

A Stop-Flow Study of Intrarenal Effects of Atrial Natriuretic Factor (42314)

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Abstract. We performed paired series of stop-flow studies on six mongrel dogs to determine a possible nephron site of action of synthetic atrial natriuretic factor (ANF). The initial free-flow response to intrarenal infusion of 5 $\mu\text{g}/\text{min}$ of synthetic ANF into mannitol-expanded dogs resulted in an increased urine flow rate (6.81 ± 0.88 to 9.00 ± 1.17 ml/min, $P < 0.05$) and a 40% increase in sodium excretion (496 ± 110 to 694 ± 166 meq/min, $P < 0.025$) when compared to paired control periods. Renal blood flow did not change, but the glomerular filtration rate increased 4% (47 ± 5 to 49 ± 6 ml/min, $P < 0.05$). The filtered load of sodium increased 4% ($P < 0.05$), and the fractional sodium excretion increased by 35% ($P < 0.01$). Stop-flow experiments showed no difference in tubular sodium concentration or in the fractional sodium-to-inulin ratio at the nadir of sodium concentration, suggesting that no differences existed in distal tubular sodium handling. Further, no apparent differences were detected in collections representing the more proximal portions of the nephron. While we were able to demonstrate marked natriuresis in response to synthetic ANF, no tubular effect was discernible, and the natriuresis obtained appears to be predominantly a function of hemodynamic effects. © 1986 Society for Experimental Biology and Medicine.

Mammalian atria contain peptides located in granules of the atrial cardiocytes. This class of peptides exerts powerful natriuretic and diuretic effects upon the kidney, and is therefore commonly referred to as atrial natriuretic factor or ANF. The mechanism of ANF-induced natriuresis and diuresis remains controversial. Some studies have suggested that ANF acts by means of hemodynamic changes, increasing the glomerular filtration rate (GFR) and the filtered load of sodium to the nephron (1-3) while others have proposed that ANF acts directly upon tubular sodium reabsorption (4, 5) localizing the site of action in either the proximal tubule (1) or in the collecting duct (6, 7).

The stop-flow technique (8) has been used to localize sites of action or the production of various substances within the kidney. Our study was designed to use this technique to localize a possible tubular site of action of ANF upon tubular sodium reabsorption. The stop-flow method seems well suited to study this controversial subject because it can determine

segmental nephron handling of sodium and water without the confounding effects of glomerular filtration.

Methods. We performed paired stop-flow experiments on 10 mongrel dogs weighing between 17 and 23 kg, using the technique of Malvin *et al.* (8). Before surgery, all dogs were fasted overnight. The dogs were then anesthetized with sodium pentobarbital (30 mg/kg) and sustained with supplemental anesthesia throughout the study. Mechanical ventilation was maintained with a Harvard respirator (Harvard Apparatus, Millis, Mass.) based in reference to the nomogram of Kleinman and Radford (9). A femoral artery was cannulated to collect arterial blood and to continuously measure blood pressure via a pressure transducer (Statham P23Db, Hato Rey, P.R.) connected to a Brush 440 recorder (Gould Instruments, Cleveland, Ohio). A femoral vein was cannulated to facilitate a continuous maintenance infusion of 3.0 ml/min 0.9% saline and to administer supplemental anesthesia. Body temperature was maintained with a circulating water heating pad (Hamilton Aquamatic K-20, Hamilton Ind., Cleveland, Ohio). The dogs were suspended from a metal frame to approximate their normal upright position.

The right and left ureters were exposed through retroperitoneal flank incisions and

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cannulated with PE-240 catheters (Clay Adams, N.Y.), which were carefully inserted and advanced until the tip was just distal to the renal pelvis. The right ureter was catheterized to ensure free flow in the contralateral kidney during the stop-flow. The dead space in the left catheter was 0.5 ml.

To measure renal blood flow, the left renal artery was cleaned and fitted with a noncannulating electromagnetic flow probe (Carolina Electronics, King, N.C.). A zero-flow baseline was established upon fitting and confirmed at the end of each experiment by mechanical occlusion of the artery distal to the probe. Accuracy of the probes was controlled by calibration *in situ* using timed collections from a cannulated renal artery. A modified 23-gauge butterfly needle placed into the left renal artery for delivery of ANF or vehicle was maintained patent by a continuous infusion of 0.5 ml/min normal saline. Arterial blood samples for hematocrit (Hct), a plasma inulin blank, and plasma sodium were collected, and each dog was allowed to equilibrate for 30 min.

ANF dose-response experiments. We performed experiments using a 24-amino acid (Atriopeptin-III) synthetic atrial natriuretic factor from Peninsula Laboratories (Belmont, Calif.). To establish its potency, a dose-response experiment was carried out in a euvolemic dog. First, three consecutive 2-min collection periods were run with the saline vehicle. Then, seven sequentially increasing doses of ANF were tested. The test started at 0.05 $\mu\text{g}/\text{min}$, and was increased to 0.125, 0.25, 0.5, 1.0, 2.6, and 5 $\mu\text{g}/\text{min}$. The resultant natriuresis and diuresis were monitored, and a dose which provoked a marked though not overwhelming response was selected.

Free-flow experiments. We performed six experiments with ANF using a paired design. In each dog two consecutive stop-flow experiments were run, one with intrarenal infusion of ANF and the other with the ANF saline vehicle. The order was randomly assigned. In an additional four dogs, control studies (two consecutive stop-flow experiments using an intrarenal infusion of only the saline vehicle) were run to assess the effect of sequence upon the results.

A priming dose of inulin (60 mg/kg, Difco Labs, Detroit, Mich.) was administered, after which volume expansion with a 20% mannitol

solution in 0.9% saline was initiated through intravenous infusion at a rate of 10–15 ml/min. Infusion rate was adjusted to achieve a constant urine flow rate of 6–10 ml/min.

Before stop-flow, a pair of 2-min, free-flow clearance collections were taken to ensure a relatively steady state of excretion and to determine that the subsequent infusion of ANF provoked a natriuretic and diuretic effect. Blood samples were taken for sodium, inulin, and hematocrit. The ANF was infused for 3 min at a rate of 5 $\mu\text{g}/\text{min}$, and an additional 2-min clearance collection was taken to evaluate the natriuretic effect. Previously, when we had run the dose-response experiments, we found that the excretory response (sodium and water) to 5 $\mu\text{g}/\text{min}$ ANF reached a peak within 2 min and remained constant over 8 min of constant infusion. Therefore, by waiting 3 min prior to free-flow collections, and 5 min before occlusion, we presume the kidney should be in a relatively steady state.

During free-flow conditions we measured renal blood flow (RBF), GFR by the clearance of inulin, urinary sodium excretion (U_{Na^+}), and urinary potassium excretion (U_{K^+V}). We calculated the following: renal plasma flow (RPF: $\text{RBF} \times 1 - \text{Hct}$); filtration fraction (FF: GFR/RPF); the filtered load of sodium (FL_{Na^+} : $[\text{P}_{\text{Na}^+}] \times \text{GFR}$); and the fractional sodium excretion (FE: $U_{\text{Na}^+V}/\text{FL}_{\text{Na}^+}$). Also, because kallikrein has been implicated in the action of ANF (10–12), we measured free-flow urinary kallikrein excretion ($U_{\text{KK}V}$).

Stop-flow. At the conclusion of the last free-flow clearance period, we initiated stop-flow conditions by clamping the left ureter shut at a point close to the origin of the kidney for 10 min. Then, the occluding hemostats were removed, and the rapid effluent of urine was collected in 36 serial 0.5-ml samples. The intrarenal infusion of ANF (5 $\mu\text{g}/\text{min}$) or of the ANF saline vehicle was maintained throughout both the occlusion and the collection periods. After the first stop-flow procedure, each dog was allowed to recover for 30 min; then the entire cycle of free-flow and stop-flow collections was repeated.

Analysis. Urinary and plasma inulin was determined after the method of Walser *et al.* (13). Plasma and urinary sodium and potassium were measured by flame photometry (Model 143, Instrumentation Lab., Boston,

Mass.). Kallikrein was measured by its kininogenase activity using partially purified dog kininogen as substrate and a radioimmunoassay for generated kinins (14).

The serial samples of urine collected represent a profile of static tubular fluid within the nephron in the absence of filtration and tubular flow (8). The sample containing the lowest sodium concentration (the sodium nadir) reflects pure distal function. Samples from all dogs were normalized by expressing serial collections as a percentage of the cumulative urine volume, with volume at sodium nadir equaling 100%. Samples collected earlier (<100%) represent a mix of distal, collecting duct, and pelvic urine. Samples collected after the nadir (>100%) represent a mix of some distal and proximal, and ultimately, free-flow urine. The "shoulder" in the collection curve approximates the proximal effect. The urinary-to-plasma (U/P) inulin ratio reflects the handling of water at any point along the nephron.

Values obtained during free-flow conditions were analyzed using a paired student's *t* test. Each collection curve was compared against its paired control, and the differences were analyzed for trends. Values are reported as means \pm 1 SE. The values at the nadir were analyzed with a paired *t* test. *P* values of less than 0.05 were considered significant.

Results. In an initial study to determine an effective dose of ANF, a progressive increase of intrarenal infusion of the synthetic ANF

resulted in a rapid twofold increase in natriuresis and diuresis at 2.6 $\mu\text{g}/\text{min}$ and in a fourfold increase in response to 5 $\mu\text{g}/\text{min}$. This response was rapidly reversible. We chose the 5- μg dose for further experiments to ensure that changes under the expanded conditions of stop flow were sufficiently amplified.

Free-flows results. Experimental results during 2-min free-flow periods taken just before ureteral occlusion, before and during ANF, are displayed in Table I. The ANF collection period was begun after 3 min of infusing 5 $\mu\text{g}/\text{min}$ of ANF into the renal artery.

The sodium concentration in urine samples during ANF did not change when compared with controls. However, urine sodium excretion increased 40% ($P < 0.025$), while urine flow rate increased 32% over controls ($P < 0.05$).

Renal blood flow showed no significant change. The glomerular filtration rate increased 4% from control ($P < 0.05$), and consequently the filtered load of sodium increased 4% (6.6 ± 0.8 to 6.9 ± 0.9 meq/min) in response to ANF ($P < 0.05$).

Fractional sodium excretion increased 36% ($P < 0.05$). Potassium excretion during free-flow ANF infusion did not change compared to free-flow control values. Although total kallikrein excretion remained unchanged, the urinary kallikrein concentration decreased significantly ($P < 0.05$) during ANF infusion.

Comparative values for free-flow collections

TABLE I. ACUTE EFFECTS OF ATRIAL NATRIURETIC FACTOR (ANF) UPON HEMODYNAMICS AND EXCRETION UNDER FREE-FLOW CONDITIONS

	Control	ANF	<i>P</i> value ^a
UV (ml/min)	6.81 \pm 0.88	9.00 \pm 1.17	<0.05
[U _{Na+}] ($\mu\text{eq}/\text{ml}$)	69.8 \pm 12.1	72.4 \pm 11.6	n.s.
U _{Na+} V ($\mu\text{eq}/\text{min}$)	496 \pm 110	694 \pm 166	<0.025
RBF (ml/min)	250 \pm 34	281 \pm 37	n.s.
GFR (ml/min)	47 \pm 5	49 \pm 6	<0.05
FE _{Na+} (%)	7.5 \pm 1.5	10.2 \pm 2.6	<0.05
U _{K+} V ($\mu\text{eq}/\text{min}$)	84 \pm 26	86 \pm 19	n.s.
[U _{KK}] ($\mu\text{g}/\text{ml}$)	174 \pm 33	105 \pm 20	<0.05
U _{KK} V ($\mu\text{g}/\text{min}$)	1143 \pm 264	1256 \pm 260	n.s.

Note. Values expressed as means of six dogs \pm 1 SE, except for kallikrein values in which only five measurements were obtained.

^a All *P* values derived by paired *t* test comparing parameters before (control) and during (ANF) infusion of atrial natriuretic factor. n.s. = not significant, UV = urinary excretion, [U_{Na+}] = urinary sodium concentration, U_{Na+}V = urinary sodium excretion, RBF = renal blood flow, GFR = glomerular filtration rate, FE_{Na+} = fractional sodium excretion, U_{K+}V = urinary potassium excretion, [U_{KK}] = urinary kallikrein concentration, and U_{KK}V = urinary kallikrein excretion.

taken in control/control experiments showed no significant changes. Urinary sodium excretion was unchanged (518 ± 101 to 530 ± 108 $\mu\text{eq}/\text{min}$), and there was no significant increase in urine flow rate (7.23 ± 1.03 to 7.54 ± 1.05 ml/min). Likewise, there was no change in GFR (43 ± 6 to 44 ± 5 ml/min) or in the fractional sodium excretion (9.2 ± 1.5 to $8.9 \pm 1.4\%$).

Stop-flow results. The profiles of data collected from stop-flow experiments with ANF are presented in Fig. 1. No differences in urinary sodium concentration at the sodium nadir were found when control and ANF values were compared (5.2 ± 1.4 vs 4.5 ± 1.1 $\mu\text{eq}/\text{ml}$, respectively). Likewise, no difference in sodium-to-inulin ratio (U/P $\text{Na}^+/\text{U/P}$ inulin) was noted at the nadir, and no difference was found in tubular inulin or urine-to-plasma inulin ratio at the sodium nadir (8.4 ± 1.4 vs 9.9 ± 2.1). A change in U/P inulin ratio should reflect changes in water reabsorption. Potassium concentration did not differ when control and ANF values were compared, either at the sodium nadir (6.3 ± 1.4 vs 5.0 ± 1.6 meq/liter, respectively) or at the more distal potassium shoulder (at 65% of volume, representing a distal locus for distal potassium secretion).

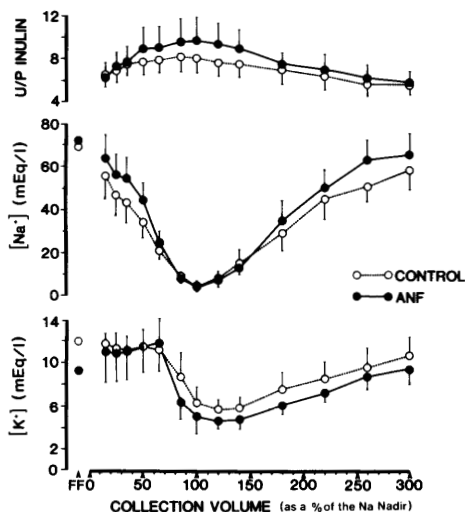


FIG. 1. Sodium and potassium concentrations and urinary inulin-to-plasma (U/P) ratio from sequential collections following stop-flow in kidneys undergoing saline infusion (control) or infusion of synthetic atrial natriuretic factor (ANF). Volume of collections is normalized to 100% of the volume found at the nadir of sodium concentration.

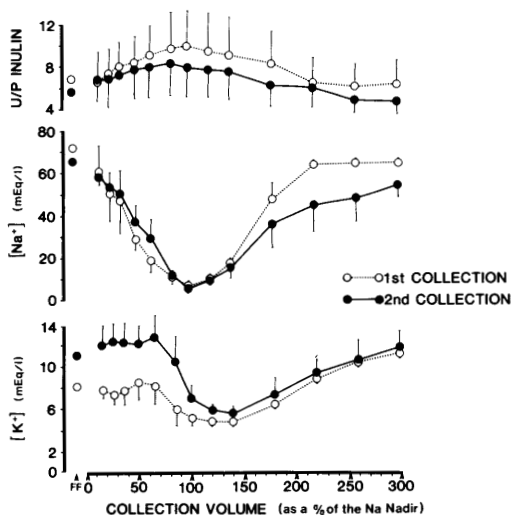


FIG. 2. Sodium and potassium concentration and urinary inulin-to-plasma (U/P) ratio from sequential collections following stop-flow in kidneys undergoing two consecutive experiments with saline infusion. Volume of collections is normalized to 100% of the volume found at the nadir of sodium concentration.

We found no difference in the profile of sodium, potassium, or the U/P inulin ratio between paired control and ANF infusion experiments. We were unable to discern any apparent proximal effect upon sodium reabsorption, as represented in the curves to the right of the sodium nadir in Fig. 1. There were no differences between the curves from ANF infusion when compared with their paired controls.

Profiles of data collected from control/control stop-flow experiments are presented in Fig. 2. No difference in urinary sodium concentration at the sodium nadir was seen when the first collection was compared with the second (6.4 ± 1.8 vs 5.4 ± 1.2 meq/liter, respectively). Likewise, there was no difference in tubular inulin or urine-to-plasma inulin ratio at the sodium nadir (10.2 ± 3.7 vs 8.2 ± 2.76 , respectively). Although not achieving statistical significance, potassium concentration tended to be less in the first collection and greater in the second collection period at the sodium nadir (5.2 ± 0.6 vs 7.0 ± 1.2 meq/liter, respectively) and in the more distal potassium shoulder (8.1 ± 1.6 vs 12.7 ± 2.1 meq/liter, respectively).

Paired double control stop-flow experiments suggested no difference in tubular sodium or water handling but did suggest a tendency for increased distal potassium secretion with the continued osmotic diuresis of the stop-flow protocol.

Discussion. Intrarenal infusion of synthetic atrial natriuretic factor in dogs undergoing a mannitol diuresis increased sodium and water excretion 40% and increased the filtered load of sodium 4% under free-flow conditions. We performed stop-flow experiments in these dogs to delineate a possible tubular site of action for ANF-induced natriuresis. Despite significant natriuresis during free-flow conditions, our studies did not localize any apparent tubular site of action of ANF.

The mechanism of ANF-induced natriuresis and diuresis remains controversial. Exogenous ANF produces hemodynamic effects resulting in an increased filtered load of sodium that can account for the natriuresis. Maack *et al.* (3) found that intravenous infusion of synthetic ANF (Auriculin A) resulted in increased GFR (28%), a fivefold increase in natriuresis and diuresis, and a 2.4-fold increase in potassium excretion. Renal blood flow increased only transiently. They suggested that the change in GFR could account for observed natriuresis. Camargo *et al.* (2) infused atrial extract into isolated perfused kidneys and found that an eightfold increase in natriuresis was accompanied by a 72% increase in GFR and a twofold rise in filtration fraction. When these authors infused the same atrial extract in reduced calcium perfusate, the change in GFR was abolished along with the significantly increased natriuresis. They concluded that the natriuretic effect of atrial extract was a function of its hemodynamic effects.

Using micropuncture during continuous infusion of ANF in rats, Huang *et al.* (15) noted a 45% increase in GFR, no change in renal plasma flow, and a 10- to 21-fold increase in sodium and chloride excretion. They noted a parallel increase in single nephron GFR at end proximal and early distal sites, and they concluded that ANF increased solute excretion predominantly by a hemodynamic mechanism.

Our free-flow results are consistent with those of Maack *et al.* (3), Camargo *et al.* (2) and Huang *et al.* (15) in that the natriuresis

we report can be accounted for by the increase in GFR and the associated increased filtered load of sodium.

While a hemodynamic effect of ANF has been established, some studies have suggested that ANF might also exert its effect by some direct tubular action. DeBold *et al.* (4) found that intravenous infusion of atrial extract into rats caused a 30-fold increase in sodium excretion without a significant increase in GFR, concluding that atrial extract contained a powerful inhibitor of renal tubular sodium reabsorption. However, GFR was increased in every rat, a mean of 28%. Keeler (16) injected a bolus of atrial extract intravenously into rats and found a transient ninefold increase in sodium excretion, and a nonsignificant 16% increase in GFR. He concluded that ANF acted by inhibiting the tubular transport of sodium. Micropuncture and microcatheterization studies (7) found that a bolus injection of atrial extract caused a 17-fold increase in sodium excretion without a significant increase in single nephron GFR. Whole kidney GFR increased 10%, although this change was apparently not significant. While the authors could not find any effect upon proximal or superficial distal sodium handling, they did find differences in sodium handling in the medullary collecting duct and papilla. They concluded that ANF inhibited sodium reabsorption in the medullary collecting duct. Borenstein *et al.* (17) have shown that ANF increases papillary blood flow, a hemodynamic alteration which could explain decreased medullary sodium reabsorption by means of medullary washout (2). Briggs *et al.* (6) also performed micropuncture studies on rats receiving intravenous atrial extract in two different doses. The lower dose induced a 13-fold increase in natriuresis, with a nonsignificant 11% increase in GFR and SNGFR ($n = 3$); the higher dose increased sodium excretion 33-fold, with a nonsignificant 16% increase in GFR and a significant 35% increase in distal single nephron GFR ($n = 3$). They proposed that the low dose of ANF could act upon the nephron, distal to their sites of measurement.

Burnett *et al.* (1) infused synthetic ANF intrarenally into dogs and found that a 13-fold increase in sodium excretion was associated with a significant 33% increase in GFR. Because the fractional excretion of both lithium

and phosphorous also increased, they suggested that these markers indicate a proximal tubular action of ANF beyond the obvious hemodynamic influence, though this could also be due to the increased filtered load of these ions.

The above studies, while suggesting that ANF may act through a tubular mechanism, do not provide any direct or conclusive evidence to support this hypothesis. In all cases a trend toward increased GFR was reported, all of which were greater in magnitude than the significant 4% increase we observed. These studies are also hampered by small *n*-values or the absence of steady-state conditions associated with bolus infusions. Hence the evaluation of real changes in filtration are difficult to interpret.

More recently, Murray *et al.* (5), working with the isolated perfused kidney model, noted natriuresis without measurable changes in GFR at low doses of continuously infused ANF. He attributed these changes to a direct tubular effect of ANF. At higher doses, increased GFR overwhelmed this effect.

Our stop-flow studies were carried out to obtain direct evidence of a tubular site of action of ANF. This technique has been well established to discriminate sites and direction of transport of particular solutes within the nephron (8). Using a paired design in which each dog served as its own control (or, additionally, comparison to untreated dogs), we were not able to determine any apparent site of action of ANF within the nephron. The tubular stasis obtained with the stop-flow technique eliminates the confounding effects of changes in hemodynamics or filtration; despite altered GFR, therefore, we should have been able to detect a secondary discrete tubular action of ANF on sodium reabsorption.

Since we could not clearly discriminate a tubular site of action of ANF in the nephron, our results do not support the possibility of a direct tubular action of ANF. The increased fractional sodium excretion induced with ANF may represent a diminution of glomerular-tubular balance and inability of the nephron to adapt to increased filtered load. It may be that the natriuresis is due to a combination of both hemodynamic and tubular effects, although the site for such a tubular action remains obscure.

The stop-flow technique has some technical limitations which should be considered when our data are interpreted (8). Because we must induce a mannitol diuresis, we are looking for a natriuretic effect superimposed upon an existing natriuresis. If production of endogenous ANF in response to volume expansion were to saturate tubular ANF receptors, we would be able to see only the effect of renal hemodynamic changes. The osmotic effect of the filtered mannitol may also nonspecifically obscure a tubular action of ANF. It should be noted that the magnitude of our natriuretic response was not as great as those described in other reports, nor as great as seen with the same concentration of ANF given to euvolemic dogs in our initial dose-response studies. Likewise, sodium and water excretion are greater than would be expected in euvolemic dogs prior to ANF administration. However, we still found a significant increase in absolute and fractional sodium excretion with ANF in these expanded animals.

We purposefully chose a dose of ANF which would induce a modest change in GFR, so that we could discriminate any separate tubular action of ANF. The concentration of ANF we've used is probably far greater than any circulating concentration which might occur under physiologic conditions (18). A pharmacologic dose should amplify any tubular action which ANF might exert. Our results do not eliminate the possibility of a nephron site of action of ANF. However, if such a tubular site exists, it is obscured by the action of mannitol or is minor in comparison with hemodynamic changes.

It has been suggested that the serine protease kallikrein may interact with ANF to modify its action upon the nephron. Recently it has been suggested (10, 12) that kallikrein may inactivate ANF by degrading it in the nephron to an inactive metabolite. Another study (11) has suggested that kallikrein may cleave Pro-ANF to form the active peptide. We measured the excretion of urinary kallikrein during control and ANF infusion during the free-flow state. We found that ANF infusion did not change the net kallikrein excretion. This does not support any obvious correlation between kallikrein excretion and ANF infusion.

In conclusion, we performed stop-flow studies to determine if we could localize any

discrete nephron site of action of ANF associated with the dramatic increase in natriuresis and diuresis. While ANF clearly provoked natriuresis and diuresis, we were not able to discern any direct tubular action of ANF. The resultant excretory effects could be attributed solely to the changes in GFR, and the resultant increased sodium load to the nephron. While these results do not rule out a tubular mechanism of ANF, if such a mechanism exists, it is minor compared to the effects of ANF upon renal hemodynamics.

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