JCI The Journal of Clinical Investigation

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J Clin Invest. 1974;53(6):1695-1708. https://doi.org/10.1172/JCI107721.

Research Article

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ABSTRACT Microperfusion experiments have shown that increases in flow rate of tubule fluid through the loop of Henle are followed by reductions in single nephron glomerular filtration rate (SNGFR) and stop-flow pressure (SFP) measured in the proximal tubule of the same nephron. Because changes in luminal sodium concentration are not consistently related to changes in SNGFR and SFP, we explored the possibility that a transport step at a flow-dependent distal-sensing site might be involved in feedback control of SNGFR. Because the macula densa cells of the distal tubule are adjacent to the glomerular vessels of the same nephrons, they could be the distal-sensing mechanism. We perfused superficial loops of Henle from late proximal to early distal segments in three groups of rats while measuring SFP in the proximal tubule of the same nephron, SNGFR in the proximal tubule of the same nephron, or flow rates of fluid, Na, K, and Cl emerging from the perfused loops. Perfusion solutions used were 0.15 M NaCl, Ringer or Ringer with one of several inhibitors of electrolyte transport. Perfusion rates were 10 or 40 nl/min (also, zero during measurements of SFP and SNGFR). With Ringer alone the loop-flow rate increased from 10 to 40 nl/min, caused a decrease in SFP from 37.6 to 32.1 mm Hg, and a decrease in SNGFR from 29.9 to 18.7 nl/min. Concentrations of Na, K, and Cl in early distal fluid and absorption of Na and Cl along the loop segment were also increased when loop perfusion rate was increased. Decreasing the perfusion rate to zero had little effect on SFP or SNGFR. The SFP response to increased flow rate did not occur when the perfusion solution contained furosemide (10^{-4} M). No reduction of the SFP response was seen with other diuretics tested (amiloride, acetazolamide, ethacrynic acid, mercaptomerin) or with 0.15 M NaCl alone. The SNGFR response to increased perfusion rate was reduced by furosemide, triflocin, and cyanide but not by amiloride. Na and Cl absorption by the perfused segment were inhibited by furosemide, triflocin, cyanide, and amiloride. Amiloride and acetazolamide, probably do not act in the ascending limb. Ethacrynic acid and mercaptomerin are known to be ineffective in rat nephrons. Thus, agents that could have inhibited NaCl absorption by macula densa cells interfered with the feedback mechanism.

INTRODUCTION

Both the close anatomical association of the vascular pole of the glomerulus with the macula densa cells in the distal tubule of the same nephron (1, 2) and evidence of a functional relation between distal flow rate and glomerular filtration rate (GFR) (3) suggest that a feedback system within individual nephrons may contribute to the control of filtration rate. Recently Schnermann, Persson, and Ågerup (4) showed that increased flow rates through the loop of Henle caused decreases in proximal stop-flow pressure (SFP).[1] Further, consistent with the finding of Blantz, Israelit, Rector, and Seldin (5) that blocking in flow to the loop did not increase glomerular capillary pressure (P_{GC}), Schnermann et al. (4) observed that interruption of loop flow is not associated with a reciprocal increase in SFP. Because SFP and single nephron filtration rate (SNGFR) did not respond to increased flow rate when the loop was perfused with mannitol or Na_2SO_4 solutions, Schnermann and co-workers (3, 4) suggested that the rate of sodium absorption by macula densa cells could be the sensed signal in a feedback mechanism. The pres-

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Received for publication 2 October 1973 and in revised form 7 January 1974.

[1] *Abbreviations used in this paper:* AR, absolute rate of reabsorption; P_{GC}, glomerular capillary pressure; SFP, stop-flow pressure; SNGFR, single nephron filtration rate.

TABLE I

Calculated Perfusion Rate, $\dot{V}_0$

Perfusion solution	Pump setting 10	Pump setting 40
	nl/min	
Ringer (8)	9.5±0.4	45.1±2.8
Furosemide (11)	10.7±0.4	41.3±1.1
Cyanide* (2)	13.4	39.6
Triflocin (9)	11.0±0.5	43.6±1.1
Amiloride (9)	10.9±0.4	42.8±1.0
Mean (39)	10.7±0.27	42.9±0.75

Values are means±SE, parentheses indicate number of tubules.
* 9 of 11 cyanide-perfused tubules are not included because a lab accident prevented our measuring the radioactivity of [^{3}H]inulin in that perfusion fluid. [^{14}C]Inulin was used in the cyanide experiments shown.

ent experiments were designed to examine further the nature of the signal by exposing the presumed distal-sensing site to inhibitors of salt transport. Using micropuncture techniques, we measured proximal SFP, SNGFR, and changes in loop function while varying the rate of fluid delivery from a late proximal puncture site. The effects of increased flow when the loop was perfused with Ringer solution were compared with responses with transport inhibitors in the perfusion fluid.

METHODS

Three types of experiments, which for convenience will be designated (*a*) SFP, (*b*) proximal collection, and (*c*) distal collections were performed on 34 male Long-Evans and Sprague-Dawley rats weighing from 120 to 300 g. All rats were fed a commercial chow diet (Ralston Purina Co., St. Louis, Mo.) containing 0.2 meq/g Na and 0.29 meq/g K. They were allowed water but no food for 15 h before being anesthetized by intraperitoneal injection of Inactin (Promonta, Hamburg) 115 mg/kg. Sometimes additional Inactin (1–4 mg) was given intravenously during the surgical preparation; inadequate depth of anesthesia was usually indicated by cyclic variations (each 2–5 min) in arterial blood pressure.

Polyethylene catheters were placed in the external jugular vein for infusing 0.15 M NaCl solution at 0.5 ml/h per 100 g body wt and in the carotid artery for monitoring blood pressure and collecting blood samples. A short polyethylene tube was placed in a tracheal incision. A heated table was used to maintain body temperature at 37–38°C. The left kidney was exposed through a flank incision and supported in a plastic cup. Mineral oil, warmed to 38°C, bathed the surface of the kidney. The left ureter was catheterized with polyethylene tubing and urine was collected under mineral oil in tared tubes. Blood pressure was measured continuously using a Statham transducer (Statham Instruments, Inc., Oxnard, Calif.) and recorded on a Grass polygraph (Grass Instrument Co., Quincy, Mass.).

All experiments involved perfusion of the loop of Henle with either a solution resembling end-proximal tubule fluid or with this solution modified by addition of an inhibitor of ion transport. The control solution, which will be referred to as Ringer solution, consisted of 140 mM NaCl, 4 mM $NaHCO_3$, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 7 mM urea and was colored by adding to each milliliter 0.01 ml of a 10 g/100 ml solution of FD & C Green no. 3 (Keystone Aniline & Chemical Co., Chicago, Ill.). Inhibitors added to this solution in separate experiments were furosemide (Lasix, Hoechst Pharmaceuticals, Inc., Somerville, N.J.) triflocin (supplied by Lederle Laboratories, Pearl River, N. Y.), sodium cyanide, amiloride (supplied by Merck & Co., Inc., West Point, Pa.), ethacrynic acid (supplied by Merck & Co., Inc.), acetazolamide (Diamox, Lederle Laboratories), mercaptomerin (supplied by Wyeth Laboratories, Philadelphia, Pa.) and poly-L-lysine, mol wt 70,000 (Sigma Chemical Co., St. Louis, Mo.). All perfusion fluids were delivered through sharpened micropipets with 8-μm OD tips by a displacement-type perfusion pump with a servo-regulated motor (Hampel, Frankfurt, Germany). Pumping a solution of known radioactivity into counting vials showed that delivery ranged from 10.5 to 12.5 nl/min when the pump was set at 10 and from 35 to 39 when the pump was set at 40. The performance of the pump was checked in vivo in experiments that included collection of the perfused fluid from early distal tubule segments. As shown in Table I the perfusion rate, calculated as $\dot{V}_0 = \dot{V}_{TF} \cdot (TF/PF)_{inulin}$ from the collected volume flow and the concentration ratio of [^{3}H]- or [^{14}C]-inulin in the collected and perfused fluids, was reproducible in separate groups of experiments.

With the pump set at 10 nl/min, a long surface proximal tubule segment was punctured. When passage of the dye into downstream segments indicated an early proximal puncture site, the perfusion pipet was then withdrawn and placed in the last surface proximal segment of that nephron. In the SFP experiments, a sharpened pipet with a 15-μm tip filled with a high viscosity (30,000 cs) silicone oil (Dow Corning 200 fluid, Dow Corning Corp., Midland, Mich.) colored with 10% Automate black no. 1 (Morton Chemical Co., Chicago, Ill.) was inserted into the initial early proximal puncture site. A 3–5-μm pipet filled with 1 M NaCl plus 0.1 g/100 ml FD & C Green and attached to a servo-nulling pressure recording system (built in the electronics and machine shop of the Department of Physiology [6]) was introduced into the same segment just proximal to the oil-filled pipet. Free-flow tubules pressure was measured and recorded on the second channel of the Grass recorder and then oil was injected to block the tubule between the pressure recording pipet and the perfusion pipet. Additional oil was injected as needed to keep the proximal end of the oil column between the pressure and oil pipets. After recording a stable SFP the perfusion pump setting was increased from 10 to 40 nl/min. The pump rate was then repeatedly reduced to 10 and increased to 40 after being kept at each setting for about 1 min. Finally, with the oil and pressure pipets still in place, the perfusion pipet was withdrawn to check the effect on SFP of zero distal perfusion. Repeated measurements at zero perfusion were not interspersed with those at 10 and 40 nl/min because we were not confident of the pump's reproducibility in the range below 5 nl/min. The zero position of the pressure recording system was then checked by withdrawing the pressure pipet and holding its tip in the surface fluid above the punctured tubule. These steps were carried out in one to four tubules in every animal using

Ringer solution as the perfusate. The perfusion pipet was then changed to one containing one of the inhibitors listed above and the procedure was repeated in tubules not previously punctured.

For proximal collection experiments, eight rats were prepared identically to those in SFP experiments except that 200 μCi of [^{3}H]inulin was injected intravenously and additional [^{3}H]inulin was infused continuously at 200 μCi/h. Late proximal segments were identified as above and perfusion of the loop of Henle with Ringer solution was begun at 10 nl/min. Silicone oil was injected and the SFP response to increased perfusion rate was measured in one or two nephrons. Late proximal segments of other nephrons were then identified and loop perfusion established at 10 nl/min. Silicone oil was injected into the mid-portion of the proximal tubule and pipets with 8-μm tips filled with stained mineral oil were then used to collect fluid from a more proximal part of this segment. The portion of this segment between the collection pipet and the silicone oil was blocked with a column of stained mineral oil 200–300 μm long. Timed samples of tubule fluid were collected spontaneously or with slight aspiration when necessary to keep the oil block in place. Collection times ranged from 2 to 5 min. After the first sample was obtained, the perfusion rate was increased to 40 nl/min and the second sample was taken from the same site or one slightly more proximal in the same tubule. Aspiration was rarely necessary during collections at the high perfusion rate. After this collection the perfusion pipet was withdrawn and a third collection was made from the same or a slightly more proximal site in the same tubule. One to three nephrons were studied with Ringer perfusion in each rat. The perfusion pipet was then exchanged for one containing either furosemide, triflocin, cyanide, or amiloride. The SFP response was again measured during perfusion of the loop at 10, 40, and 0 nl/min. Additional triple collections were then made in three to six fresh tubules. Two solutions (Ringer and Ringer plus an inhibitor) were tested in each rat. Arterial blood samples of approximately 100 μl were obtained after every second set of tubule fluid collections. The volume of each tubule fluid sample was measured in a constant bore capillary and the radioactivity of tubule fluid and plasma samples was measured in a scintillation counter using Aquasol (New England Nuclear, Boston, Mass.) as the counting solution.

For distal collection experiments six rats were prepared as for the SFP experiments. 50 μCi of [^{3}H]- or [^{14}C]inulin was added to 0.5 ml of the stained perfusion fluids that had been used in the proximal collection experiments. Late proximal tubule segments were identified and punctured with the perfusion pipet. Perfusion of the loop of Henle was begun at either 10 or 40 nl/min. An oil block was then inserted in the upstream part of the proximal tubule and a hole was made more proximally to allow filtrate to drain. Pipets with 8-μm tips filled with stained mineral oil were used to collect fluid from an early segment of the distal convoluted tubule of the nephron being perfused. Collection sites were identified as early when two or more distal segments were colored by the perfusion fluid. The earliest of these was punctured. Timed samples were obtained with occasional aspiration after placing an oil block distal to the puncture site. Collection time was 3–5 min. After collecting the first sample the perfusion rate was either increased to 40 nl/min or reduced to 10 and a second sample was collected from the same distal site. Two or three of the five perfusion fluids were tested in each rat. The volume of each tubule fluid sample was measured and then portions of approximately 1 nl were analyzed for Na and K using a micro-flamephotometer (7) and for Cl by electrometric titration (8). Radioactivity of 5-nl portions of the tubule fluid samples and 5-μl portions of plasma was measured in a scintillation counter using Aquasol as the counting solution.

SFP at 10 and 40 nl/min, usually two or three measurements at each perfusion rate, were averaged for each nephron. SNGFR was calculated as $V_{TF} \cdot (TF/P)_{inulin}$, the product of proximal flow rate and the ratio of tubule fluid and plasma inulin concentrations. Values for plasma inulin were corrected by adding 6% so they expressed concentration per volume of plasma water. The absolute rate of fluid absorption along the perfused segment, AR, was calculated as $V_0 - V_{TF}$. Reabsorptive rates for Na, Cl, and K were determined as the differences between perfused load and collected amounts. Differences between mean values were tested using the t distribution.

RESULTS

Increased flow rate through the loop of Henle was associated with decreased proximal SFP with some perfusion fluids but not others. The responses to high and low perfusion rates with Ringer solution as well as three solutions containing inhibitors of transport are shown in Fig. 1. The intratubular pressure tracings show the establishment of a steady SFP between 30 and 40 mm Hg after placement of an oil block in the mid-proximal tubule while the loop of Henle is perfused at 10 nl/min. In the top tracing, showing the results with Ringer perfusion, an increase in the perfusion rate to 40 nl/min was followed within seconds by a decline in SFP that was reversible when the perfusion rate was decreased again. This result is similar to that reported by Schnermann et al. (4). Little further change in SFP occurred when loop perfusion was stopped by withdrawing the perfusion pipet. The three lower tracings show arterial and tubular pressures during perfusions with solutions containing furosemide, triflocin, and cyanide. In these experiments increases in perfusion rate were not accompanied by changes in SFP. However, it is possible to see, for example in the furosemide experiment, that transient changes in arterial pressure were associated with parallel changes in SFP.

Data from all the SFP experiments using these solutions are shown in Fig. 2. In nephrons perfused with Ringer solution, SFP decreased an average of 5.5 mm Hg ($P < 0.001$) when the perfusion rate was increased from 10 to 40 nl/min. At zero perfusion the SFP was 0.6 mm Hg higher ($P < 0.01$) than the value at 10 nl/min. When 10^{-4} M furosemide was added to the Ringer perfusion solution, increases in perfusion rate were not accompanied by decreases in SFP. The average values at 10 and 40 nl/min are not different ($P < 0.05$). Again, a slightly higher ($P < 0.05$) SFP was seen after withdrawal of the perfusion pipet. The measurements at zero perfusion rate are for single observations at the end of a series during which SFP often tended to rise

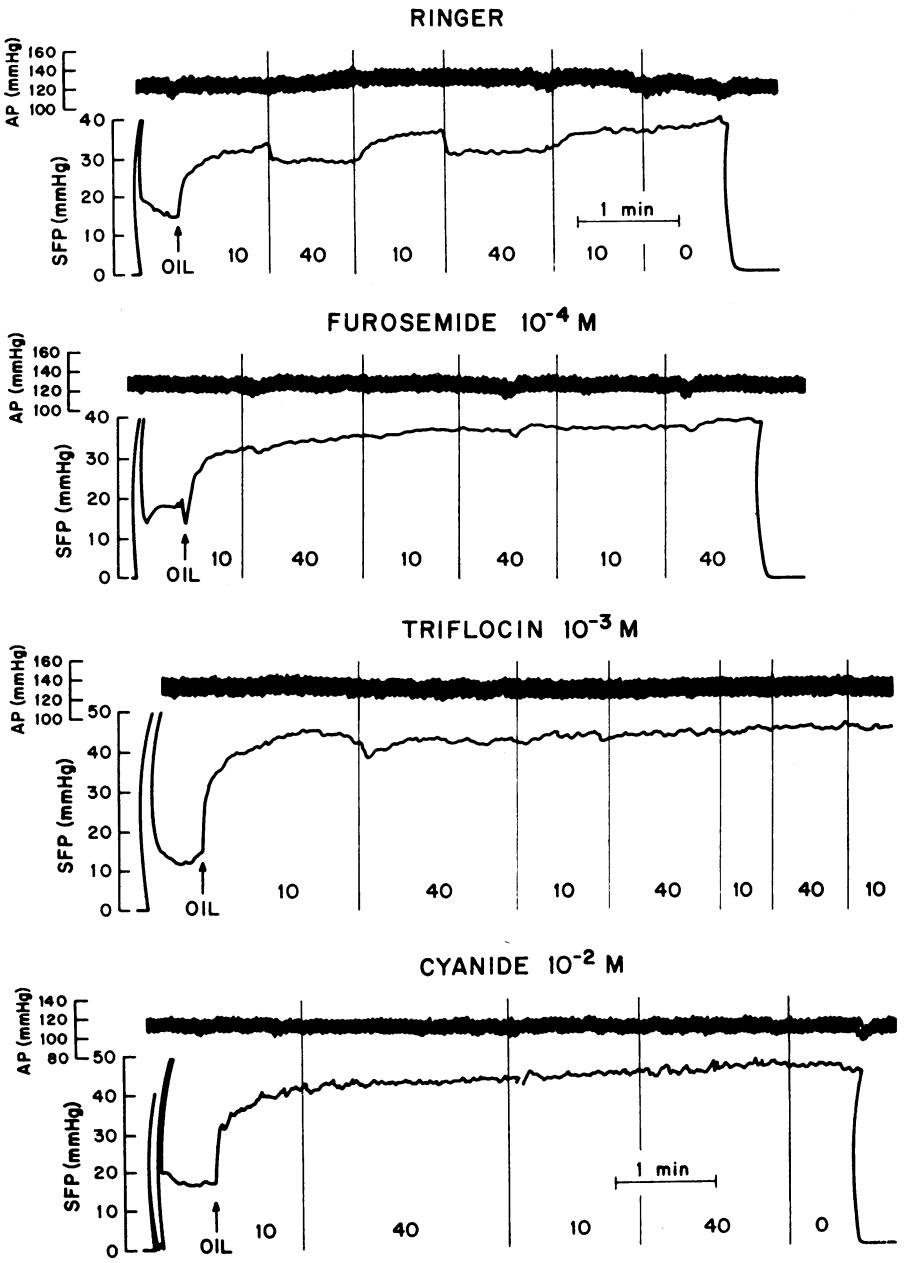

FIGURE 1 Effects of loop perfusion rate and transport inhibitors on SFP. Simultaneous tracings show arterial pressure (AP) and proximal intratubular pressure during perfusion of the loop of Henle at 10, 40, and 0 nl/min. Single examples are shown from experiments with Ringer solution alone and with furosemide, triflocin, or cyanide.

continuously over a period of several minutes. It is, therefore, likely that the SFP values in some experiments at zero perfusion are spuriously high because of the order of measurement.

Triflocin was initially added to the Ringer perfusion at a concentration of 10^{-4} M and did not appear to block SFP changes as completely as furosemide did. In four tubules in two rats changes in perfusion rate from 10 to 40 nl/min were associated with a decrease in SFP from 34.0 mm Hg±2.5 SE to 31.4±2.6 ($P < 0.05$). At zero perfusion SFP was 2.6 mm Hg higher than at 10 nl/min ($P < 0.05$). Testing 10^{-3} M triflocin, however, we observed results similar to those with a 10 times lower concentration of furosemide (Fig. 2). When the

perfusion rate was increased to 40 nl/min SFP was not different from SFP at 10 nl/min ($P < 0.1$). The mean SFP at zero perfusion was 1.7 mm Hg greater than the SFP at 10 nl/min ($P < 0.025$).

In our initial experiments with cyanide we added 2×10^{-3} M NaCN to the perfusion fluid in nine tubules in two rats. Increasing the perfusion rate to 40 nl/min was accompanied by a reduction of SFP from 34.8 mm Hg±1.1 SE to 32.3±1.5 ($P < 0.025$). A fivefold increase in cyanide concentration to 10^{-2} M NaCN not only prevented a fall in SFP at higher perfusion rates but caused an 11% increase in SFP ($P < 0.001$) when perfusion rate was increased from 10 to 40 nl/min. At zero perfusion the SFP was less than at 40 nl/min but was slightly higher ($P < 0.025$) than the value at 10 nl/min.

As with the lower concentrations of triflocin and cyanide, four other potentially inhibitory agents did not block the pressure response to increases in loop perfusion rate. Representative pressure tracings from these experiments with amiloride, acetazolamide, ethacrynic acid, and mercaptomerin are shown in Fig. 3. Data from all the SFP experiments with these agents are shown in Fig. 4. When the loop was perfused with Ringer solution containing 10^{-4} M amiloride the perfusion rate increased to 40 nl/min and was followed by a reversible decrease in SFP of 5.0 mm Hg ($P < 0.001$). At zero perfusion the SFP was 0.6 mm Hg higher ($P < 0.025$) than at 10 nl/min. An attempt to test a higher concentration of amiloride was not successful. When the loop was perfused with 10^{-3} M amiloride increasing the perfusion rate to 40 nl/min caused the SFP to increase approximately 7 mm Hg. It is possible that this parallel change in SFP was due to blockage of the loop by precipitated amiloride. Deetjen (9) and others have reported such precipitation at high blood levels. Measurements of proximal tubule pressure with no oil block between the pressure pipet and the perfusion pipet provided additional evidence that the loop was blocked during perfusion with 10^{-3} M amiloride. Immediately after increasing the loop perfusion rate from 10 to 30 nl/min, the proximal tubule free-flow pressure increased from 15 to 30 mm Hg whereas in a Ringer-perfused nephron, proximal free-flow pressure increased only 5 mm Hg. That the loop was blocked by this solution, was further suggested by our failure to observe the slight distention of the surface distal convolution that normally followed increases in perfusion rate.

With solutions containing either 10^{-4} or 10^{-3} M acetazolamide increases in perfusion rate elicited similar decreases in SFP (Fig. 4). The mean decrease in SFP was 5.6 mm Hg ($P < 0.001$). Similarly there was no indication that mercaptomerin could block the response to Ringer perfusion. In experiments with this mercurial diuretic at 10^{-3}, 5×10^{-3}, and 10^{-2} M the average SFP

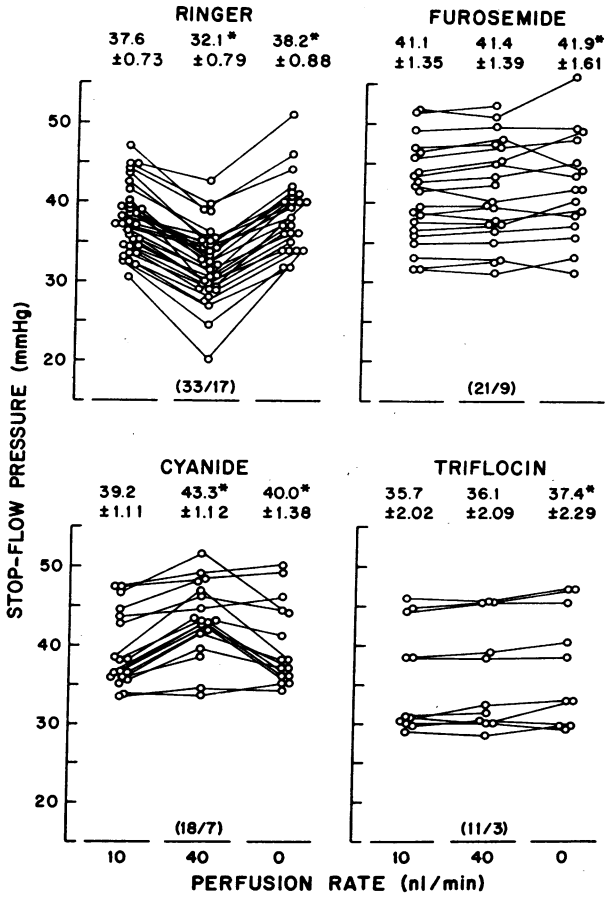

FIGURE 2 Effects of loop perfusion rate and transport inhibitors on SFP. Data are from all experiments with the perfusion solutions shown in Fig. 1. Points are averages of results in a single nephron at each perfusion rate. Values above the points are means ±SE. (*) indicates mean is different from the SFP at 10 nl/min ($P < 0.05$). Numbers in parentheses indicate tubules/rats.

at 40 nl/min was 5.6 mm Hg lower ($P < 0.001$) than the SFP at 10 nl/min. Ethacrynic acid was tested at 10^{-3} M without indication that it interfered with the SFP response to higher perfusion rates (Fig. 4). Higher concentrations of ethacrynic acid were not tested because of this compound's limited solubility.[2]

[2] We have performed additional experiments recently the results of which show inhibition of the SFP response when ethacrynic acid is added to the perfusion solution either in combination with cysteine in a molar ratio of 1:1.1 (24) or as sodium ethacrynate (Edecrin, Merck Sharp & Dohme), a more soluble form of ethacrynic acid, in a higher concentration. No interference with feedback suppression of SFP was seen in these additional experiments with Ringer perfusion solution alone, with ethacrynic acid 10^{-3} M, with ethacrynic acid-cysteine 10^{-4} M, or with Na-ethacrynate 10^{-3} M. The feedback response was completely blocked by a perfusion solution containing ethacrynic acid-cysteine 10^{-3} M or Na-ethacrynate $5 \times$

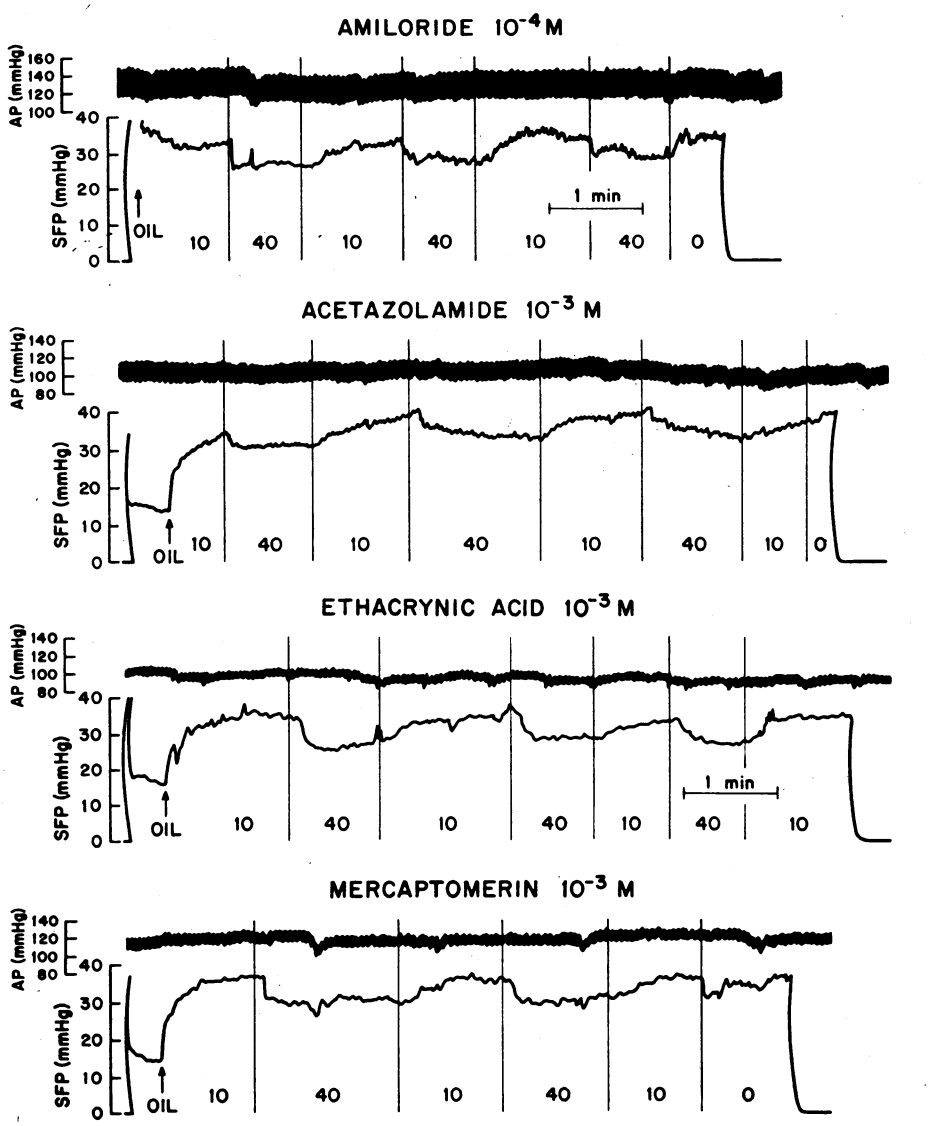

FIGURE 3 Effects of loop perfusion rate and transport inhibitors on SFP. As in Fig. 1 single examples are shown. The perfusion solutions contained amiloride, acetazolamide, ethacrynic acid, and mercaptomerin.

Results from SFP experiments with two additional solutions are shown in Fig. 5. With 0.15 M NaCl as the perfusion solution increasing the flow rate from 10 to 40 nl/min was followed by a decrease in SFP. The mean decrease ($P < 0.001$) of 4.6 mm Hg during NaCl perfusion was not different ($P < 0.2$) from the decrease seen during Ringer perfusion. There was a suggestion, however, in the individual tracings that the response was slower. When 10^{-4} M poly-L-lysine was added to Ringer solution stained with FD & C Green, SFP decreased when loop flow was increased. However, when the poly-L-lysine solution was not stained, SFP increased with increases in perfusion rate. The average increase in SFP seen when flow into the loop was increased to 40 nl/min was 1.6 mm Hg ($P < 0.005$). We tested the effect of using the unstained solution because of the possibility that the anionic dye would bind and inactivate the cationic poly-amino acid (10, 11). An attempt to use a cationic dye, capri blue, was not successful because the dye appeared to bind to the tubule wall at the perfusion site. Without dye in the perfu-

10^{-3} M. Also, distal collection experiments showed that these last two solutions, but not ethacrynic acid 10^{-3} M, reduced fluid and chloride absorption along Henle's loop by 40% and increased emerging chloride concentration to 100 mM (at 10 nl/min perfusion) and 140 mM (at 40 nl/min).

sion solution, however, we were unable to observe flow in the distal convolution. Because of this lack of visual control over the perfusion, and because the poly-L-lysine solution caused a spreading pallor of the renal surface in the vicinity of the perfusion site, we did not use this compound in further experiments.

To determine whether the agents that prevented the SFP response to increased flow of Ringer solution through Henle's loop would also interfere with the reciprocal change in SNGFR observed in a previous investigation (3), we performed a second set of experiments involving simultaneous perfusion of the loop of Henle and collection of proximal tubule fluid using three of the agents that blocked the SFP response and one that did not. Perfusion fluids were either Ringer solution or Ringer solution with furosemide, triflocin, cyanide, or amiloride. The results of these experiments are shown in Fig. 6 and Table II. When single loops were perfused with Ringer solution, the volume flow rate in the proximal tubule of that nephron was decreased 37% when the perfusion rate was increased from 10 to 40 nl/min. When perfusion of the loop was stopped, measurements of proximal flow were higher than during perfusion at 10 nl/min. Since the three successive collections in these experiments were made by puncturing slightly closer to the glomerulus at each perfusion rate, somewhat higher collected volumes and lower $(TF/P)_{inulin}$ ratios might be expected in the second and third collections. Increases in perfusion rate to 40 nl/min were associated with an increase in $(TF/P)_{inulin}$, whereas at zero perfusion $(TF/P)_{inulin}$ was lower than at 10 nl/min. Increasing loop perfusion from 10 to 40 nl/min was, therefore, associated with a mean decrease in SNGFR of 31%.

When 10^{-4} M furosemide was added to the perfusion fluid proximal flow rate was 10% lower at the higher perfusion rate. $(TF/P)_{inulin}$ was not different. The decline in SNGFR that occurred when loop flow rate was increased was significantly smaller ($P < 0.001$) than the decrease seen in the Ringer perfusion experiments. Addition of 10^{-3} M triflocin to the perfusion fluid blocked the effects of increased perfusion rate even more completely. Proximal flow rate, $(TF/P)_{inulin}$ and SNGFR were not different at the two perfusion rates.

A 19% increase in the rate of proximal flow was observed when the perfusion rate of Ringer solution containing 10^{-3} M NaCN was increased from 10 to 40 nl/min. A slightly smaller decrease in $(TF/P)_{inulin}$ in these samples indicated a 6% higher value for SNGFR, which was not, however, significantly different from the value at the low perfusion rate ($P > 0.2$).

Amiloride, which did not block the SFP response to increased perfusion rate, also did not appear to interfere with the SNGFR response. With 10^{-4} M amiloride

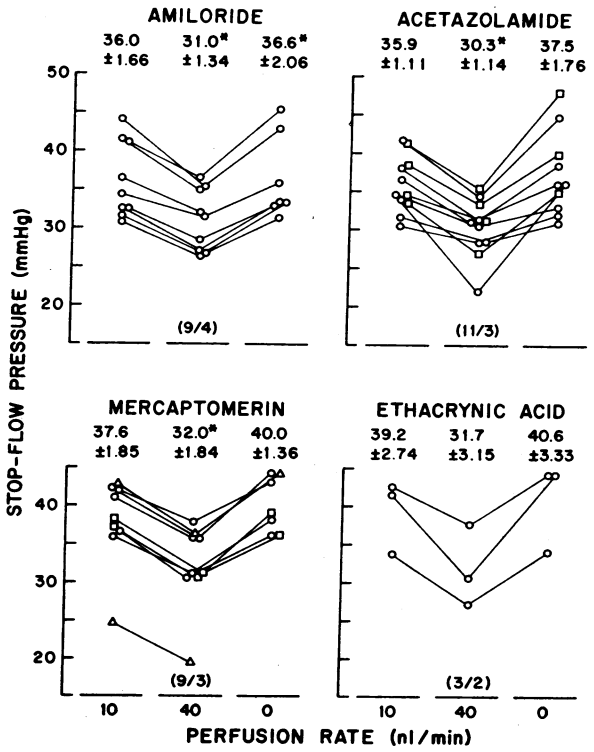

FIGURE 4 Effects of loop perfusion rate and transport inhibitors on SFP. Data are from all experiments with the perfusion solutions shown in Fig. 3. Points are averages of results in a single nephron at each perfusion rate. Values above the points are means ±SE. (*) indicates mean is different from the SFP at 10 nl/min ($P < 0.05$). Numbers in parentheses indicate tubules/rats. Acetazolamide was tested at 10^{-4} M (circles) and 10^{-3} M (squares). Mercaptomerin was tested at 10^{-3} M (circles), 5×10^{-3} M (triangles), and 10^{-2} M (squares).

increases in loop flow to 40 nl/min were associated with a 35% decrease in proximal flow rate and no significant change in $(TF/P)_{inulin}$. The 28% decrease in SNGFR observed at the higher perfusion rate was not different from the decrease in SNGFR observed with Ringer solution alone.

Since furosemide, triflocin, and cyanide, but not amiloride, prevented flow-dependent decreases in both SFP and SNGFR seen in Ringer-perfused nephrons, we examined in a third set of experiment changes in the volume and composition of these fluids during their passage through the loop of Henle. In these experiments two successive collections were made from the same early distal segment while perfusing from a late proximal site at either 10 or 40 nl/min. In 22 of 48 experiments the higher rate was tested first. The results of these experiments are shown in Figs. 7 and 8 and Table III. When the loop was perfused at 10 nl/min, the volume collected at the early distal site was 60–80% larger

when 10^{-4} M furosemide, 10^{-5} M triflocin, and 10^{-4} M amiloride were added to the perfusion fluid. The collection rate in cyanide experiments was not different from that with Ringer's perfusion. No difference in collection rate was detected with any perfusion solution when the pump rate was 40 nl/min. The rate of fluid reabsorption along the loop segment, increased with all perfusion solutions when the perfusion rate was increased. With Ringer solution a fourfold increase in perfusion rate was associated with a doubling of absolute reabsorption. The calculated changes in fluid absorption were from 1.6 to 4.2 nl/min less with the other four perfusion solutions, however, only the change with the cyanide solution was significantly smaller ($P < 0.05$) than that with Ringer solution. In the 9 cyanide perfusions where we could not calculate V_o the perfusion rate was taken as the average value for the 39 tubules in Table I.

During its passage through loop segments at the lower perfusion rate Ringer solution was diluted (Figs. 7 and 8). Fluid emerging from Ringer-perfused loops had a mean sodium concentration of 51 meq/liter ±5 SE, a chloride concentration of 48±4, and a potassium concentration of 2.5±0.4. The loops' capacity to lower ionic concentrations was diminished when the perfusion rate was increased from 10 to 40 nl/min. At the high perfusion rate early distal Na, Cl, and K were 116±6, 121±4, and 4.8±0.8.

Addition of 10^{-4} M furosemide to the perfusion fluid impaired the ability of the loop segment to lower the concentrations of Na, Cl, and K. With furosemide the concentrations of these ions were about three times higher at the low perfusion rate and about 30% higher at the high perfusion rate. The concentrations at 10 and 40 nl/min were as follows: 132 meq/liter ±8 SE and

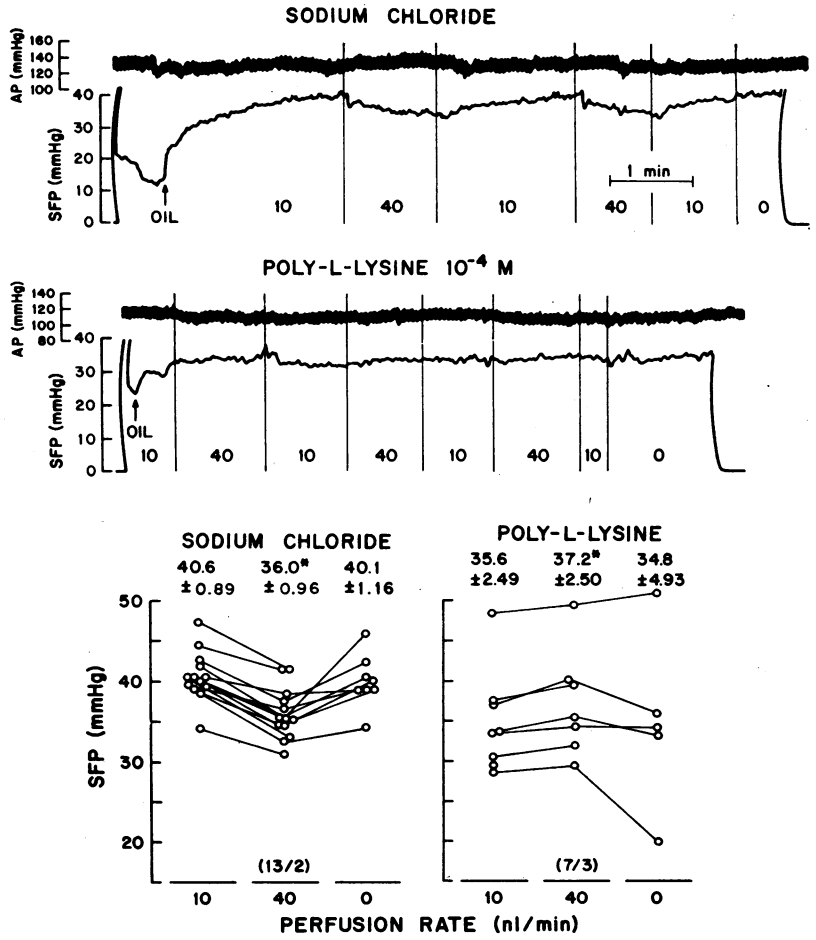

FIGURE 5 Effects of loop perfusion rate and transport inhibitors on SFP. Data with two perfusion solutions, 0.15 M NaCl and 10^{-4} M poly-L-lysine, are shown. The top tracings are for single experiments as in Figs. 1 and 3. The lower panels include all data as in Figs. 2 and 4.

147±6 for Na; 143±7 and 159±4 for Cl; and 8.1±1 and 7.9±0.5 for K. There were no differences between values measured at 10 and 40 nl/min.

Addition of 10^{-3} M triflocin to the perfusion fluid also reduced the degree to which Na, Cl, and K concentrations were lowered during passage through the loop of Henle. At 10 nl/min perfusion emergent Na and Cl concentrations were approximately two times higher with triflocin than with Ringer solution alone. At 40 nl/min perfusion the Na and Cl concentrations were about 30% higher with triflocin-containing solution. Potassium concentrations were not significantly different with triflocin and Ringer solutions at either perfusion rate. With triflocin the ionic concentrations at low and high perfusion rates were as follows: Na, 106±3 and 151±5; Cl, 101±4 and 150±2; K, 3.2±0.6 and 5.6±0.6. The concentration of each ion was significantly higher at the higher perfusion rate.

In nephrons perfused with 10^{-2} M NaCN the early distal Na concentration at the low perfusion rate was slightly higher ($P < 0.025$) than in Ringer-perfused loops. Chloride concentrations were not different from those found during Ringer perfusion at either flow rate. The perfusion fluid in these experiments had a higher sodium concentration than that of the Ringer solution: 154 meq/liter vs. 144. Chloride concentrations in the perfusion fluids were 145 meq/liter. Early distal potassium concentrations were not different in cyanide and Ringer-perfused nephrons. With cyanide perfusion ionic concentrations in distal fluid were increased when the perfusion rate was increased from 10 to 40 ml/min: Na, 68±4 and 126±4; Cl, 57±4 and 121±4; K, 2.6±0.4 and 4.4±0.6.

Perfusion with fluid containing 10^{-4} M amiloride yielded results simliar to those with Ringer and cyanide solutions. Average ionic concentrations at low and high perfusion rates were: 56±4 and 122±6 for Na; 50±5 and 121±5 for Cl; and 1.7±0.2 and 3.0±0.5 for K. The concentrations increased significantly with perfusion rate and in no case were different from the concentra-

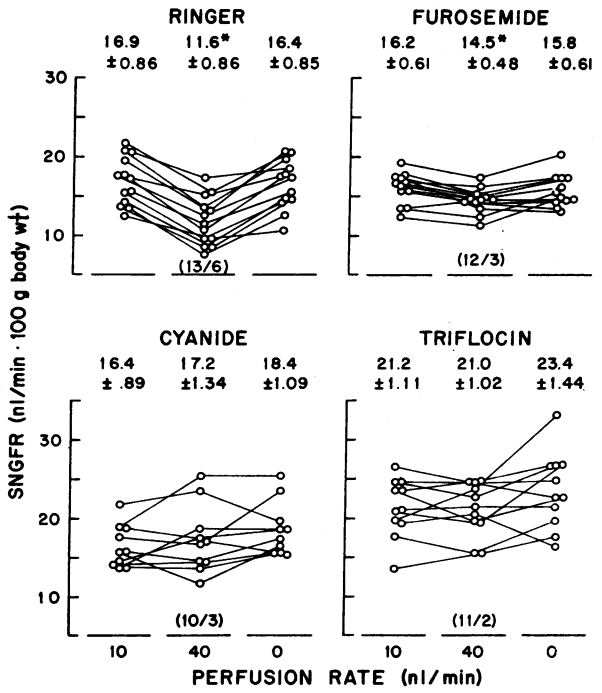

FIGURE 6 Effects of loop perfusion rate and transport inhibitors on SNGFR. Data are from all experiments with the perfusion solutions in Figs. 1 and 2. Points indicate single measurements.

TABLE II

Proximal Collection Experiments

	Perfusion solution														
	Ringer			Furosemide			Triflocin			Cyanide			Amiloride		
Perfusion rate, *nl/min*	10	40	0	10	40	0	10	40	0	10	40	0	10	40	0
Collected $\dot{V}_{TF}$, *nl/min*	29.9 ±1.9	18.7 ±1.7	32.6 ±1.8	28.5 ±1.6	25.6 ±1.4	29.3 ±1.6	41.1 ±2.3	40.5 ±2.5	45.5 ±2.4	24.0 ±1.9	28.5 ±2.8	25.6 ±2.2	23.7 ±4.6	15.5 ±3.1	22.9 ±4.8
P		<0.001	<0.05		<0.05	NS		NS	NS		<0.05	NS		<0.005	NS
n	13			12			11			10			4		
$(TF/P)_{IN}$	1.38 ±0.08	1.51 ±0.09	1.23 ±0.08	1.38 ±0.05	1.36 ±0.07	1.29 ±0.07	1.42 ±0.06	1.44 ±0.07	1.42 ±0.08	1.34 ±0.07	1.14 ±0.03	1.36 ±0.06	2.01 ±0.25	2.24 ±0.37	1.89 ±0.20
P		<0.01	<0.005		NS	NS		NS	NS		<0.025	NS		NS	NS
n	13			12			11			10			4		
SNGFR, *nl/min*	40.6 ±3.1	28.1 ±2.9	39.3 ±2.7	37.8 ±1.4	33.9 ±1.2	36.9 ±1.6	57.5 ±2.7	57.0 ±2.6	63.2 ±3.0	30.1 ±1.3	32.2 ±3.1	34.1 ±2.5	44.1 ±4.2	31.9 ±5.0	41.5 ±6.7
P		<0.001	NS		<0.001	NS		NS	NS		NS	NS		<0.05	NS
n	13			12			11			10			4		

Values are means±SE.
P is for significance of difference from value at perfusion rate of 10 nl/min.

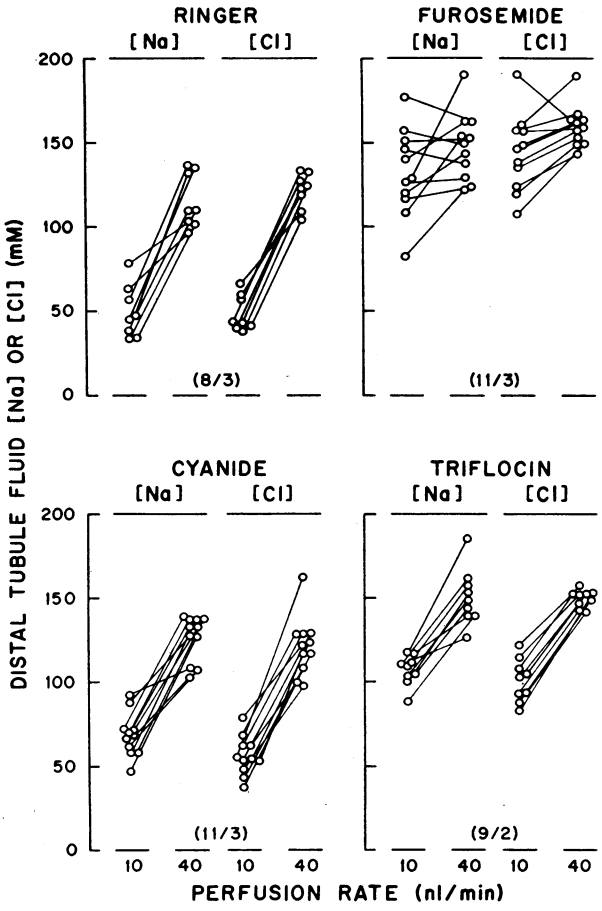

FIGURE 7 Effect of loop perfusion rate and transport inhibitors on early distal concentrations of Na and Cl. Data are from all experiments with the perfusion solutions in Figs. 1, 2, and 6. Points indicate single measurements.

tions observed with Ringer perfusion. The average quantities of Na, Cl, and K absorbed (or secreted) during passage through the loop of Henle are shown in Table III. As with absolute fluid absorption, a fourfold increase in the perfusion rate of Ringer solution resulted in a doubling of the amounts of Na and Cl absorbed. Potassium reabsorption was not different at the two perfusion rates.

Sodium and chloride absorption were only slightly different when 10^{-4} M amiloride was added to the perfusion fluid. At 10 nl/min perfusion Na and Cl absorption were not different in Ringer and amiloride experiments. The increase in Na and Cl absorption when the perfusion rate was increased to 40 nl/min, however, was less than twofold and the amounts absorbed at the higher perfusion rate were significantly less with amiloride than with Ringer alone. Another effect of amiloride appears to be a greater degree of net potassium absorption along the perfused segment. In contrast to the results with Ringer perfusion, with fluid containing amiloride the K absorption is significantly higher at 40 than at 10 nl/min.

With 10^{-3} M NaCN in the perfusion fluid both sodium and chloride absorption were reduced. Na and Cl absorption in cyanide experiments were not different from the values in Ringer experiments at the low perfusion rate, but when the pump rate was increased to 40 nl/min, the reabsorption of both ions was less with cyanide than with Ringer solution. Potassium absorption was not different at the two perfusion rates.

A reduction in the reabsorptive rates of sodium, chloride, and potassium was seen at both pump rates when 10^{-4} M furosemide was added to the perfusion solution. At 10 nl/min about half as much Na and Cl were absorbed from the furosemide-containing solution compared with Ringer solution. The reduction in reabsorption of these ions was even more marked at 40 nl/min. There was a small increase in sodium absorption when the perfusion was increased but this was only a third of the increase with Ringer perfusion. Potassium reabsorption appears to have been abolished by furosemide. At the lower perfusion rate potassium was neither added or absorbed. At 40 nl/min the direction of net K transport was reversed and net secretion between the perfusion and collection sites is evident. Addition of 10^{-5} M triflocin to the perfusion fluid resulted in a similar impairment of sodium and chloride absorption. No

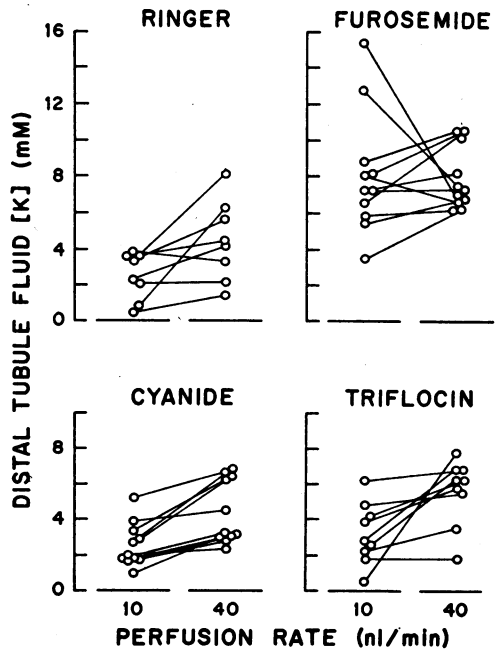

FIGURE 8 Effect of loop perfusion rate and transport inhibitors on early distal K concentration. Data are from the same collections as in Fig. 7.

TABLE III
Distal Collection Experiments

	Perfusion solution									
	Ringer		Furosemide		Triflocin		Cyanide		Amiloride	
Perfusion rate, *nl/min*.....	10	40	10	40	10	40	10	40	10	40
$\dot{V}_{TF}$, *nl/min*	3.4 ±0.3	32.9* ±2.0	6.2‡ ±0.3	32.3* ±1.0	5.9‡ ±0.5	34.0* ±0.6	4.4 ±0.5	33.6* ±1.1	5.4‡ ±0.3	33.7* ±0.7
AR, *nl/min*	6.1 ±0.2	12.2* ±1.9	4.5‡ ±0.3	8.9* ±0.8	5.1‡ ±0.4	9.6* ±1.0	6.8 ±0.7	8.7* ±0.8	5.5 ±0.3	9.0* ±0.5
FR	0.65 ±0.02	0.25* ±0.03	0.41‡ ±0.01	0.22* ±0.02	0.46‡ ±0.02	0.22* ±0.02	0.60 ±0.05	0.20* ±0.02	0.51‡ ±0.02	0.21* ±0.01
AR Na, *pmol/min*	1201 ±48	2727* ±207	677‡ ±64	1174‡* ±218	952‡ ±48	1156‡ ±141	1337 ±93	1974‡* ±226	1291 ±59	2064‡* ±96
AR Cl, *pmol/min*	1216 ±48	2574* ±267	629‡ ±54	764‡ ±147	931‡ ±65	993‡ ±165	1234 ±59	1537‡ ±179	1286 ±62	2033‡* ±91
AR K, *pmol/min*	31 ±1.6	44 ±25.2	−6‡ ±6.2	−66‡* ±24.8	26 ±5.0	−11 ±23.0	30 ±2.9	−11 ±22.5	34 ±2.1	68* ±14.7
n	8		11		9		11		9	

$\dot{V}_{TF}$ is volume collected at early distal site, AR is absolute reabsorptive rate of fluid along the perfused segment, FR is fractional reabsorption of fluid, AR Na is absolute reabsorptive rate for sodium.
Values are means±SE.
* Indicates mean at 40 different from mean at 10 ($P < 0.05$).
‡ Indicates mean is different from Ringer value ($P < 0.05$).

increase in reabsorption of either Na, Cl, or K occurred when the perfusion rate of triflocin solution was increased.

DISCUSSION

In addition to confirming the results of two previous investigations in which increased rates of flow through the loop of Henle were associated with decreased SNGFR (3) and with decreased P_{Go} (4), the present experiments show that furosemide, triflocin, and cyanide are capable of interfering with this feedback response. In the earlier experiments neither the proximal SFP (4) nor the SNGFR (3) responses to increased perfusion rate in the loop of the same nephron were seen when solutions of mannitol or sodium sulfate were substituted for a Ringer perfusion solution. These findings led to the suggestion (3, 4) that feedback regulation of GFR might depend on the rate of sodium absorption by the macula densa cells of the distal nephron. That chemical inhibitors of transport also interfere with the feedback response, provides additional evidence that the transport of some constituent of distal fluid is involved in its mediation.

Another property of the feedback mechanism appears to be that, starting with normal flow rates of tubule fluid, it responds more to increases in delivery of fluid from the proximal tubule than to decreases in fluid delivery. Hierholzer, Butz, Müller-Suur, and Lichtenstein (12) found that early proximal tubule pressure and volume flow decreased when flow rate through the loop of Henle was increased from 7 to 15 nl/min but that pressure did not change when loop flow was decreased. Similarly, Blantz et al. (5) and Israelit, Rector, and Seldin (13) have found no persisting change in P_{Go} when flow into the loop was blocked. Schnermann et al. (4) examined a wide range of loop perfusion rates and found the greatest responsiveness at rates between 15 and 35 nl/min, that is, at and above normal end-proximal flow rates. Reduction of loop flow below normal rates had little effect on SFP.

Stop-flow pressure. Continuous measurement of proximal SFP while varying the rate of loop perfusion was used as a screening procedure to identify agents that might interrupt the feedback response. In contrast to the result with Ringer perfusion, increases in loop perfusion rate were not followed by decreases in SFP when the perfusion fluid contained either the diuretic agents furosemide and triflocin or cyanide or poly-L-lysine. The ascending limb of Henle's loop is considered to be the major site of action of furosemide (14–16) and of triflocin (17–19). An action in the ascending limb has also been attributed to cyanide (20–21), however, its action and that of poly-L-lysine are probably not limited to this nephron segment. Diuretic drugs that did not appear to interfere with the response of SFP to changes in loop flow rate were amiloride, acetazolamide, mercaptomerin, and ethacrynic acid. Amiloride and acetazolamide are agents that do not inhibit transport in the loop of Henle. Burg and Green (22) found no effect of either drug when they were added to the bath surround-

ing, or the solution perfusing, isolated ascending limbs of rabbit nephrons. In similar experiments with isolated rabbit-ascending limbs Burg and Green have found that mersalyl 10^{-5} M (23) and ethacrynic acid 10^{-3} M (24) do inhibit active Cl transport. The failure of mercaptomerin and ethacrynic acid at 10^{-3} M to interfere with the feedback response in the present experiments is probably due to a true species difference. Cafruny (25), Cafruny, Cho, and Gussin (26) Heidenreich (27), and Baer and Beyer (28) have pointed out that mercurials do not increase urine flow as promptly or as much as they do in other species. Cafruny attributes the small diuretic effect that is seen to renal injury. Similarly, Goldberg has concluded (29) that, since the dose of ethacrynic acid that is needed to produce diuretic effects in rats is 20 times larger than the maximally effective dose in dogs and humans (28, 30–32), the effects that are seen in rats are probably due to changes unrelated to their usual action in other species at lower doses. Although removal of potassium, calcium, magnesium, bicarbonate, and urea from the perfusion solution did not block the feedback response, involvment of one of these substances in signaling a feedback mechanism is not clearly ruled out by this experiment, since any of these constituents of normal tubule fluid could have entered the perfusion fluid during its passage through the descending limb.

To avoid some of the variability that attends comparisons among either different groups of animals or individual nephrons, we tested the effect of increasing and decreasing loop flow rate in the same nephron. Because we were attempting to identify agents that would or would not interfere with feedback control, the experiments were designed to yield maximum changes. To best distingush between perfusion solutions we chose a large change in flow, increasing the pump rate from a rate slightly below normal to a rate at which a maximum response could be expected (4). Using this procedure we found clear differences between Ringer solution and solutions containing furosemide, triflocin, or cyanide in the SFP responses to increased perfusion rate. We did not find that SFP was initially higher when the loop was being perfused at 10 nl/min with the agents capable of blocking feedback. This is as expected in view of the unresponsiveness of the feedback mechanism at flow rates lower than normal (4, 12).

Filtration rate. Furosemide, triflocin, cyanide, and amiloride were examined in further experiments to determine whether they would affect the SNGFR response to increased loop flow in a manner similar to their effects on the SFP response. When the perfusion rate of solutions containing furosemide, triflocin, or cyanide was increased, SNGFR was not reduced to the extent that it was when the loop was perfused with either Ringer or amiloride solutions. The 10% decrease in SNGFR of furosemide-perfused nephrons was significantly less than the 37 and 28% reductions seen with Ringer and amiloride. No decrease in SNGFR occurred with triflocin perfusion. With cyanide SNGFR increased in 6 of 10 tubules.

Reductions of perfusion rate from 10 nl/min to 0 did not cause increases in filtration rate. Since increases in proximal tubule pressure (12), SFP (4), and P_{GC} (5) have been found to be small or nonexistent when loop perfusion is reduced below normal, increases in SNGFR would not be expected to be large. Because of this apparent nonlinearity of the feedback response, and because 10 nl/min is a lower than normal rate of fluid delivery, it is not surprising that higher filtration rates were not observed at zero perfusion.

Function of the loop of Henle. Because of its close anatomical association with the vascular structures of the glomerulus of the same nephron, the macula densa cells of the early distal tubule are prime suspects as a sensing site in the feedback mechanism. As a first step toward understanding functional changes that might have occurred in the macula densa segment, we analyzed fluid emerging from the perfused loops and calculated changes in reabsorption of fluid, sodium, chloride, and potassium between the perfusion and collection sites.

Although we have speculated (3, 4) that changes in macula densa sodium transport might signal a feedback mechanism, the present experiments do not rule out a role for some other constituent of distal tubule fluid in mediating feedback. Chloride appears to be the ionic species actively transported by cells of the ascending limb (33, 34) and may also be actively transported by macula densa cells. Calcium has recently been implicated as a mediator of feedback (13, 35). Since agents that inhibit NaCl transport interfere with the SFP and SNGFR response to increased perfusion rate, we think it preferable to refer to this inhibitable portion of the feedback pathway as salt transport, leaving open for the present the question of just which transported substance is the crucial one.

Whereas we have not measured macula densa salt transport directly, it is probable that in Ringer-perfused tubules NaCl transport by macula densa cells increased when loop flow rate did. This is because increases in the rate of Ringer perfusion resulted in a threefold increase in the concentration of Na and Cl in the fluid emerging from the loop of Henle (Fig. 7) and in a doubling of the rates of Na and Cl absorption along the loop segment (Table III). At low perfusion rates Na and Cl concentrations are low because water absorption is less complete than salt absorption. Faster flow rates limit the time available for any portion of the ascending limb to lower NaCl concentration and thus cause the fluid to move on to the next portion

at a higher than normal concentration. In each more distal portion of the ascending limb the higher luminal NaCl concentration adds to the driving forces for salt absorption. Thus, although we cannot say with certainty where along the loop segment Na and Cl absorption were increased, it is likely that much of the observed change occurred in the more distal parts of the ascending limb. If macula densa cells absorb Na and Cl, the higher luminal NaCl concentration at higher perfusion rates should have increased their rate of salt transport.

Furosemide and triflocin clearly inhibited Na, Cl, and fluid absorption along the perfused segment at both perfusion rates (Table III). Compared with Ringer-perfused tubules, Na and Cl absorption were reduced more than volume reabsorption was. The early distal Na and Cl concentrations were, therefore, higher than in Ringer-perfused nephrons at both 10 and 40 nl/min and would be expected to have exerted a stimulating effect on macula densa NaCl absorption. However, because of the direct effect of these agents on salt transport, we think it likely that increasing the perfusion rate of solutions containing furosemide or triflocin resulted in less stimulation of macula densa NaCl transport than occurred with Ringer solution. The observed interference with the SFP and SNGFR responses to increased perfusion rate would follow from this postulated reduction in macula densa salt reabsorption.

When cyanide was added to the perfusion fluid, NaCl absorption was not reduced at the low perfusion rate and at the high perfusion rate, Na and Cl concentrations increased only to the same extent as with Ringer perfusion. This happened because Na, Cl, and fluid absorption were all about 28% less than with Ringer perfusion. In contrast to the Ringer's experiments, however, increases in loop perfusion rate were not followed by decreases in SFP or SNGFR. Since cyanide has been found to act in the ascending limb (20, 21) it is possible that a direct inhibitory action of cyanide on macula densa cells prevented NaCl absorption from increasing. In addition, since at physiological pH cyanide is largely in the undissociated HCN form and penetrates membranes rapidly (36) and has been found to diffuse rapidly through renal tissue (37, 38), it is also possible that cyanide diffused from the tubule lumen to the vascular structures at the glomerular pole and directly relaxed the vascular tone of the afferent arteriole. Cyanide has a direct vasodilator action on vascular smooth muscle (39). PAH clearance is increased by arterial infusion of KCN (40). The increases in SFP and SNGFR that were seen in most nephrons after increasing the perfusion rate of cyanide solution, therefore, could have been due to the combined effects of inhibition of macula densa NaCl transport and direct vasodilation of the afferent arteriole.

With amiloride in the perfusion fluid, Na and Cl concentrations at the early distal collection site were increased to the same extent as with Ringer perfusion. However, since amiloride does not affect salt transport in isolated ascending-limb segments in vitro (22), these changes probably occurred beyond the ascending limb in the first part of the distal convolution. Thus, a flow-dependent signal accounting for the similar SFP and SNGFR responses, could have been the same in Ringer- and amiloride-perfused nephrons.

These experiments do not establish which constituent of ascending limb fluid is sensed by the macula densa, however, they do indicate that increased absorption of a transported species is involved in the pathway that links increases in flow through the loop of Henle to decreased rates of glomerular filtration.

ACKNOWLEDGMENTS

We thank Miss Michelina Basile for her excellent technical assistance.

This work was supported by U. S. Public Health Service grant AM 14423 and by American Heart Association grant 760.

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