대한생리학회지 : 제22권 제1호

Effect of Cadmium on Renal Organic Anion Transport *In vitro*

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= 국문초록 =

신장의 유기음이온 이동에 대한 카드뮴의 영향(*In vitro* 실험)

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가토 신피질 절편에서 p-aminohippurate (PAH) 이동에 대한 카드뮴 이온(Cd)의 영향을 조사하여 다음과 같은 결과를 얻었다. 조직절편과 용액내의 PAH 농도비(S/M PAH)는 용액내에 Cd이 0.1 mM이상 존재할 때에 현저히 감소되었다. PAH influx에 대한 동력학적 분석결과 Cd에 의하여 influx의 최대이동율(즉 V_{max})은 심하게 감소되지만 운반체와 기질간의 친화력(즉 K_m)에는 변화가 없었으며, 수동적 influx 역시 변화되지 않았다. 신피질조직의 산소 소모율은 1 mM Cd에 의하여 35% 가량 억제되었으며, 신피질 microsome 분획의 Na-K-ATPase 활성도는 Cd 농도가 10^{-7} M 이상일 때 의의있게 억제되었다.

이상의 결과로 미루어 볼 때 신장조직이 카드뮴이온에 직접 노출될 경우 유기음이온의 능동적 이동능이 심하게 저해된다고 사료된다.

Key Words: Cadmium intoxication, Renal function, Organic anion transport

INTRODUCTION

Several studies in the past have indicated that the renal transport system for organic anions is impaired in animals exposed to inorganic cadmium (Cd). Nomiyama et al. (Nomiyama et al., 1973; Nomiyama, 1978) observed in rabbits that the PAH clearance reduced after acute administration of $CdCl_2$ (2-12 mg Cd per animal, intra-arterial injection) and the Tm_{PAH} decreased gradually during chronic treatment with $CdCl_2$ (0.5-15 mg Cd/kg·day, subcutaneous injection). In a kinetic study of PAH transport in renal cortical slices of rats exposed to $CdCl_2$ (2 mg Cd/kg·day for 3-16 days), we have observed a significant reduction in the maximum rate of active influx (V_{max}) (Kim et al., 1988). While this observation indicates that the capacity of renal tubules for organic anion transport is attenuated by Cd treatment, the underlying mechanism remains unresolved.

The capacity of transfer will be determined by the total number of active carriers in the tissue. A morphological study on kidneys from Cd-intoxicated rats (Scott et al., 1977) has shown a loss of basal infoldings of proximal tubules. Since the carrier system for active PAH transport resides in the basolateral membrane (Tune et al., 1969; Shimomura et al., 1981), a decrease in basal infoldings with a consequent loss of basolateral membrane area may

constitute a decrease in available carriers to mediate the transfer of PAH. However, in our study mentioned above, the passive influx and efflux of PAH were not apparently changed in renal tissue of Cd-treated animals, suggesting that the area of basolateral membrane in unit mass of tissue was not reduced. Furthermore, it is not known whether cadmium ions directly alter the density of carrier in the membrane.

We therefore undertook the present series of experiments to evaluate an acute effect of cadmium ions on the PAH transport system.

MATERIALS AND METHODS

Male rabbits were sacrificed by injecting air through an auricular vein. The abdomen was opened through a middle incision, and the blood in the kidneys was washed out by a perfusion of normal Ringer solution. The kidneys were promptly removed and placed in an ice-cold medium containing 95 mM NaCl, 40 mM KCl, 1.5 mM $CaCl_2$, 5 mM sodium acetate, and 40 mM Tris-HCl (pH 7.5 at 25℃). Slices of renal cortex of approximately 0.5 mm thick were cut using a Stadie-Riggs microtome and stored in the same solution until used.

For the measurement of PAH uptake, approximately 100 mg slices were placed in a reaction vessel which contained 10 ml of either normal incubation medium or $CdCl_2$-containg medium oxygenated and equilibrated at 25℃. In experiments with metabolically inhibited slices the medium contained 1 mM iodoacetate (IAA) and gased with nitrogen. After 15 min preincubation, reaction was started by adding small volume of PAH stock solution. At the end of incubation period (60 min in distribution studies and 15 min in kinetic studies) tissues were removed from the vessel, rinsed with PAH-free medium for about 2 seconds to remove PAH adhering to the slice, weighted and transfered to samll test tube containing 2 ml of distilled water. Tissues were then leached out of PAH by being kept in the refrigrator overnight at 4℃. The tubes were mixed thoroughtly to insure equal dispersion of leached PAH and they were centrifuged for 10 min at 3,000 rpm. PAH concentration in the supernatant and in the final incubation medium were determined by the method of Smith et al. (1945).

The oxygen consumption of slices was determined using a polarographic oxygen monitor system (Yellow Springs Instrument, Model 53). Approximately 20 mg of slices were placed in a reaction chamber containing 2.5 ml of incubation medium saturated with air at 37℃. After 15 min of preincubation in the presence or absence of 1 mM $CdCl_2$, changes in Po_2 in the medium was measured with a Clark-type oxygen electrode (Yellow Springs Instrument, Model 5331) and recorded on a potentiometric recorder (Kipp & Zonen, Model BD40). From the initial slope of Po_2 vs. time curve the rate of oxygen consumption (Qo_2, μl/hr mg wet tissue) was calculated.

For the measurement of sodium, potassium-activated adenosine-triphosphatase (Na-K-ATPase) renal cortical microsomes were prepared by a method similar to that described by Jørgensen and Skou (1971). Slices of kidney cortex were homogenized in 10 volumes of imidazole-sucrose buffer (0.03 M imidazole, 0.25 M sucrose, pH 7.6 at 25℃). The homogenates were then centrifuged at 1,200×g for 15 min in a refrigerated centrifuge (Sorvall, Model RC-5B). The supernatant was centrifuged at 9,500×g for 15 min and the resulting supernatant was centrifuged again at 25,000×g for 30 min. The pellet was suspended in 2 ml of imidazole-sucrose buffer (0.03 M imidazole, 0.25 mM sucrose, pH 7.6 at 25℃) to a concentration of approximately 2 mg protein per ml and stored at −60℃. Protein concentration of this suspension was determined by the method of Lowry et al. (1951).

Prior to ATPase assay, aliquots of microsomal preparations were treated with deoxycholate by incubating them in a solution containing 60 mg%

deoxycholate, 2 mM EDTA and 25 mM imidazole (pH 7.6 at 25℃) for 30 min at 25℃, and the mixture was adjusted to contain 0.25 mg protein per ml. The ATPase activity was estimated by measuring inorganic phosphate (Pi) liberated by ATP hydrolysis during 10 min incubation of deoxycholate-treated microsomes with 1 ml of appropriate medium containing Na_2-ATP as the substrate. The total ATPase activity was determined in the presence of Na^+, K^+ and Mg^{++}, and the Mg-ATPase in the absence of K^+ and presence of ouabain (1 mM) in the incubation medium. The difference between the total and Mg-ATPase activity was taken as the measure of Na-K-ATPase activity. Concentrations of Na^+, K^+, Mg^{++}, and ATP in the incubation medium were 150, 20, 1, and 1 mM, respectively. The medium pH was adjusted to 7.4 at 37℃ with imidazole-HCl. After 20 min preincubation at 37℃ in the presence or absence of $CdCl_2$ (10^{-7}-10^{-3} M) the reaction was initiated by adding ATP stock solution. The reaction was terminated by adding 0.2 ml of ice-cold 6% perchloric acid, and the mixture was centrifuged at 3,500×g for 15 min. Inorganic phosphate in the supernatant was measured according to Fiske and SubbaRow (1925). The enzyme activity was expressed as μmoles Pi/mg of microsomal protein per hr.

Statistical evaluation of the data was done using the Student's t-test and all results were presented as the mean±SE.

RESULTS

In the first series of experiments, the PAH accumulation by renal cortical slices was determined as a function of Cd concentration in the medium. Slices were incubated in media containing 0.1 mM PAH and various concentrations of Cd for 1 hr. The results, expressed as the distribution ratio, μmoles PAH/g tissue to μmoles PAH/ml medium (S/M PAH), and depicted in Fig. 1, indicated that tissue accumulation of PAH is significantly inhibited by Cd at concentrations above 0.1 mM. The value of S/M PAH at 1 mM Cd was approximately 60% lower and those at 10-50 mM Cd were 80% lower than the control level (4.5±0.7, N=5).

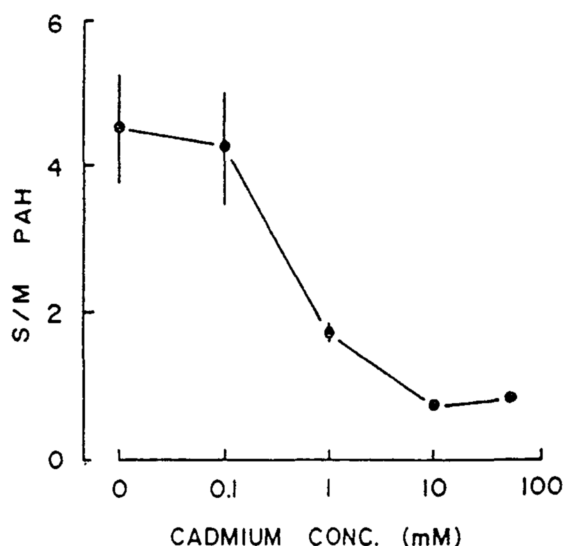

Fig. 1. Slice-to-medium (S/M) ratios for PAH distribution as a function of cadmium ($CdCl_2$) concentration in the initial incubation medium. Incubation time was 1 hr. Each point and vertical bar represent the mean ± SE of 5 experiments.

Since the distribution ratio merely represents a concentration gradient which depends on the influx and efflux across the basolateral membrane of the proximal tubular cell, the above data may not reflect Cd-sensitive components of PAH transport. We therefore investigated in the next series of experiments the effect of Cd on the PAH influx.

Fig. 2 illustrates PAH influx into renal slices as a function of PAH concentration in the medium. Incubations were carried out for 15 min in the presence and absence (control) of 1 mM Cd. In both control and Cd-treated tissues, the total influex (V_t, i.e., active+passive influx) increased curvilinearly as the medium concentration of PAH increased, although the value at a given PAH concentration was much higher in the former than in the latter. On the other

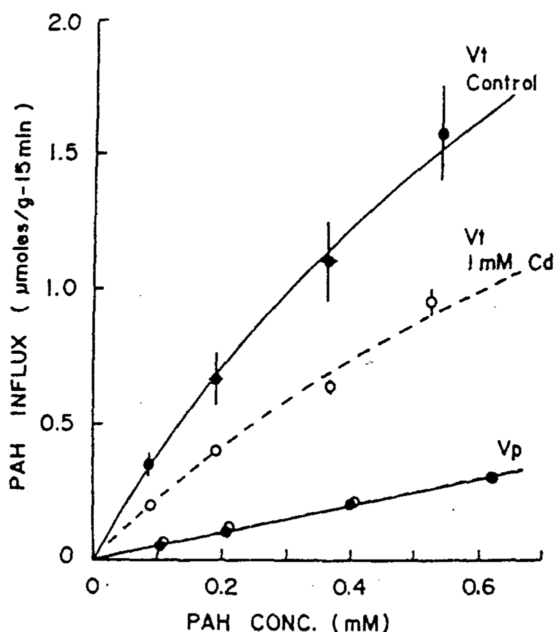

Fig. 2. Influx of PAH into rabbit renal cortical slices as a function of final medium concentration of PAH. Vt and Vp represent the total and passive influx of PAH. Data represent the mean ± SE of 6 experiments.

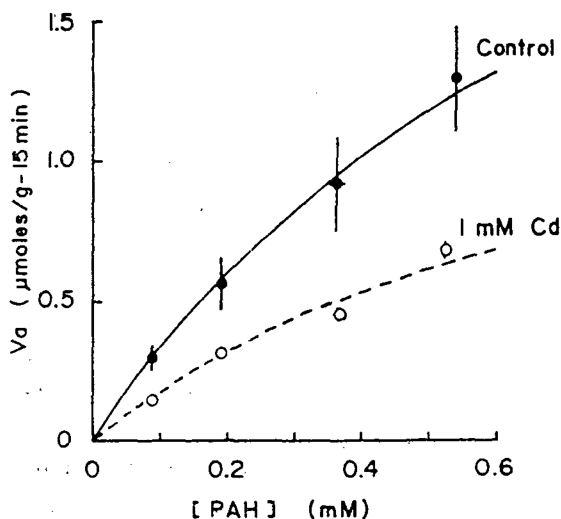

Fig. 3. Active influx of PAH (Va) into rabbit renal cortical slices as a function of final medium concentration of PAH. Data represent the mean ± SE of 6 experments.

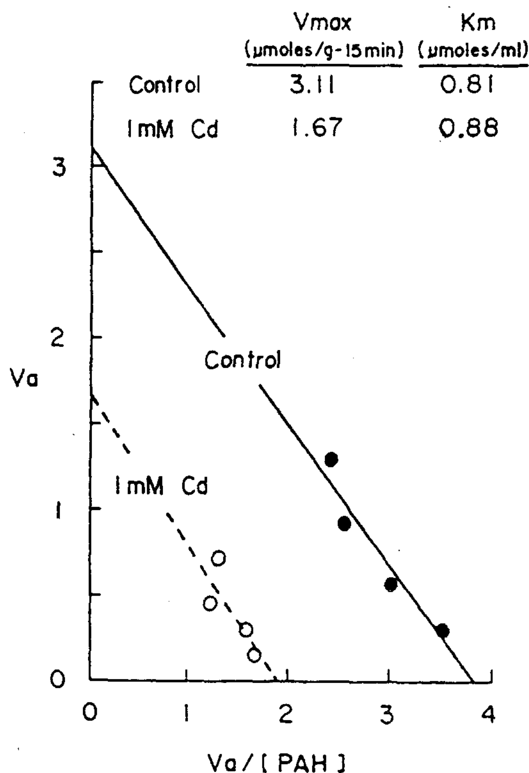

Fig. 4. Hofstee kinetic analysis of active PAH influx. Values are based on the data shown in Fig. 3. The intercept of the line with Y-axis represents the Vmax and the slope represents-Km.

hand, the passive influx (V_p), measured in metabolically poisoned slices, increased directly with the medium concentration of PAH and was independent of Cd.

Fig. 3 presents the active influx (V_a) estimated by subtracting the passive influx from the total influx in each situation. Hofstee plot (Hofstee, 1959) of the data (Fig. 4) indicated that in both control and Cd-treated tissues the active influx followed simple Michaelis-Menten kinetics, i.e.,

$$V_a = V_{max} \times [PAH] / (Km + [PAH])$$

where V_{max} is the maximal influx (i.e., capacity of influx) and Km is the PAH concentration ([PAH]) for $V_{max}/2$. Thus, the total influx (V_t) can be expres-

sed as:

$$V_t = V_{max} \times [PAH]/(Km + [PAH]) + D \times [PAH]$$

where D is the coefficient for passive influx (slope of the V_p lines in Fig. 2). Since D was not affected by Cd, any change in PAH influx in the Cd-treated tissue must have been due to alteration of carrier-mediated transport component. As shown in Fig. 4 insent, the V_{max} was 46% reduced (3.11 μmoles/g·15 min in the control vs. 1.67 in Cd-treated tissues), but the Km was not apparently alterd (0.81 mM in the control, 0.88 in the Cd-treated tissues) by Cd.

In another series of experiments, the Cd effect of tissue respiration was studied. As summarized in Table 1, oxygen consumption of renal cortical slices was approximately 35% reduced by 1 mM Cd (1.14±0.07 and 0.78±0.07 μl/mg·hr in the control and Cd-treated tissues, respectively).

Table 1. Effect of cadmium on oxygen consumption of renal cortical slices

	N	Q_{O_2} (ul/mg·hr)	P
Control	12	1.140 ± 0.065	< 0.01
$CdCl_2$ (1 mM)	4	0.775 ± 0.068	

Values represent the mean ± SE.

Table 2. Effect of cadmium on Na-K-ATPase activity of renal cortical microsomes

$CdCl_2$ conc. (M)	N	Na-K-ATPase activity (% of control)	P
0	10	100	
10^{-7}	4	71.7 ± 4.6	< 0.001
10^{-6}	4	34.7 ± 2.5	< 0.001
10^{-5}	6	6.8 ± 0.9	< 0.001
10^{-4}	6	2.8 ± 1.1	< 0.001
10^{-3}	4	2.3 ± 0.8	< 0.001

The average specific activity of Na-K-ATPase in control (0 M $CdCl_2$) preparations was 37.1 ± 4.3 umoles Pi/mg·hr. Values represent the mean ± SE.

In the last series of experiments, we examined the effect of Cd on the renal cortical Na-K-ATPase system. The enzyme activity was determined in renal cortical microsomes as a function of Cd concentration in the medium. The results summarized in Table 2 indicated that the enzyme system was drastically inhibited by Cd of above 10^{-7} M.

DISCUSSION

Chronic exposure to inorganic cadmium produced proximal tubular nephropathy (Axelsson & Piscator, 1966; Axelsson et al., 1968; Kajikawa et al., 1981) and impaired renal functions (Kjellström, 1986). It has been proposed that (Friberg, 1984; Kjellström, 1986) during chronic exposure Cd is gradually accumulated in the kidney mainly in the proximal tubules, because the Cd in plasma is transported bound to metallothionein, a protein ligand for Cd, which is readily filtered through glomeruli and reabsorbed into proximal tubular cells by endocytosis. After entering lysosomes, the Cd-metallothionein complex is catalyzed, liberating free Cd, which in excess amount induces nephrotoxicity.

According to the above hypothesis, the amount of free Cd in renal tissue may be an important determinant of the acute renal toxicity of Cd. It follows that functional impairment should result if the kidney is acutely exposed to large doses of $CdCl_2$ and hence increase the amount of free Cd in the renal tissue.

In the present study we have directly exposed renal cortical slices to Cd-containing media. Although in isolated kidney tissues tubular lumens are collapsed (Bojesen & Ceyssac, 1965; Evan et al., 1978), Cd may enter the cells through the peritubular route. In fact, Diamond et al. (1986) have shown that in isolated perfused rat kidneys Cd is accumulated into the cortical cells from the peritubular capillary fluid. The tissue concentration of Cd was not determined in the present study, but it must be sufficiently high to produce functional

changes, as evidence by impaired PAH transport (Figs 1-4) and tissue respiration (Table 1).

The change in PAH transport system by Cd in the present study was comparable to that observed in renal cortical slices of rats chronically treated with Cd in our previous study (Kim et al., 1988). In the later study, the tissues were incubated in Cd-free medium. In both studies the V_{max} of PAH influx was attenuated without any change in the Km. In kinetic analysis of carrier-mediated transfer, the term K_m is determined by the substrate affinity of the carrier, and the V_{max} by the number of carriers and the proportion of absorbed molecules which dissociate in forward direction in unit time (Neame & Richards, 1972). Since Km was not changed it is unlikely that carrier-substrate dissociation was retarded by Cd. It is more likely that Cd reduced active carrier sites, thereby attenuated the V_{max}.

The number of carriers per unit mass of tissue will be determined by the total area of basolateral membranes and the density of carrier in the membrane. Histological studies (Scott et al., 1977) indicated that the area of basolateral membrane is decreased in the proximal tubule of long-term Cd-exposed animals. If similar change occurred in our studies, it would attribute to the reduction of V_{max}. However, it is hard to imagine that the area of cell membrane can be changed so drastically during such a short period (30 min) of Cd exposure, as in the present study. We therefore speculate that, at least in acute exposure, the major effect of Cd is not on the area but on the density of carriers involved in PAH transfer in the proximal tubular basolateral membrane.

The mechanism by which carreir density was altered is not understood. Cd may inactivate or immobilize certain fraction of carriers perhaps through its binding to the membrane, or indirectly by inhibiting mechanism(s) energizing the carrier system. Although direct alteration of membrane properties by Cd has not been reported in the kidney, studies in other tissues suggest such a possibility. For instance, Kunimoto et al. (1986) observed in red cells that Cd incorporated into the cells is accumulated in the membrane fraction and modifies membrane properties such as filterability. With respect to the latter possibility, a number of studies have suggest that the renal organic anion transport system may be energized by the activity of Na-K-ATPase (Gerencser & Hong 1975; Spencer et al., 1979; Maxild et al., 1981). In the present study, the Na-K-ATPase activity of renal cortical microsomes appeared to be significantly reduced by Cd (10^{-7}-10^{-3} M) (Table 2), in accordance with other studies (Nechay & Saunders, 1977). If the enzyme system is involved in energy-linking step of organic anion transport, such an inhibition of the enzyme activity by Cd would reduce the fraction of activated carriers. In this connection, it is important to point out that incubations of renal cortical slices in low sodium (Gerencser et al., 1973; Misanko et al., 1977; Park & Lee, 1980) or ouabain (Spencer et al., 1979) containing media, which restrain the Na-K-ATPase activity, also reduce the V_{max} but not the Km for PAH influx, as with Cd in the present study.

In the present study, oxygen consumption of renal cortical slices was significantly inhibited in the presence of 1 mM Cd (see Table 1). It is therefore possible that ATP supply to the transport system was restricted. This effect of Cd would also attribute to the reduction of PAH transport in renal tissues directly exposed to Cd. This, however, may not be true in the kidney of Cd-intoxicated animals. We have observed in kidney preparations of rats chronically exposed to Cd that the capacity of PAH transport and the Na-K-ATPase activity were significantly attenuated although the ability to utilize oxygen was not apparently altered (Kim et al., 1988).

In summary, the results of the present study indicate that a direct exposure of renal tissues to inorganic cadmium impairs PAH transport capacity

without altering the biochemical property of the transport system. This change in transport capacity is most likely due to reduction of active carriers in the basolateral membrane of the proximal tubular cells.

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REFERENCES

Axelsson B & Piscator M (1966). Renal damage after prolonged exposure to cadmium. *Arch Environ Health* 12, 360-373

Axelsson B, Dahlgren SE & Piscator M (1968). Renal lesions in the rabbit after long-term exposure to cadmium. *Arch-Environ Health* 17, 24-28

Bojesen E & Leyssac PP (1965). The kidney cortex slices technique as a model for sodium transport in vivo. A qualitative evaluation. *Acta Physiol Scand* 65, 20-32

Diamond GL, Cohen JC & Weinstein SL (1986). Renal handling of cadmium in perfused rat kidney and effects on renal function and tissue composition. *Am J Physiol* 251, F784-F794

Evan AP, Park YS & Solomon S (1978). Changes in structure and function of rat kidney slices produced by low sodium. *Nephron* 21, 209-220

Fiske CH & SubbaRow Y (1925). The calorimetric determination of phosphorus. *J Biol Chem* 66, 375-400

Friberg L (1984). Cadmium and the kidney. *Environ Health Perspect* 54, 1-11

Gerencser GA & Hong SK (1975). Roles of sodium and potassium ions on p-aminohippurate transport in rabbit kidney slices. *Biochim Biophys Acta* 406, 108-119

Gerencser GA, Park YS & Hong SK (1973). Sodium influence upon the transport kinetics of p-aminohippurate in rabbit kidney slices. *Proc Soc Exp Biol Med* 144, 440-444

Hofstee BHJ (1959). Non-inverted versus inverted plots in enzyme kinetics. *Nature* 184, 1296-1298

Jφrgensen PL & Skou JC (1971). Purification and characterization of (Na^+-K^+)-ATPase in preparations from the outer medulla of rabbit kidney. *Biochim Biophys Acta* 233, 366-388

Kajikawa K, Nakanishi I & Kuroda K (1981). Morphological changes in the kidney and bone of rats in chronic cadmium poisoning. *Exp Mol Pathol* 34, 9-24

Kim YK, Choi JK, Kim JS & Park YS (1988). Changes in renal function in cadmium-intoxicated rats. *Pharmacol & Toxicol* (in press)

Kjellström T (1986). Renal effects. In: Friberg L, Elinder C-G, Kjellström T & Nordberg GF (ed). *Cadmium and Health: A Toxicological and Epidemiological Appraisal*, Vol II. Effects and Response. Chapter 9. CRC Press, Boca Raton. Florida, p 21-109

Kunimoto M, Miyasaka K & Miura T (1986). Changes in membrane properties of rat red blood cells induced by cadmium accumulating in the membrane fraction. *J Biochem* 99, 397-406

Lowry OH, Rosebrough NJ, Farr AL & Randall RJ (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem* 193, 265-275

Maxild J, Mφller JV & Sheikh MI (1981). Involvement of Na^+-N^+-ATPase in p-aminohippurate transport by rabbit kidney tissue. *J Physiol* 315, 189-201

Misanko BS, Park YS & Solomon S (1977). Effect of hypophysectomy on p-aminohippurate transport kinetics in rat renal cortical slices. *J Endocr* 74, 121-128

Neame KD & Richards TG (1972). *Elementary Kinetics of Membrane Carrier Transport.* Wiley, New York

Nechay BR & Saunders JP (1977). Inhibition of renal adenosine triphosphatase by cadmium. *J Pharmacol Exp Ther* 200, 623-629

Nomiyama K (1978). Experimental studies on animals, in vivo experiments. In; Tsuchiya K (ed). *Cadmium Studies* in Japan, Tokyo, Kodansha Ltd. p 45-97

Noimyama K, Sato C, Yamamoto A (1973). Early signs of cadmium intoxication in rabbits. *Toxicol Appl Pharmacol* 24, 625-635

Park YS & Lee SM (1980). Role of sodium ion in renal transport of p-aminohippurate in vitro. *Yonsei Med J* 21, 123-128

Scott R, Aughey E & Sinclair J (1977). Histological and ultrastructural changes in rat kidney following cadmium injection. *Urol Res* 5, 15-20

Shimomura A, Chonko AM & Grantham JJ (1981). Basis for heterogeneity of para-aminohippurate secretion in rabbit proximal tubules. *Am J Physiol* 240, F430-F436

Smith HW, Finkelstein N, Aliminosa L, Crawford B & Graber M (1945). The renal clearances of substituted hippuric acid derivatives and other aromatic acids in dog and man. *J Clin Invest* 24, 388-404

Spencer AM, Sack J & Hong SK (1979). Relationship between PAH transport and Na-K-ATPase acitivity in the rabbit kidney. *Am J Physiol* 236, F126-F130

Tune BM, Burg MB & Patlak CS (1969). Characteristics of p-aminohippurate transport of proximal renal tubules. *Am J Physiol* 217, 1057-1063