

Studies on a Putative (Na⁺, K⁺)-ATPase Inhibitor in Chronic Renal Failure Patient's Serum[†]

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= Abstract =In order to identify and purify a putative substance which might inhibit (Na⁺, K⁺)-ATPase *in vitro* assay system, serum extracts from normal persons and C.R.F.(chronic renal failure) patients were chromatographed and active peaks were compared with each other. Rat kidney membrane was partially purified with a sucrose step density gradient ultracentrifugation and used for (Na⁺, K⁺)-ATPase source. Deproteinized serum obtained by acidification and boiling was gel chromatographed on Sephadex G-25 column in order to remove salts and inorganic phosphate in the sample which interfered with the assay system. Two active peaks appeared just prior to the total volume and in the total volume fractions, and the former was further investigated.

Collected fractions were concentrated with freeze-dryer and rechromatographed on Sephadex G-75-120. One major active peak at total volume fractions was separated to several active peaks by using cation exchange CM-Sephadex C-25 column. These peaks from normal and C.R.F. serum were compared with each other respectively. The eluted patterns were similar to each other except that one peak appeared at three fifths of total elution volume was shown to be more active in C.R.F. serum than that in normal serum.

Key Words: *Circulating inhibitor, (Na⁺, K⁺)-ATPase, Chronic renal failure, Rat kidney membrane, Naturetic hormone, Hypertension*

INTRODUCTION

The exchangeable sodium in patients with chronic renal failure (C.R.F.) is known to be increased and sodium is retained not only in extracellular fluid but also in the cell. Currently the mechanism for this increased intracellular Na is poorly explained. Decreased (Na⁺, K⁺)-ATPase activity in the cell membrane was one of the proposed mechanisms and this was in turn proposed due to the presence of an inhibitor of (Na⁺, K⁺)-ATPase in the blood of C.R.F. patients. There is another factor, namely middle molecule, which causes toxicity in C.R.F. patient. This material is known to be a peptide having molecular weight of 300-2,000 daltons

(Babb *et al.* 1972), and is also known to cause widespread cellular toxicity including inhibition of (Na⁺, K⁺)-ATPase (Dzurik *et al.* 1982).

Inhibition of (Na⁺, K⁺)-ATPase in kidney is also the proposed mechanism of "naturetic hormone" (Dewardner 1977; Haddy 1982). Partially purified naturetic hormone is not sensitive to proteolytic digestion, resistant to heat, lacks amino acids, and has molecular weight less than 500, sharing common features with an ATPase inhibitor. Apparently different atrial naturetic factor is recently defined but it shows no ATPase inhibitor property (Currie *et al.* 1984).

This study was aimed to identify an inhibitor of (Na⁺, K⁺)-ATPase in serum of the patients with chronic renal failure, utilizing the heat stable, acid resistant property and low molecular weight of this putative substance.

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METHODS

Deproteinization of serum: Normal and C.R.F. serum, each of 330 ml, were acidified with 1.0 N HCl to pH 5.5 and boiled for 2 minutes, respectively. The slurry was centrifuged for 1 hour at 29,000g. The resultant supernatant was used for further investigation. All the following steps were done at 4°C except otherwise indicated.

Gel permeation chromatography on Sephadex G-25-80: Deproteinized samples were lyophilized and applied on a column of Sephadex G-25-80. Limited by column capacity, samples were divided into two equal portions before gel chromatography and processed separately. The column (2.6 × 60 cm) was previously equilibrated with 10 mM acetate solution and developed with the same solution. Fractions of 4 ml each were collected at a flow rate of 12 ml per hour.

Gel permeation chromatography on Sephadex G-75-120: Active fractions collected from Sephadex G-25-80 chromatography were lyophilized and applied on a column of Sephadex G-75-120. The column (2.5 × 52 cm) was previously equilibrated with 10 mM ammonium acetate buffer, pH 5.0 and developed with the same buffer. Fractions of 3 ml each were collected at a flow rate of 12 ml per hour.

Ion-exchange chromatography on CM-Sephadex C-25-120: Active fractions collected from Sephadex G-75-120 chromatography were applied on a column of CM-Sephadex C-25-120 at a flow rate of 15 ml per hour at room temperature. The column (2.9 × 4 cm) was previously equilibrated with 10 mM ammonium acetate buffer, pH 5.0. Without washing, the column was eluted with a linear gradient of ammonium acetate buffer (500 ml from 10 mM, pH 5.0 to 500 mM, pH 7.5). Fractions of 5 ml each were collected at a flow rate of 15 ml per hour.

(Na⁺, K⁺)-ATPase inhibitor assay: Standard reaction mixture (1.0 ml) for total ATPase activity contained 110 mM NaCl, 20 mM KCl, 4 mM MgCl₂, 30 mM imidazole, pH 7.2, 1 mM Tris-ATP (Sigma), rat kidney membrane and fraction obtained by chromatography. Standard reaction mixture for Mg⁺⁺-ATPase activity was the same as above except that this did not contain NaCl and KCl. Reaction was started by adding Tris-ATP at 37°C. After 10 minutes, reaction was stopped by adding 0.2 ml of ice-cold 40% TCA and reaction tubes were centrifuged. Produced inorganic phosphate

was determined by measuring the absorbance of 0.8 ml each of the supernatants at 870 nm after color reaction and compared with controls which contained no inhibitory fractions. (Na⁺, K⁺)-ATPase activity was calculated by subtracting Mg⁺⁺-ATPase activity from total ATPase activity.

Miscellaneous methods: The method of Lebel *et al.* (1978) was used for the determination of inorganic phosphate. The method of Maeda *et al.* (1983) was used for the preparation of rat kidney membrane.

RESULTS

1. **Gel permeation chromatography on Sephadex G-25-80:** 0.05 ml each of collected fractions was used for ATPase inhibitor assay. Elu-

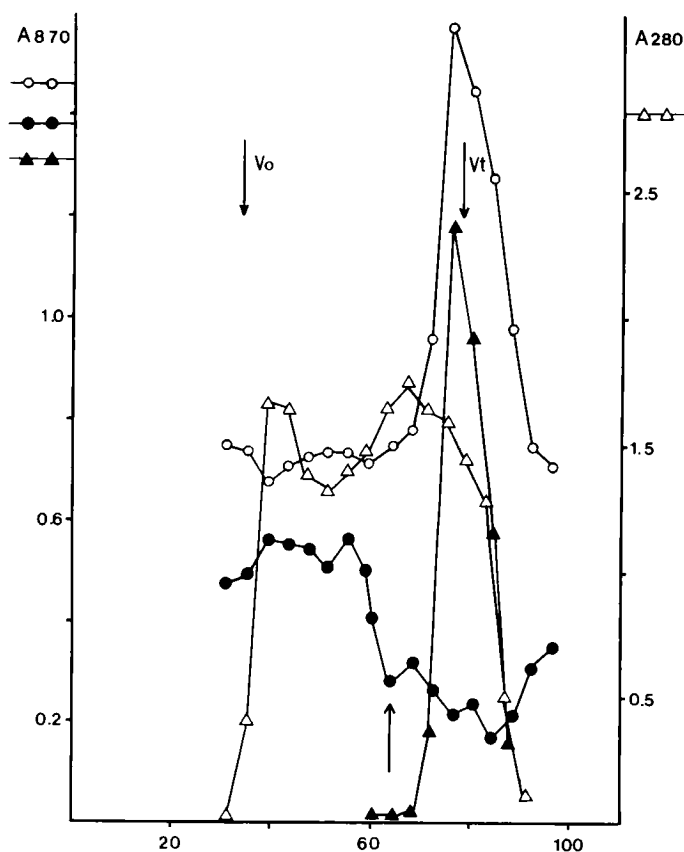


Fig. 1. Gel permeation chromatogram of deproteinized normal serum on a column of Sephadex G-25-80. Fractionation was done as described in the Methods. The arrow indicates a peak which showed inhibitory effect on (Na⁺, K⁺)-ATPase activity. These fractions were used at the next fractionation step.

- : (Na⁺, K⁺)-ATPase activity
- : Mg⁺⁺-ATPase activity
- △—△— : UV absorbance of reactions at 280 nm
- ▲—▲— : Relative concentration of inorganic phosphate in fractions

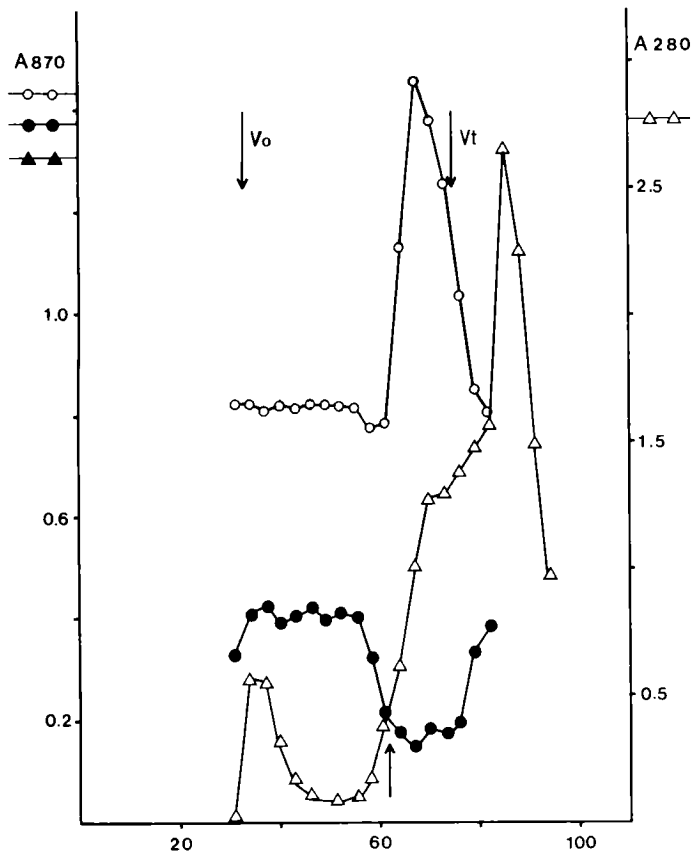


Fig. 2. Gel permeation chromatogram of deproteinized C.R.F. serum on a column of Sephadex G-25-80. Experimental conditions were the same as described in Fig. 1.

tion profiles of normal and C.R.F. deproteinized serums are shown in Fig. 1 and Fig. 2, respectively. Active fractions marked by arrows were pooled for the next step.

2. Gel permeation chromatography on Sephadex G-75-120: 0.1 ml each of collected fractions was used for ATPase inhibitor assay. Elution profiles of normal and C.R.F. samples are shown in Fig. 3 and Fig. 4, respectively. There were major activities in total volume fractions in both elution profiles. Compared with normal sample, C.R.F. sample showed some differences. First, there was no active peak in void volume fractions of C.R.F. sample. But it was not important because those fractions also inhibited Mg^{++} -ATPase activity. Second, there was no Mg^{++} -ATPase activating activity in total volume fractions of C.R.F. sample. Active fractions marked by arrows were pooled for the next step.

3. Ion-exchange chromatography on CM-Sephadex C-25-120: Chromatography was done at room temperature. 0.1 ml each of col-

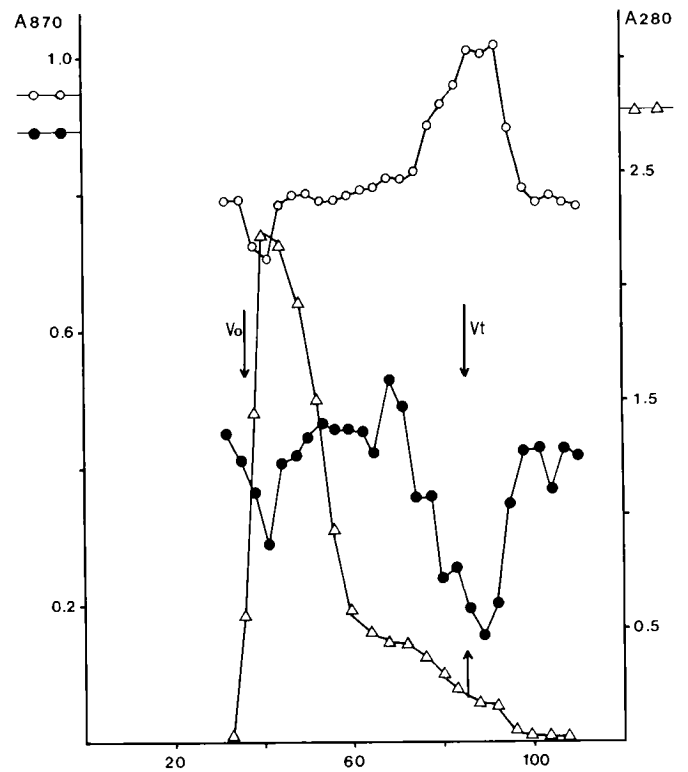


Fig. 3. Sephadex G-75-120 gel permeation chromatogram. Pooled inhibitory fractions obtained from Sephadex G-25-80 chromatography of deproteinized normal serum were applied. Fractionation was done as described in the Methods. The arrow indicates a peak which showed inhibitory effect on (Na^+, K^+) -ATPase activity. These fractions were used at the next fractionation step.

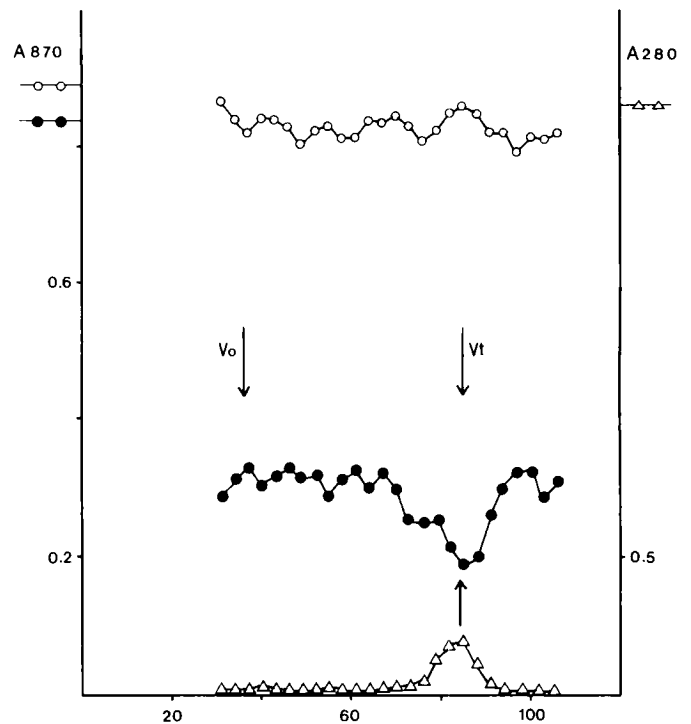


Fig. 4. Sephadex G-75-120 gel permeation chromatogram. Pooled inhibitory fractions obtained from Sephadex G-25-80 chromatography of deproteinized C.R.F. serum were applied. Experimental conditions were the same as described in Fig. 3.

lected fractions was used for ATPase inhibitor assay. Elution profiles of normal and C.R.F. samples are shown in Fig. 5 and Fig. 6, respectively. Many active peaks were shown in both samples. Three peaks were eluted during sample loading.

There was no homology between two samples up to fraction number 35, and there was no candidate for the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ specific inhibitor. From fraction number 36 to the end of the elution, the elution profiles of two samples were not so different each other. Although it was difficult to conclude that each peak in C.R.F. sample was not resulted from random fluctuation, one peak around fraction number 60 was more prominent in comparing with that of normal sample.

DISCUSSION

With above results, we conclude that the active peak eluted around the fraction number 60 in CM-Sephadex C-25-120 chromatogram might be a reasonable candidate for the specific $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ inhibitor. The peak at the end of sample loading of C.R.F. sample might be also a good candidate. As these peaks have relatively higher inhibitory activity against $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ than their counterparts in normal sample and these peaks have no effect on $\text{Mg}^{++}\text{-ATPase}$ activity, it may be concluded that these are specific to C.R.F. status. However, the material should be tested further for its activity in human system and purified further before any reasonable conclusion could be drawn.

There are many inhibitors of $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ in nature such as Mg^{++} , ATP, K^+ (Fagan and Racker 1977), vanadate (Cantley *et al.* 1977), candida extract (Satre *et al.* 1979). A circulating inhibitor of $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ associated with essential hypertension was identified by Hamlyn *et al.* (1982), but another ATPase inhibitor activity from pig brain was turned out to be an artefact (Kracke 1983). In our assay system, the pH and other salt contents also appeared to influence the activity, even though these conditions did not contribute to our study results as these factors were carefully matched or avoided.

Therefore our statement that there are specific $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ inhibitor peaks in C.R.F. sample eluate does not necessarily mean that these peaks contain a specific inhibitor of middle molecule nature, or "naturetic factor".

However, as the inhibitor property was measured after the treatment of samples with heat and as the activity just prior to the salt peak was found to have low molecular weight nature, it is also possible that these peaks might contain the putative substance(s). Further purification of these material is under progress.

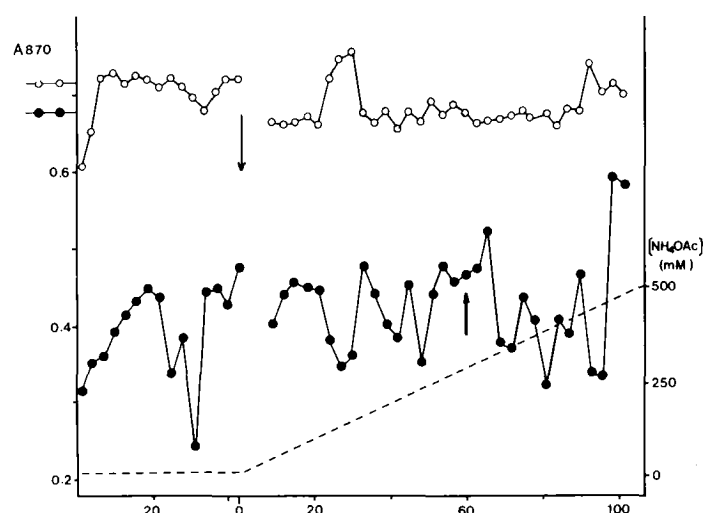


Fig. 5. CM-Sephadex C-25-120 cation-exchange chromatogram. An inhibitory peak eluted at the total volume fractions of Sephadex G-75-120 column chromatography of deproteinized normal serum was applied. Fractionation was done as described in the Methods. The arrow indicates an inhibitory peak which is considered to be a possible candidate for the true $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ specific inhibitor.

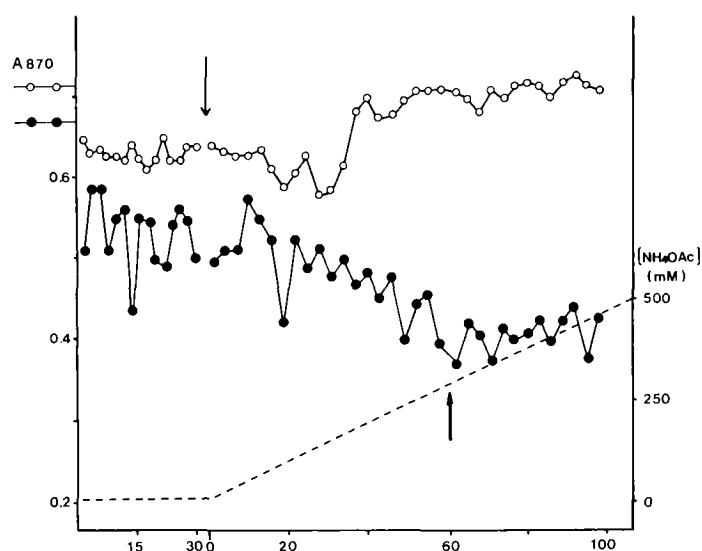


Fig. 6. CM-Sephadex C-25-120 cation-exchange chromatogram. An inhibitory peak eluted at the total volume fractions of Sephadex G-75-120 column chromatography of deproteinized C.R.F. serum was applied. Experimental conditions were the same as described in Fig. 5.

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= 국문초록 =

만성 신부전증 환자혈액내의 (Na⁺, K⁺)-ATPase 억제물질에
 관한 연구

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In vitro 반응계에서 (Na⁺, K⁺)-ATPase를 억제하는 물질을 동정 및 순수분리하기 위해 정상인과 만성 신부전증 환자의 혈청 추출물을 각각 크로마토그래피하고 효소 억제 활성을 갖는 분획을 서로 비교하였다. (Na⁺, K⁺)-ATPase로는 쥐 신장의 원형질막을 sucrose step 밀도 구배 초원심분리 방법으로 분리하여 사용하였다. 혈청을 산성화하고 끓여 단백질을 제거한 후 효소반응에 영향을 미치는 염과 무기인을 제거하기 위해 Sephadex G-25 컬럼으로 전개시켰다. 그 결과 컬럼 총부피의 바로 앞과 총부피의 분획에서 각각 효소 억제 활성이 나타났으며, 전자의 분획을 계속 연구하였다.

활성을 갖는 분획을 냉동건조기로 농축한 다음 Sephadex G-75-120 컬럼으로 전개시켰다. 주된 활성은 컬럼 총부피 분획에서 나타났는 바, CM-Sephadex C-25컬럼으로 양이온 교환 크로마토그래피를 한 결과 여러 분획으로 분리되었다. 이 분획들은 정상인과 만성 신부전증 환자를 서로 비교했을 때 전체적으로 비슷했으나 전체 용출 부피의 3/5에 해당하는 위치의 분획에서 정상인의 경우보다 만성 신부전증 환자의 경우 활성이 더 높았다.