the reference substances and urine from test animal to test animal, we made repeated alternate injections of the same preparation of urine, and of methacholine in each of five dogs' legs. Table 2 shows the result of this comparison, using in each case 0.4 ml. of a dialyzed urine preparation (60D) and 0.4 ml. of the standard methacholine solution (0.2 μg. per ml.). The responses to both methacholine and urine varied considerably from time to time in each of the experiments. However the ratio of the response to methacholine to the response to urine remained relatively constant so long as pairs of injections, made near the same time in the experiment, were compared. Furthermore, as shown in table 2, despite the considerable fluctuation in the various responses from time to time in the same experiment, and from experiment to ex-

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Urine Preparation</th>
<th>Methacholine</th>
<th>Ratio C/F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of Injections Range Av.</td>
<td>Number of Injections Range Av.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>16 103–410 167</td>
<td>8 110–287 182</td>
<td>0.92</td>
</tr>
<tr>
<td>6</td>
<td>3 152–246 190</td>
<td>3 184–198 192</td>
<td>0.90</td>
</tr>
<tr>
<td>7</td>
<td>15 96–357 211</td>
<td>15 147–366 233</td>
<td>0.91</td>
</tr>
<tr>
<td>8</td>
<td>5 26–63 48</td>
<td>6 26–65 48</td>
<td>1.0</td>
</tr>
<tr>
<td>9</td>
<td>3 146–182 160</td>
<td>3 109–122 115</td>
<td>1.39</td>
</tr>
</tbody>
</table>

In all experiments the injections of urine preparation and methacholine were given alternately.

Table 2.—Comparison of responses to 0.4 ml. of a dialyzed urine preparation (60D) and to 0.4 ml. of a solution of 0.2 μg. of methacholine per ml. All responses are expressed as percentile increment in flow (per cent ΔF).
periment, the ratios of the average response to the urine preparation, to the average response to the methacholine solution were relatively constant from experiment to experiment.

(6) Relationship of Dose to Response. In six experiments the responses were sufficiently stable so that an approximation of the dose-response relationship was attempted. Plots of the data from these six experiments were made on linear and on single and double logarithm paper. In general over the range of 0.2, 0.4, 0.8 and 1.6 ml. the closest approximations to straight lines were obtained on double logarithm paper. Figure 4 shows a plot for the data for a typical experiment. The slopes were such that the percentile increments in flow were related to the dose according to the formula $R^x = K \cdot D$, where $x$ varied between 1 and 3. The average value for $x$ was 1.8.

(6) Effect of Filtration and Dialysis on Vasodilator Activity of Urine. Urines, prepared as described under Methods, were injected into test preparations. The vasodilator responses of the treated preparations were related to that on the original untreated urine. In seven experiments six different urines were tested. The average of the results of 15 sets of injections of each of the four kinds of urine preparations were as follows: filtered urine possessed 80 per cent, dialyzed urine 80 per cent, and filtered and dialyzed urine 70 per cent, of the vasodilator activity of the untreated urine. In all but one of the 15 trials, the vasodilator effect of the plain urine was as great as or greater than that of the treated urines. When pairs of responses to filtered and dialyzed urine were compared the latter was on the average 23 per cent less effective. However this average difference was not statistically significant and in four of the 15 pairs of injections the dialyzed urine gave a greater response than the filtered urine.

**Discussion**

Using the Dumke and Schmidt bubble flowmeter as a measure of control femoral artery blood flow, Kemp, Paul and Hines found average flows of 40 ml. per minute and 74 ml. per minute for two dogs. Higher average flows of 120 ml. per minute and 106 ml. per minute for two different dogs were later described. The lower flows previously recorded by these authors were thought by them to be the result of experimental error in that they had not waited a sufficient time for stabilization. Siems, Kosman and Osborne,\textsuperscript{13} using a modified Leden bubble flowmeter, noted average control flows of 42 ml. per minute. Richardson and co-workers,\textsuperscript{12} using the electromagnetic flowmeter, found control flow values of 59 to 66 ml. per minute. In our series the average control flows ranged from 9 ml. per minute to 35 ml. per minute or approximately one-half of that found by other investigators. Our lower flows may have been due: (1) to the use of smaller animals (the dogs in our control experiments weighed 10 to 13 Kg.); (2) to perfusion of a smaller fraction of the leg mass, or (3) to development of a shocklike state with vasconstriction as a result of trauma incident to the preparation of the leg for flow measurement.

We have been unable to find any literature primarily devoted to study of blood flow responses to methacholine with which to compare our results. An injection of 0.4 ml. of methacholine solution (0.08 $\mu$g.) produced a wide range of change of flow in the intact control experiments (+44 per cent to +287 per cent). The variation in response was considerably less within a single animal, but still such as to give coefficients of variation of 4 to 48 per cent.

The possible causes of the variability in flow following drug injections are numerous. They include changing vasomotor tone in the per-
fused leg due to spontaneous alterations in the activity of the vasomotor center and to the occasional injections of additional anesthetic, fluctuations in viscosity, alterations in mean arterial pressure, variations in the rate of drug injection, and development of refractoriness to the drug. No obvious refractoriness to either the methacholine or the urine preparations was noted. The rate and time of the drug injections was kept as constant as possible. Elimination of the effects of variations in activity of the pressor receptor organs and of the direct influence of the vasomotor center upon the leg by section of the femoral and sciatic nerves did not significantly reduce the variability of response to methacholine. The variability was considerably reduced by selection of experiments in which vasomotor tone was fairly constant. This suggests that part of the variability was due to irregular fluctuations in the concentration of circulating vasotropic substances.

The range of variation of the responses to methacholine was fairly large with the assay method described in this paper, but it was less than that obtained by recording the effects of vasodilator drugs on mean arterial pressure. Furthermore, when the vasodilator effect of urine was related to that of methacholine in pairs of injections given closely together in the same experiment, the measure of relative potency of the urine was much more constant. Since the method also demonstrates the precise peripheral vasodilator effects of the drug, we believe this procedure possesses advantages over registration of mean arterial pressure in the study of vasotropic substances.

A previous report has shown the presence of a depressor substance in human urine which may be the same as that described by Wollheim, but probably different from that described by Westerfield and co-workers. The vasotropic principle causes an increase in cardiac output and a decrease in mean arterial pressure. This paper demonstrates that urine and urine extracts possess a direct peripheral vasodilator action. This vasodilator effect is probably responsible, at least in part, for the depressor effect of urine.

The vasodilator effect is not due to the presence of pyrogenic substances since it is obtained with preparations of urine which are demonstrated to have been sterile throughout their process of handling and, furthermore, such preparations do not induce pyrogenic reactions when injected into rabbits. Pyrogen-free saline, handled in the same way as the urine, also does not develop significant vasodilator properties.

A vasodilator effect equivalent to that induced by 0.08 μg. of methacholine injected intra-arterially is produced by 0.4 ml. of plain urine obtained from many but not all subjects. The dose of urine or methacholine usually has to be increased roughly as the square (average 1.8 power) of the response, when the response is expressed as the percentile increase in maximum flow per cycle of the flowmeter. The vasodilator substance is reduced in potency by only 20 to 30 per cent by filtration and dialysis. This loss may be due to adsorption.

**Summary**

We have studied the blood flow response to methacholine injected intra-arterially in an isolated, blood-perfused leg. In a series of six control experiments, graphic and statistical studies were made of control flows, and of the changes in flow produced in the intact and in the denervated isolated leg by intra-arterial injections of methacholine. The responses to repeated injections of the drug varied considerably (coefficients of variation were 18 to 48 per cent). No tachyphylaxis to methacholine was demonstrated.

Section of the vagus nerves and ligation of the carotid arteries consistently reduced the control flows; section of the sciatic and femoral nerves and injections of the anesthetic regularly increased control flow, particularly if performed when vasomotor tone was high as a result of the preceding procedures. Neither removal of the active reflex homeostatic mechanisms (section of the vagi and ligation of the carotid arteries) nor section of the sciatic and femoral nerve supplying the perfused leg decreased the variability of the flow responses to methacholine. Elimination of data in which control flow varied considerably from the mean of the group resulted in a significant decrease in the variability of the responses to methacholine.

A vasodilator substance is present in normal...
human urine which cannot be attributed to bacterial contamination. The substance is only partially removed by Seitz filtration and dialysis through cellophane bags in distilled water. Its action appears to be peripheral, since it produces its effect when given directly intra-arterially in the intact or denervated extremity. No tachyphylaxis is noted to repeated injections. The concentration of this substance varies considerably in different urine specimens. On the average the amount present in 0.4 ml of urine is approximately equivalent in vasodilator effect to that of 0.08 µg of methacholine.

Despite the variability of the responses to methacholine and to urine, the ratio of the response to urine and the response to methacholine in closely adjacent pairs of injections remained quite constant. This method appears to offer certain advantages over registration of the effects of vasotropic substances on mean arterial pressure in the bioassay of vasotropic substances, particularly when their potency is related to that of a standard vasotropic substance.

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