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DIURESIS FROM LEFT ATRIAL RECEPTORS: EFFECT OF PLASMA ON THE SECRETION OF THE MALPIGHIAN TUBULES OF *RHODNIUS PROLIXUS*

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SUMMARY

1. Stimulation of left atrial receptors by distension of a balloon in the lumen of the left atrium of anaesthetized dogs was shown to result in an increase in urine flow. Plasma samples obtained from these dogs during control periods and during periods of stimulation were applied to the Malpighian tubules of *Rhodnius prolixus*.

2. It was found that the tubules suspended in test plasma secreted at a significantly lower rate than those suspended in control plasma.

3. These differences were also evident in extracts of plasma prepared using the solvent *n*-butanol.

4. Cutting or cooling the cervical vagi abolished these differences along with the increase in urine flow. It is argued that this preparation of the Malpighian tubule of *Rhodnius prolixus* could be used as a means of detecting the diuretic agent responsible for the increase in urine flow.

INTRODUCTION

Distension of a balloon in the lumen of the left atrium of anaesthetized dogs produces a diuresis (e.g. Henry, Gauer & Reeves, 1956; Ledsome, Linden & O'Connor, 1961) which has been shown to result from the stimulation of atrial receptors (Ledsome & Linden, 1968). The diuresis has also been observed in the denervated kidney (Ledsome *et al.* 1961) and in the isolated perfused kidney (Carswell, Hainsworth & Ledsome, 1970). From these experiments it was suggested that the diuretic response to distension of a balloon in the left atrium is mediated at least in part by a blood-borne agent. There has been considerable support for the suggestion that this response is mediated by a reduction in the concentration of circulating antidiuretic hormone (e.g. Share & Claybaugh, 1972). This suggestion has been disputed and an unknown humoral agent postulated (Kappagoda, Linden, Snow & Whitaker, 1974, 1975).

The present investigation was undertaken to determine the effect of plasma obtained from dogs during control periods and during stimulation of atrial receptors on the secretion of the Malpighian tubules of the blood-sucking bug *Rhodnius prolixus* and, thereby, to ascertain whether this preparation could be used as a method of detecting the humoral agent. A preliminary report of this investigation has already been given (Kappagoda, Knapp, Linden & Whitaker, 1976*a*).

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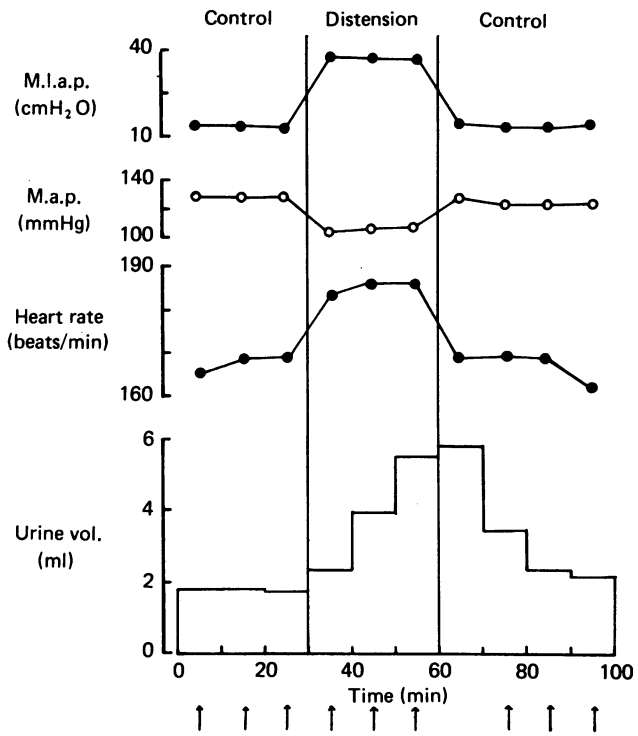
METHODS

Stimulation of atrial receptors

The experimental methods used in the present investigation relating to stimulation of atrial receptors in anaesthetized dogs have been described in detail previously (Kappagoda, Linden & Snow, 1972*a*, *b*) and only a brief summary is given below. Dogs weighing 18–24 kg were anaesthetized with chloralose and artificially respired. Soon after the induction of anaesthesia 500,000 i.u. benzyl penicillin (Crystapen, Glaxo Laboratories Ltd, Greenford) were administered I.V. The chest was opened in the fifth intercostal space on the left side. The pericardium was opened and a latex balloon attached to a nylon catheter (1 mm i.d.) was inserted into the lumen of the left atrium through the atrial appendage. The left atrium could then be distended by injecting warm saline (a solution of NaCl, 0·9 g/100 ml. at 38 °C) into the balloon. The volume injected was sufficient to increase the mean pressure in the left atrium by approximately 15 cm H_2O.



Text-fig. 1. The effect of distension of the balloon in the left atrium in one dog. From above down: mean left atrial pressure (m.l.a.p.); mean arterial pressure (m.a.p.); heart rate; urine volume collected over 10 min periods. The values for the atrial pressure, arterial pressure and the heart rate refer to the average values during the corresponding collection periods. The arrows indicate the times at which blood samples were collected for assay.

Pressures in the cardiovascular system and in the trachea, the e.c.g. and the end-tidal P_{CO_2} were recorded as described previously (Kappagoda *et al.* 1972*a*). The oesophageal temperature and the acid-base status of the animal were measured and maintained within normal limits.

Each ureter was catheterized through a suprapubic incision and the urine volume measured every 10 min. The sodium concentration in the urine was measured as described previously (Kappagoda *et al.* 1975).

After the surgical procedure had been completed and a steady state reached for at least 40 min

with respect to heart rate, arterial blood pressure and urine flow, the balloon in the left atrium was distended for a period of 30 min. The precise experimental protocol and the method of calculating responses were the same as those adopted previously (Kappagoda *et al.* 1972*b*).

In five dogs the cervical vagi were cooled to 6 °C (5 °C in the thermode) using a silver thermode (Kappagoda *et al.* 1972*b*) and in three dogs the cervical vagi were cut.

Plasma samples

Samples (5–10 ml.) of blood were collected from the femoral artery midway through each period of urine collection as indicated by arrows in Text-fig. 1. Three samples were obtained during each of the two control periods and during the period of stimulation respectively. The blood withdrawn was immediately replaced with an equal volume of Dextraven 150 (Dextran 150 in 5% Dextrose, Fisons Pharmaceuticals Ltd, Loughborough). The blood was collected into syringes chilled to − 15 °C and immediately centrifuged at 5000 *g* at 4 °C for 10 min. Plasma was removed from each 10 min collection and equal proportions were pooled to form three samples: a control sample before distension; a test sample taken during distension and a second control sample after release of the distension.

Technique for extraction of plasma

Samples of pooled plasma were shaken for 20 sec with 2 volumes of *n*-butanol (Chromopak-Vickers Analar grade) previously chilled to − 15 °C. This procedure was repeated three times. The aqueous and organic layers were separated by centrifugation at 700 *g* at 4 °C for 20 min; the butanol layer was removed and the aqueous layer re-extracted twice more in the manner described above. The butanol layers were pooled and dried under nitrogen at 38 °C. The dried residues were reconstituted by vortexing in *Rhodnius* Ringer solution (NaCl 129 mmole.l.$^{-1}$, KCl 8·6 mmole.l.$^{-1}$, $MgCl_2$ 8·5 mmole.l.$^{-1}$, $CaCl_2$ 2·0 mmole.l.$^{-1}$, $NaHCO_3$ 10·2 mmole.l.$^{-1}$, NaH_2PO_4 4·3 mmole.l.$^{-1}$ and glucose 34 mmole.l.$^{-1}$) for 20 sec, the final volume being a tenth the volume of the plasma. The aqueous solutions were centrifuged at 105,000 *g* at 4 °C for 45 min and the supernatant removed and stored at − 15 °C until tested on the tubules.

Secretion of Malpighian tubules

The investigation was performed on the distal region of Malpighian tubules of the fifth instar *Rhodnius prolixus* (Maddrell, 1969; Kappagoda, Knapp, Linden & Whitaker, 1976*b*). The tubules were dissected away from the other tissues in the abdominal cavity. A section of the distal end (about 20 mm) of each tubule was cut free and transferred to a drop of plasma or plasma extract suspended under liquid paraffin in a petri dish (Pl. 1). The petri dish contained a wax base into which small steel pins were inserted and these pins served to hold the suspended drops of plasma or plasma extract in place. The cut end of the tubule was drawn out from the drop and secured around another pin 2–3 mm away; fluid secreted by the tubule accumulated at this cut end. The petri dish was illuminated from below and a dissecting microscope with a micrometer scale attachment was used to measure the diameter of the secreted drop. The volume secreted was calculated on the basis that the drop was spherical in shape.

The following protocol was adopted for studying the effect of control and test plasma. Two of the four tubules from any one insect were transferred to two drops of control plasma while the other two were placed in test plasma. The mean volume secreted at the end of 60 min by the two tubules from one insect suspended in test plasma was compared with the mean volume secreted by the corresponding tubules in control plasma and the difference was expressed as a percentage. For any one pair of samples, this procedure was performed four times. A procedure similar to the above was used to examine butanol extracts of control and test plasma. Throughout the study the investigators were unaware of the identity of the test and control samples.

RESULTS

Experiments were performed on sixteen dogs. The heart rate and mean blood pressure at the commencement of recording was 98 beats/min (mean; range 50–186) and 124 mmHg (mean; range 97–155). The pH, P_{CO_2} and P_{O_2} of arterial blood were

7·41 (mean; range 7·36–7·46), 34·7 mmHg (mean; range 31·0–37·0) and 197 mmHg (mean; range 150–228) respectively.

Effect of distending balloons in the left atrium on urine flow

When a steady state with respect to heart rate, mean arterial pressure, urine flow and anaesthetic dose had been achieved, 2–4 hr after completion of the surgical procedures, the balloon in the left atrium was distended. The volume injected was 16 ml. (mean; range 9–23). The response to distension in one dog is shown in Text-fig. 1. In this experiment the mean urine flow during the control period before distension was 1·8 ml./10 min and it increased to 5·2 ml./10 min during distension. The mean urine flow during the control period after distension was 2·6 ml./10 min. The pressure in the left atrium was increased by 24·7 cm H_2O and the heart rate increased by a mean value of 22 beats/min during the period of distension. There was a decrease of 20 mmHg in the mean arterial pressure.

In sixteen dogs balloons in the left atrium were distended sixteen times. The urine flow increased from a mean value of 0·37 ml./min (S.E. of mean, ± 0·04) during control periods to a mean value of 0·74 ml./min (S.E. of mean, ± 0·13) during the test period. This response represented a mean increase in urine flow of 105 % (range 9–236 %).

In eight dogs the balloon in the left atrium was distended after cutting or cooling the vagi. There was no increase in urine flow during distension. The mean urine flow during the control period was 0·40 ml./min (S.E. of mean, ± 0·08) and during the test period was 0·33 ml./min (S.E. of mean, ± 0·04).

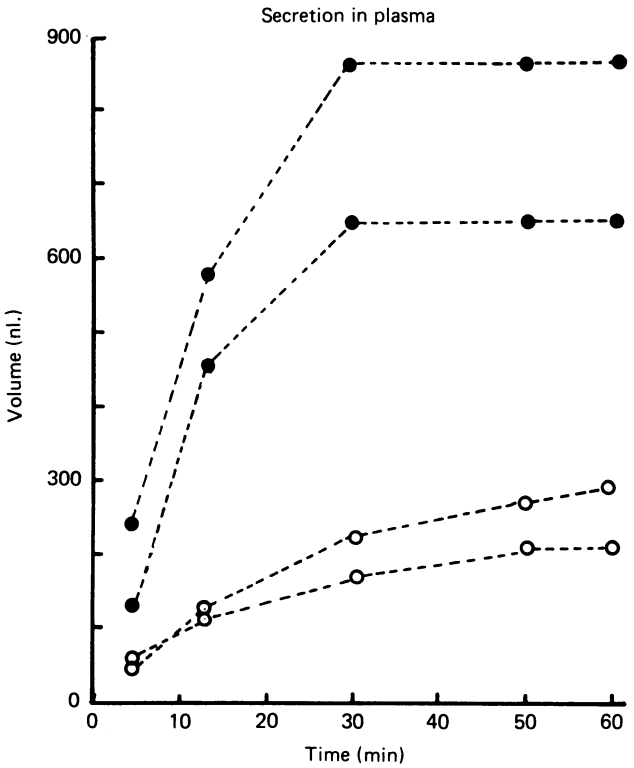
The cardiovascular changes and the diuresis observed in this investigation are within the ranges of changes observed in the previously reported investigations of this group (e.g. see References).

Effect of plasma on the secretion of Malpighian tubules

Malpighian tubules were suspended in control and test plasma and the volume of fluid secreted was measured at the end of 60 min. Text-fig. 2 is a representative example of the secretions of four tubules from one insect. Two of the tubules were suspended in test plasma obtained during balloon distension and two in control plasma obtained before the distension. At the end of 60 min both tubules suspended in test plasma secreted a considerably smaller quantity of fluid than those suspended in control plasma. This consistency in the behaviour of the tubules from any one insect was a persistent feature throughout this investigation. For the purpose of calculating response, the mean secretion of the two tubules suspended in test plasma was compared with the mean secretion of the two tubules suspended in control plasma. For any one pair of samples this procedure was performed four times so as to obtain four mean values for the test and control samples respectively. The average of these four values was taken for quantifying the differences between the two samples of plasma.

In five of the sixteen experiments, test plasma was compared with control samples from both the period before and the period after diuresis. There was a significant difference between the results obtained from the tubules suspended in test plasma and an average of the results obtained from those suspended in control plasma obtained before and after the diuresis (n, 5; mean difference, 27 %; S.E. of mean,

$\pm 7{\cdot}6$; $P < 0{\cdot}02$). During five of the remaining eleven experiments, test plasma was compared with control samples from the period before diuresis and with pooled control samples obtained by pooling equal volumes of the control samples obtained before and after diuresis. The tubules suspended in test plasma secreted less than those suspended in plasma from the control period before diuresis (mean difference, 34%; S.E. of mean, $\pm 9{\cdot}7$); the same test samples also secreted less than the pooled



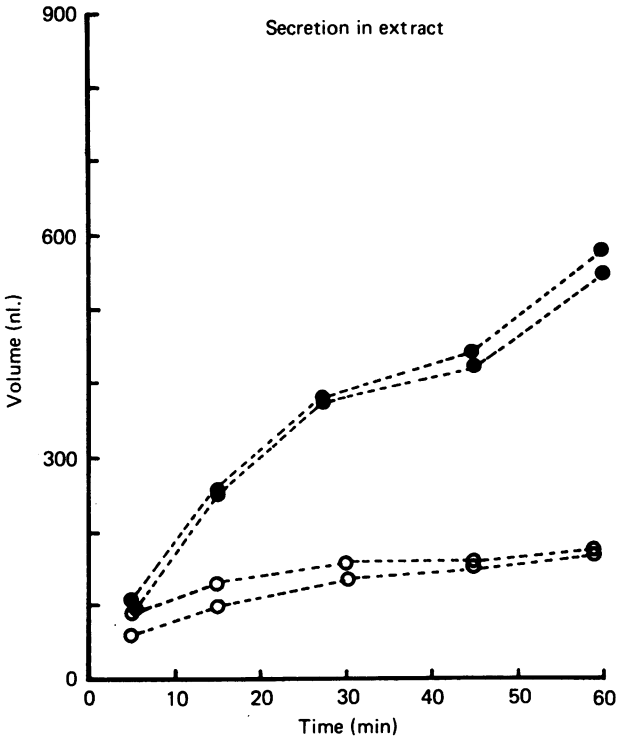
Text-fig. 2. Secretions of four Malpighian tubules from one *Rhodnius*, suspended in plasma. Ordinate: cumulative volume of fluid secreted (nl.). Abscissa: time (min); ○, secretion of the tubules suspended in test plasma; ●, secretion of tubules suspended in control plasma.

control samples (mean differences, 41%; S.E. of mean, $\pm 19{\cdot}6$). If the results from the experiments in which the pooled control samples were used are combined with the results of the first five experiments in which the averages were obtained mathematically the differences between test and average control secretions is significant in the ten animals (mean, 34%; S.E. of mean, $\pm 10{\cdot}3$; $P < 0{\cdot}01$).

During the remaining six experiments, test plasma was compared only with the initial control plasma. In all sixteen experiments the comparison of test and initial control plasma on the tubules again showed that the tubules suspended in the test plasma secreted significantly less than those suspended in control plasma, the mean difference being 26% (S.E. of mean, $\pm 6{\cdot}2$). These results are presented in Table 1.

In eight dogs the cervical vagi were sectioned or cooled to 6 °C (5 °C in the thermode) and the balloons in the left atrium distended. An increase in urine flow was not

observed in any of these experiments. The plasma obtained during the initial control period was compared with that obtained during the test period using Malpighian tubules. The secretions of the tubules suspended in test plasma were not significantly



Text-fig. 3. Secretions of four Malpighian tubules from one *Rhodnius*, suspended in *n*-butanol extracts of plasma. Ordinate: cumulative volume of fluid secreted (nl.). Abscissa: time (min); ○, secretion of tubules suspended in text extract; ●, secretion of tubules suspended in control extract. These extracts were prepared from the plasma used in the study depicted in Text-fig. 2.

different from those of tubules suspended in control plasma (Table 1). Though the difference between test and control before vagal blockade was not significant in these eight dogs ($P > 0{\cdot}1$) the difference after was much smaller and significantly different from that observed before (see Table 1).

Effect of butanol extracts on the secretion of Malpighian tubules

In eight of the sixteen experiments in which an increase in urine flow was observed, the plasma was extracted with butanol and applied to the tubules (Text-fig. 3). The protocol adopted for this part of the study was identical with that used for the plasma. In two of these eight experiments, extracts of the control plasmas obtained both before and after diuresis were compared with the test plasma extract. The tubules suspended in test extract secreted less than those suspended in extracts of control plasma obtained before diuresis (differences: 68 and 36%) and after (differences: 36 and 86%). In the eight experiments the tubules suspended in extracts prepared from the initial control samples were found to secrete significantly more than

those suspended in extracts prepared from test plasma. The mean difference was 45% (S.E. of mean, ± 17·6). These results are presented in Table 2.

In four of the eight experiments which were performed after cutting or cooling the cervical vagi butanol extracts were prepared from the samples of plasma. Once again, the secretions in tubules suspended in extracts prepared from control plasma were not significantly different from those of tubules suspended in extracts of test plasma though before vagal blockade the difference was significant. These results are presented in Table 2.

TABLE 1. The effect of dog plasma on the rate of secretion of the Malpighian tubules of *Rhodnius prolixus*. The rate of secretion is expressed as the volume produced in 60 min (nl.)

	Intact vagi (V+)			Vagi cut or cooled 5 °C (V−)		
Dog no.	Control (C) (nl.)	Test (T) (nl.)	Difference (%)	Control (C) (nl.)	Test (T) (nl.)	Difference (%)
107/73	985	275	−72*	—	—	—
1/74	358	207	−42*	—	—	—
4/74	963	576	−40*	—	—	—
62/74	750	658	−12	439	518	+15*
65/74	479	410	−14	—	—	—
3/75	528	276	−48	—	—	—
5/75	735	333	−55	464	223	−52
14/75	315	325	+3*	408	474	+14
17/75	557	242	−57	333	242	−27
23/75	492	407	−17	528	483	−9
25/75	438	305	−30	—	—	—
46/75	764	697	−9	620	620	0
52/75	588	734	+20	605	741	+18
54/75	862	854	−1	1122	1328	+16
78/75	617	455	−26	—	—	—
82/75	723	634	−12	—	—	—
Mean	635	462	−26	—	—	—
$n = 16$						
P(C *vs.* T)	< 0·01					
† Mean	633	531	−16	565	579	−3
$n = 8$						
P(C *vs.* T)	> 0·10, < 0·20			n.s.		
P(V+ *vs.* V−)	< 0·02					

Each value represents the average results from four bugs except for those marked with * where tests were performed on two bugs only. For calculating differences (%) the larger value was taken as 100%. When the test value was less than the control value the difference was expressed as − and when the test value was greater than the control value the difference was expressed as +.

† Refers to the eight experiments in which the vagi were cut or cooled.

n.s. not significant (Student's two-tailed, paired t test).

DISCUSSION

Stimulation of atrial receptors by distension of a balloon in the left atrium of anaesthetized dogs results in an increase in urine flow which is mediated by a blood-borne agent (see Linden, 1973 and 1975 for references). There has been a considerable degree of speculation as to the identity of this agent, particularly involving the

antiduretic hormone. It has recently been shown that this increase in urine flow was not accompanied by a consistent reduction in the antidiuretic activity of plasma (Kappagoda *et al.* 1974) and that it was unaffected by hypophysectomy (Kappagoda *et al.* 1975). It was therefore suggested that the increase in urine flow was not mediated by a reduction in the concentration of antidiuretic hormone. The investigations reported in this paper were undertaken on the basis that the increase in urine flow which followed stimulation of atrial receptors was mediated by an unknown humoral agent which was probably diuretic in nature: this hypothesis was supported by the

TABLE 2. The effect of butanol extracts of dog plasma on the rate of secretion of Malpighian tubules of *Rhodnius prolixus*. The rate of secretion is expressed as the volume produced in 60 min (nl.)

	Intact vagi (V^+)			Vagi cut or cooled 5 °C (V^-)		
Dog no.	Control (C) (nl.)	Test (T) (nl.)	Difference (%)	Control (C) (nl.)	Test (T) (nl.)	Difference (%)
62/74	546	280	−49**	280	278	−1
3/75	643	244	−62	—	—	—
5/75	672	66	−90	—	—	—
14/75	47	159	+70*	—	—	—
17/75	855	169	−80	317	323	+2
25/75	264	85	−68	—	—	—
46/75	451	249	−45	475	425	−11
52/75	239	153	−36	121	217	+44
Mean	465	176	−45	—	—	—
$n = 8$						
P(C *vs.* T)		< 0·05				
† Mean	523	213	−53	298	311	+9
$n = 4$						
P(C *vs.* T)		< 0·05			n.s.	
P(V^+ *vs.* V^-)			< 0·02			

Each value represents the average results from four bugs, except for those marked with * and ** where the tests were performed on one and three bugs respectively. For calculating differences (%) the larger value was taken as 100 %. When the test value was less than the control value the difference was expressed as − and when the test value was greater than the control value the difference was expressed as +.

† Refers to the four experiments in which vagi were cut or cooled.

n.s. not significant (Student's two-tailed, paired *t* test).

fact that plasma obtained during the period of stimulation of atrial receptors did not inhibit urine flow in the water loaded ethanol anaesthetized rat (Kappagoda *et al.* 1974).

It is clearly appreciated that distension of balloons to cause partial obstruction of the mitral valve also results in major changes in the circulation, e.g. a fall in systemic arterial pressure. Thus it is likely that many other cardiovascular reflexes will be affected and the concentration of other humoral agents in the blood (e.g. catecholamines and angiotensin) will be altered by this procedure. In addition the time course of the effects of the potential diuretic agent is unknown. However, at the moment, the technique of distending a large balloon in the atrium is the only method adequate to effect the changes in urine flow and allow sufficient quantities of blood and extracted material to be collected to enable the possible diuretic agent to be investigated.

Although a diuretic hormone is not known in mammals, such an agent has been extensively investigated in insects, particularly in *Rhodnius prolixus*. These investigations were carried out using the Malpighian tubules of these insects which were made to function *in vitro* (see Maddrell, 1969). It was shown that the rate of secretion of the tubules was increased by humoral agents derived from the thoracic ganglia of the insects but several mammalian hormones including the antidiuretic hormone were shown to have no significant effect on the tubules (Maddrell, 1969; Maddrell, Pilcher & Gardiner, 1971).

Since these investigations (Maddrell, 1969) had shown the existence in the Malpighian tubules of *Rhodnius prolixus* of active processes engaged in the transport of water and electrolytes, it was felt that these tubules could act as a biological 'test bed' for a humoral agent which could alter urine flow in anaesthetized dogs as suggested previously. Such an outcome was particularly attractive because the plasma of dogs remained the only potential source of the material and the Malpighian tubule required only minute quantities of plasma for its operation.

The present investigation has indicated that this preparation can detect a difference between samples of plasma obtained during stimulation of atrial receptors and during control periods. However, it does appear that the tubule secretion rate is inhibited by the test plasma (i.e. plasma obtained during an increase in urine flow in the dog) and no explanation is available for this phenomenon. At the present, this preparation exists simply as a means of detecting qualitative differences in test and control plasma and subsequent extracts. Such differences were preserved in butanol extracts. The difference between the effects of test and control plasma was not present after vagal section or cooling. With the butanol extracts the significant difference before vagal section or cooling was abolished after, and this result was significant.

No attempt has been made to correlate the diuresis and the inhibition of secretions by the Malpighian tubules; such a correlation will be necessary in the future before it is possible to claim that a hormone is involved. However, at the moment this critical relationship can not be examined for the following reasons. We do not know, and have no means of knowing, whether there are any concomitant changes during distension of the balloons which affect urine flow or the proposed substance; and any changes could have altered periodically in relation to the substance. There are many secondary effects of distending the large balloon, e.g. cardiac output, blood pressure, and these may also vary. Another large variable is the response of the *Rhodnius* tubule. Tubules from different insects respond differently to the same stimuli for many reasons, e.g. to standard doses of 5-hydroxytryptamine; one reason being the starting position in a 'dose–response' curve. Qualitative differences are the same from insect to insect but it is probably not valid to compare quantitatively. Thus there is as yet no unequivocal evidence to indicate that this material detected by the Malpighian tubules is that responsible for the increase in urine flow which follows stimulation of atrial receptors in the dog.

However one item of evidence which supports such a hypothesis is provided by the response to stimulation of atrial receptors after blockade of the cervical vagi. Previous investigations have shown that the response of an increase in urine flow was abolished by this procedure (e.g. Henry & Pearce, 1956; Ledsome & Linden, 1968) thus establishing its reflex nature. In the present investigation too, cutting or

cooling the vagi to 6 °C (5 °C in the thermode) abolished the increase in urine flow. The possible fallacies of conclusions based on vagal section are well understood: vagal cooling is slightly less hazardous in that fewer nerve fibres are rendered inactive. However the question was asked in such a way that the hypothesis could have been refuted if the difference between the tubule secretions had been maintained after vagal blockade; it was not, so the hypothesis remains.

Therefore, it is suggested that the differences detected by the tubules are related to the humoral agent responsible for the diuresis and are a means of following this potential agent through various extraction processes. These findings also provide an argument against the potential 'diuretic' agent being produced following secondary changes in response to other reflexes (e.g. from the carotid sinus) in that the substance is not detectable following vagal section or cooling, although the primary disturbance of a fall in cardiac output and systemic arterial blood pressure are still present. However, final resolution of the problem should await the outcome of two other avenues of enquiry: first, the examination of plasma during the less disturbing intervention of distending small balloons at the pulmonary vein-atrial junctions in the manner of Ledsome & Linden (1968) and secondly, the identification of substances of mammalian origin which inhibit the secretion of the Malpighian tubules.

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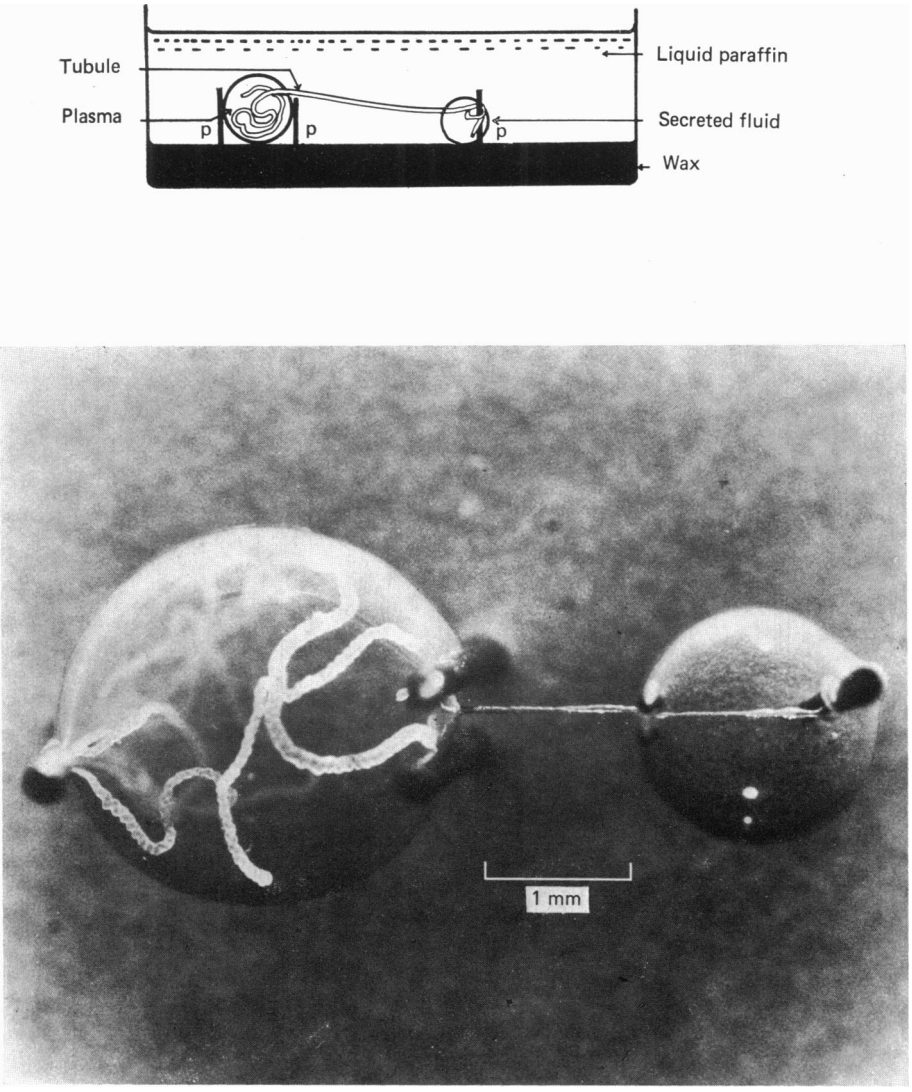
Tubule
Plasma
Liquid paraffin
Secreted fluid
Wax
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EXPLANATION OF PLATE

Preparation of the Malpighian tubules of *Rhodnius prolixus*. Top: schematic diagram of the preparation viewed from the side. The wax base of the petri dish is covered with liquid paraffin. The drop of plasma is 'attached' by surface tension to the stainless steel pins (p) embedded in the wax. The tubule is inserted into this drop and the cut end is drawn around another pin. The fluid secreted by the tubule collects at this end. Bottom: photograph of the preparation viewed from above. The drop of plasma is seen on the left and the secreted fluid on the right.