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TRANSPORT AND METABOLISM OF PANTOTHENIC ACID BY RAT KIDNEY

LARRY M. KARNITZ *, CAROL J. GROSS and LAVELL M. HENDERSON

Department of Biochemistry, University of Minnesota, St. Paul, MN 55108 (U.S.A.)

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Transport of [^{14}C]pantothenic acid was studied using brush-border membrane vesicles prepared from rat kidney. In the presence of a Na^+ gradient an accumulation of pantothenic acid 3-fold above equilibrium was observed. The K_m and V_{max} found were 7.30 μM and 23.8 pmol/mg protein per min, respectively. Isolated perfused rat kidneys were employed to study excretion of pantothenic acid at various concentrations in the perfusate. At physiological plasma concentrations, the filtered pantothenic acid was largely reabsorbed by the active process observed in the vesicles. At higher concentrations, pantothenic acid was found to undergo tubular secretion. Penicillin inhibited this secretory process indicating that both compounds share a secretory mechanism. Live animal studies indicated that the only compound excreted after injection of [^{14}C]pantothenic acid was free pantothenic acid. After 1 week only 38% of the administered dose was excreted in the urine, indicating that effective conservation was taking place in the whole animal.

Introduction

The extretion of pantothenic acid has previously been studied in dogs [1] and man [2]. In man it is believed that pantothenic acid is actively reabsorbed by the kidney at physiological plasma concentrations and at higher concentrations undergoes tubular secretion. Pantothenic acid in dog blood is believed to be bound to plasma proteins in a non-dialyzable state and therefore not filtered by the kidneys. Taylor et al. [3] reported that dogs excrete the β-glucuronide of pantothenic acid at the same rate as they excrete inulin. The purpose of the present study was to determine some of the detailed mechanisms of transport of pantothenic acid by the rat kidney and how these results relate to the whole animal.

Materials and Methods

Male Sprague-Dawley rats were purchased from Simonsen Laboratories, White Bear Lake, MN and were fed commercial diet ad libitum. [^{3}H]Inulin, mean molecular weight 5200 with a purity of 98% and a specific activity of 1.96 Ci/mmol was purchased from Amersham Corporation, Arlington Heights, IL. Sodium D-[3(n)-^{3}H]pantothenate, labelled on the β-carbon of the β-alanine moiety, with a purity of 98% and a specific activity of 48.8 Ci/mmol was purchased from New England Nuclear, Boston, MA. Sodium D-[1-^{14}C]pantothenate, labelled in the carboxyl of the β-alanine moiety, with a purity of 97% and a specific activity of 57.6 mCi/mmol was also obtained from New England Nuclear. Sodium 4'-phosphopantothenate and sodium 4'-phosphopantetheine were a gift from Dr. Y. Abiko, Daiichi Seiyaku Co., Ltd., Tokyo. Pantetheine was obtained from Vega Biochemicals, Tucson, AR. Fraction V bovine serum albumin, lot No. A-4503 and pantoyl lactone were from Sigma Chemical Company. Filters used were

* Present address: Department of Biochemistry, University of Iowa, Iowa City, IO 52242, U.S.A.

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

Whatman GF/F 2.5 cm.

Perfusions were performed by a modification of the method of Bowman and Maack [4]. Rats were anesthetized with sodium pentobarbital. The perfusate consisted of a modified Krebs-Ringers bicarbonate buffer to which 6.5 g bovine serum albumin, 90 mg glucose and 11.5 mg glutamine were added per 100 ml. Other amino acids were also added to give a perfusate similar to that used by DeMello and Maack [5]. The concentrations of these amino acids were as follows:).8 mM alanine, 0.8 mM proline, 0.4 mM isoleucine, 1.2 mM aspartate, 0.8 mM glycine and 0.4 mM arginine. 15400 units of penicillin and 15.3 mg of streptomycin were added per 100 ml of perfusate for metabolism and some clearance studies. The perfusion equipment was that used for intestinal perfusions, with the exception that no anti-siphon reservoir was used [6]. The perfusate was pH 7.4 after equilibration with 95% O_2 and 5% CO_2. The kidney was perfused in situ, covered with a gauze pad saturated with 0.9% NaCl and a heat lamp was used to maintain the organ at a temperature of 37°C. The flow rate of the perfusate was adjusted to maintain the arterial pressure between 100 and 110 mm of Hg. The kidney was perfused for 30 min after completion of the surgery to permit stabilization of the vascular pressure and urine flow before experiments were initiated. Urine collection periods were begun 10 minutes after the addition of the [^{3}H]inulin and [^{14}C]pantothenic acid to the perfusate. 2 μCi of [^{14}C]pantothenic acid and 2.5 μCi of [^{3}H]inulin were routinely used for each perfusion. At the end of the perfusion the kidney vasculature was washed with 5 ml of 0.9% NaCl, the organ removed and placed into boiling water for 10 min to destroy enzymatic activity. The kidney was then homogenized using a Polytron homogenizer. Urine flow was determined gravimetrically and the urine not used for determining ^{3}H and ^{14}C content was pooled for ^{14}C-analysis for metabolites of pantothenic acid.

The kidney homogenate was filtered through glass wool to remove large particles, and the filtrate was concentrated under reduced pressure at 40°C. The concentrate was then applied to a Bio-Rad G-10 column and eluted with dionized water. The radioactive fractions were then concentrated and dithiothreitol was added to a final concentration of 10 mM and analyzed using high-voltage electrophoresis.

Aliquots of tissue, urine and perfusate concentrates were spotted on Whatman 3MM paper and electrophoresed in a pyridine/acetic acid/water buffer (1:10:289, v/v) at pH 3.5 at 2000 volts for 90 min [7].

Kidney vesicles were prepared basically as described by Beck and Sacktor for rabbit kidney [8]. The kidney was quickly cooled in a medium containing 50 mM mannitol and 2 mM Hepes adjusted to pH 7.4 with a concentrated solution of Tris. After cooling, the renal pelvis and medulla were removed, leaving only the cortex. The cortices were then transferred to a new 40 ml portion of the mannitol buffer, which had been cooled to 4°C, and homogenized at a setting of 6 with a Polytron homogenizer for 20 s. The homogenized suspension was then kept at 0°C and sufficient 1 M $CaCl_2$ was added to make the final Ca^{2+} concentration of the homogenate 10 mM. The homogenate was then allowed to stand on ice with occasional stirring for 20 min. The resulting suspension was then centrifuged at $2000 \times g$ for 10 min. The vesicle containing supernatant was then decanted off and centrifuged at $20000 \times g$ for 30 min. The supernatant was discarded and the vesicle pellet resuspended in 20 ml of a buffer containing: 300 mM mannitol, 5 mM Hepes, adjusted to pH 7.4 with a concentrated Tris buffer. Centrifugation was repeated after suspending in the 300 mM mannitol buffer and the final pellet was resuspended in 0.5 ml of the same buffer. The final pellet suspension showed a 10-fold increase in alkaline phosphatase activity over the homogenate as determined by the methods of Heidrich et al. [9]. The method of Lowry was used to determine protein [10].

Transport assays were performed in microcentrifuge tubes containing 150 μl of vesicle suspension. The uptake assay was begun by the addition of 450 μl of a buffer containing: 100 mM NaCl, 100 mM mannitol and 5 mM Hepes adjusted to pH 7.4 with Tris. The NaCl buffer also contained 1.95 nmol of [^{14}C]pantothenic acid per 450 μl. 50 μl aliquots of the incubation mixture were removed at the indicated times and quickly filtered through Whatman glass fiber filters using a vacuum filtration manifold, then washed with

two 4 ml portions of the previously described cold 300 mM mannitol buffer. Application of sample to filter, addition of wash buffer and completion of filtering required approx. 4 s.

For inhibition experiments, transport assays were performed in microcentrifuge tubes to which 50 μl of the vesicle suspension had been added. The experiments were begun by the addition of 0.65 nmol of [^{14}C]pantothenic acid which had been diluted to 50 μl with the previously described NaCl buffer and which contained the indicated amount of inhibitor. Background values were determined by the same procedure as above, with the exception that vesicles were not used. Background values were subtracted from all data points.

Binding studies were done using 1 ml of a 10% bovine serum albumin solution dialyzed against 4 ml of 1.4 mM polyethylene glycol. Membranes used for dialysis were soaked in deionized water for 20 min before use. Dialysis was performed at 4°C for 18 to 20 h to allow complete equilibration. 6.2 pmol of [^{3}H]pantothenic acid were added to the dialysate in some cases and to the dialysant in other cases. In either case, results were the same in the time allowed for dialysis.

Binding of [^{3}H]pantothenic acid to rat plasma was also investigated. Blood was drawn from a 350 g rat into a heparinized syringe by cardiac pucture. After centrifugation, 1 ml of the plasma was dialyzed against 4 ml of 0.9 mM polyethylene glycol dissolved in 0.9% NaCl. 17 pmol of [^{3}H]pantothenic acid were added to either the polyethylene glycol or plasma. Complete equilibration also occurred in either case in the time allowed for dialysis. Dialysis conditions were the same as above.

Rats weighing 200–250 grams were lightly anesthetized with sodium pentobarbital. 3 μCi of [^{14}C]pantothenic acid was injected intraperitoneally. This amount represents approx. 10% of the daily requirement for pantothenic acid [11]. Urine was collected in a beaker cooled by solid CO_2 and kept frozen until analyzed. Analysis was performed using high-voltage electrophoresis or by DEAE-cellulose columns. The urine was concentrated under reduced pressure and then loaded onto a 1.3 × 47.5 cm DEAE cellulose column and eluted with 0.005 M NH_4OH to remove all neutral or positively charged compounds. The radioactivity was then eluted with a gradient of 100 ml of 0.1 mM NaOH to 100 ml of 10 mM HCl. The radioactivity co-eluted with authentic pantothenic acid.

Results

The perfused kidneys were determined to be functional by the following criteria: urine flow, glomerular filtration rate and metabolism. The mean urine flow was 49.0 ± 8.8 μl/min and decreased gradually during the experiments. Data for individual concentrations are shown in Table I. Glomerular filtration rates were more constant, but they also gradually decreased. Kidney mass ranged from 1.0 to 2.4 g and size did not affect the fractional reabsorption. The use of penicillin in the perfusate inhibited the secretion of pantothenic acid at higher concentrations. The fractional reabsorption of pantothenic acid was constant throughout the course of the experiment and the kidneys were stable and functional for at least 1 h, therefore, it was feasible to study the excretion of three concentrations of pantothenic acid during each perfusion. At 2 μM, the physiological concentration of pantothenic acid in rat plasma [12], the fractional reabsorption was 88.0 ± 3.6 with penicillin and 81.8 ± 4.3 without penicillin (Table I and Fig. 1). Fractional reabsorption at the 0.135 μM was 87.0 ± 4.4 with penicillin and 81.0 ± 3.7 without, and not different from the 2 μM concentration. This indicated that secretion may have taken place at physiological concentrations and below. As the concentration was increased, secretion became the dominant process, although it was more apparent and began at a lower concentration in the absence of penicillin.

Recoveries of ^{14}C after the perfusion ranged from 78% to 103%, with a mean value of 93%. Metabolism was studied at the same two low concentrations used for excretion. At 0.27 μM metabolism of pantothenic acid to its intermediates was observed. At the end of the perfusion the kidney contained 7.1 ± 0.3% of the dose per gram of kidney. The concentration of ^{14}C in the kidney was determined by assuming that the fresh kidney contained 75% moisture [13]. High-voltage electrophoresis and paper chromatography were used to confirm that only free pantothenic acid appeared in the urine. Free pantothenic acid

TABLE I

PHYSIOLOGICAL CHARACTERISTICS OF KIDNEY PERFUSIONS

Number in parentheses represents number of experiments at each concentration. Results are expressed as means of averages for 5-min urine collection periods for each concentration ± S.D. of indicated number of experiments. PaA, pantothenic acid.

PaA (μM)	Additions	Glomerular filtration		
		Urine flow (μl/min)	Rate (μl/min)	Fractional reabsorption (%)
0.135(4)	Penicillin	48.5 ± 9.8	460 ± 102	87.0 ± 4.4
2.0 (4)	Penicillin	52.6 ± 12.9	431 ± 87	88.0 ± 3.6
10.0 (4)	Penicillin	63.2 ± 11.7	350 ± 129	79.6 ± 2.9
20.0 (4)	Penicillin	53.0 ± 7.7	320 ± 140	33.0 ± 7.0
30.0 (4)	Penicillin	53.0 ± 10.0	414 ± 112	−5.2 ± 6.9
0.135(4)		32.7 ± 7.6	424 ± 143	81.0 ± 3.7
2.0 (4)		38.5 ± 15	437 ± 136	81.8 ± 4.3
5.0 (4)		51.7 ± 17.0	448 ± 109	72.8 ± 5.2
10.0 (4)		42.4 ± 9.5	437 ± 115	27.6 ± 10.8
30.0 (4)		53.6 ± 15.7	389 ± 106	−33.8 ± 7.4

was the only labelled compound detected in the perfusate. The ratio of tissue concentration to perfusate concentration of ^{14}C (T/P) was 10.7.

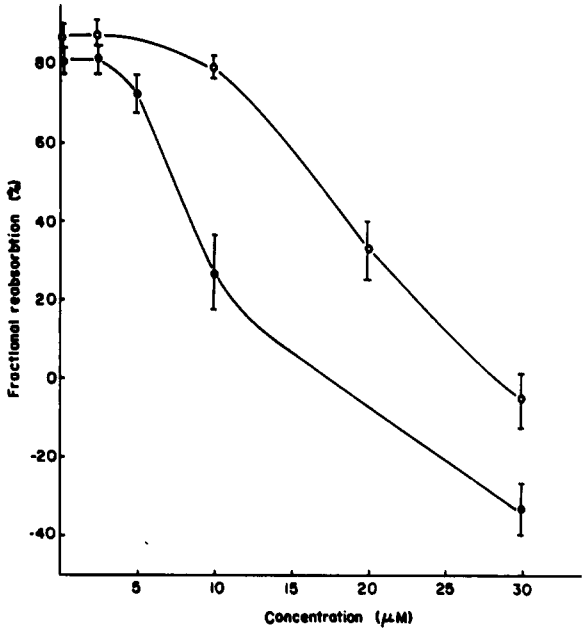

Fig. 1. Fractional reabsorption of pantothenic acid by the perfused kidney with and without penicillin. A negative value for fractional reabsorption represents net secretion of pantothenic acid. A value of zero represents no net secretion or reabsorption. Values are means ± S.D. of 4 experiments. ○——○, Penicillin added, 20000 units/130 ml perfusate; ●——●, no penicillin added.

The kidney contained labelled free pantothenic acid, CoA, dephospho-CoA, pantetheine, and 4′-phosphopantetheine at the end of the 1 h perfusion with 0.27 μM pantothenic acid. The amounts of label in the intermediates was determined after separation by high-voltage electrophoresis. The kidney contained 72.7 ± 12.3% of the label as free pantothenic acid. 8.0 ± 1.2 was found as pantetheine, 6.3 ± 3.1 as dephospho-CoA, 9.5 ± 2.9 as 4′-phosphopantetheine and 4.9 ± 0.9 as CoA after the 1 h perfusion.

At the 2 μM concentration no labelled metabolites of pantothenic acid were found in the kidney. The T/P ratio remained high at 9.97 ± 2.3, not significantly different from the ratio at the lower concentration studied. No labelled metabolites, other than free pantothenic acid, could be detected in the perfusate or urine.

Equilibrium dialysis was employed to determine if binding of pantothenic acid to bovine serum albumin could be a complication in determining excretion rates and to see if this binding could be a method of conservation in the whole animal. The concentration of [^{3}H]pantothenic acid in the dialysis apparatus was 1.24 nM. A 10% solution of bovine serum albumin was used to see if binding occurred. Rat plasma was also dialyzed with a final concentration of 3.14 nM [^{3}H]pantothenic acid and again no binding was detected.

Results of transport experiments using kidney

brush-border membrane vesicles showed that transport of pantothenic acid across the brush-border membrane of the proximal tubule is a Na^+-dependent, active transport process. This is shown in Fig. 2A. Here [^{14}C]pantothenic acid accumulated against a concentration gradient in the isolated vesicles. The curve tails off to an equilibrium value in 20 min. In the initial stages of uptake, maximal accumulation of pantothenic acid occurred at 30 s after the application of a Na^+ gradient directed out to in. This corresponds to an intravesicular concentration three times the equilibrium value.

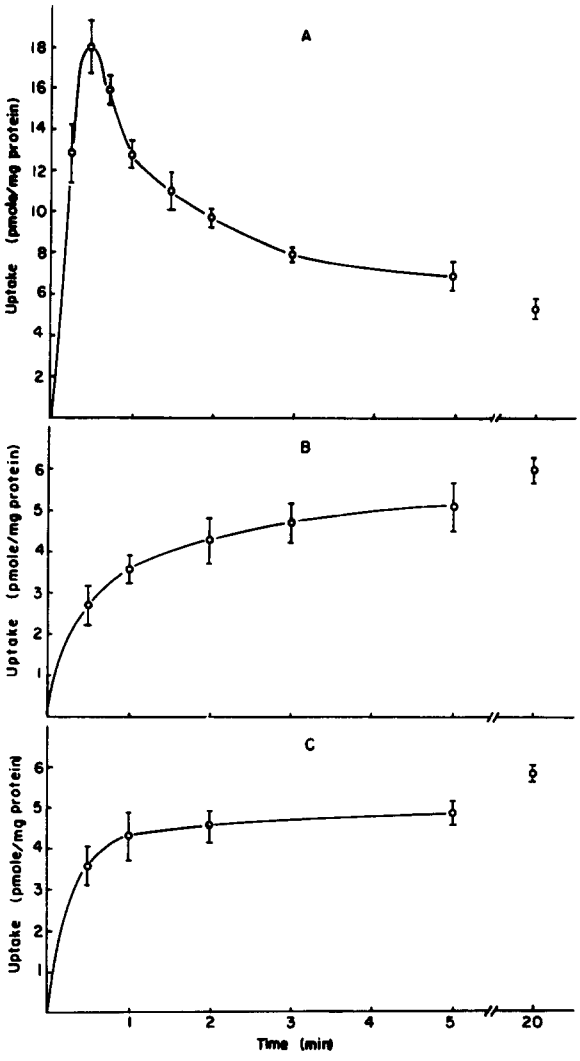

Fig. 2. Uptake of pantothenic acid by kidney brush-border membrane vesicles as a function of time. Membrane vesicles were incubated with [^{14}C]pantothenic acid and the indicated buffer. At the indicated times, aliquots were filtered and the filters analyzed for radioactivity. The final concentration of pantothenic acid in the incubation tube was 2 μM. Results are expressed as means ± S.E. of five experiments; (A) in the presence of an initial 100 mM NaCl gradient, (B) 100 mM KCl gradient and (C) no ion gradient.

A gradient of K^+ directed out to in did not cause a transient accumulation of [^{14}C]pantothenic acid above equilibrium and equilibrium was slowly reached by 20 min (Fig. 2B). No transient accumulation of pantothenic acid was seen when no gradient was used (Fig. 2C) and pantothenic acid was allowed to diffuse into the vesicles. A vesicle suspension was incubated with [^{14}C]pantothetic acid for 15 s or 15 min and spotted for electrophoresis. All of the radioactivity migrated to the pantothenic acid position indicating that metabolic trapping did not play a role in the transient accumulation observed.

The effect of varying the concentration of pantothenic acid on its uptake by vesicles studied. True initial rates were not obtainable, since incubation periods of less than 30 s caused extensive point scatter. As the concentration of pantothenic acid in the incubation medium increased, the absolute uptake of pantothenic acid leveled off, indicating saturation kinetics, with a K_m of 7.30 μM and a V_{max} of 34.8 pmol/mg protein per min.

Using vesicles it was possible to examine the inhibiting effect of structurally similar compounds to determine if they inhibit transport of pantothenic acid. The results are seen in Table II. Again incubation time was 30 s, to minimize varia-

TABLE II

INHIBITORS OF PANTOTHENIC ACID UPTAKE BY MEMBRANE VESICLES

Uptake was measured 30 s after the addition of [^{14}C]pantothenic acid and the inhibitor. All inhibitor standard solutions (0.5 mM) contained 1 mM dithiothreitol.

Inhibitor	Absolute uptake (dpm/mg protein)	: of control
Control	724 ± 64	100
β-Alanine	780 ± 13	107
Pantetheine	750 ± 33	104
4'-Phosphopantothenate	228 ± 22	31.5
4'-Phosphopantetheine	556 ± 35	77.0
Pantoyl lactone	704 ± 67	97.2

tion in data. Dithiothreitol (1 mM) was preincubated with each analog to insure they were in the sulfhydryl form for the experiment. The same concentration of dithiothreitol was added to the control uptake experiments. The final concentration of all inhibitors in the incubation tube was 0.5 mM. The dithiothreitol in the inhibitor and control solutions did not appear to alter transport in the 30 s period it was in contact with the vesicles. 4′-Phosphopantetheine and 4′-phosphopantothenate inhibited transport. All other compounds tested did not.

3 μCi of [^{14}C]pantothenic acid was administered to rats to determine which radioactive metabolites were excreted in the urine. In 24 h 10 to 16% of the dose was excreted, with decreasing amounts appearing in the urine every day thereafter. After 1 week, approx. 38% of the dose had been excreted. The radioactivity co-eluted with authentic pantothenic acid from DEAE-cellulose columns. Electrophoresis confirmed that the only labelled metabolite excreted was free pantothenic acid. Approx. 6% of the ^{14}C appeared in the feces during a 7-day collection.

Discussion

Reabsorption, transport, metabolism and excretion of pantothenic acid by the kidney has not been studied previously. We have used a variety of techniques to examine these processes. Using a combination of live animals, kidney perfusions and brush-border membrane vesicles it was possible to determine how the rat kidney manages pantothenic acid.

Ono et al. [14] have demonstrated that free pantothenic acid is the major circulating form of the vitamin. They also found that approx. 10% of the circulating vitamin was pantetheine. Smith [15] found that 90 min after intravenous injection of [^{14}C]pantothenic acid, all the radioactivity in the serum remained as free pantothenic acid.

The only excretory product of intraperitoneally administered [^{14}C]pantothenic acid was found to be free pantothenic acid. This is in contrast to the results of Taylor et al. [3], showing that the excretory product of the dog is the β-glucuronide; very little free pantothenic acid was excreted. No pantetheine was found in the urine of our rats given pantothenic acid, which agrees with the results of Ono et al. [14]. Their results also showed that no panthetheine was excreted with pantothenic acid was given orally, but pantetheine accounted for 20% of the total pantothenic acid-containing compounds when a large dose of pantetheine was administered intravenously. These results indicate that pantothenic acid is the only excretory form in rats under normal conditions and that pantetheine may be very efficiently reabsorbed when it is present in the plasma.

Since binding of pantothenic acid to human serum albumin has been reported [16], it was important to know, if any, fraction of the pantothenic acid added to the perfusate was bound to the bovine serum albumin. It would then be possible to correct for the binding, when determining the amount of pantothenic acid filtered. Equilibrium dialysis was performed using a high concentration of albumin and a small amount of [^{3}H]pantothenic acid, which had a high specific activity. Any significant binding should have been detected using these techniques. Since no binding was found, no corrections need be made when determining fractional reabsorption. Binding of pantothenic acid by dog plasma has also been reported [1]. These results would indicate that one method of conservation of pantothetic acid could be by a binding phenomenon. Equilibrium dialysis again showed no binding of [^{3}H]pantothenic acid to rat plasma proteins and, therefore, indicated that the only mechanism of conservation for free pantothenic acid in the rat is tubular reabsorption. These results are similar to the observations of Roholt and Smith [2] who found no binding of pantothenic acid to human plasma proteins.

Transport of pantothenic acid in kidney brush border membrane vesicles is a Na^{+}-dependent active transport process. These data provide the first direct evidence for an active transport mechanism for pantothenic acid in a mammalian system. The process is saturable which is consistent with the idea of a carrier protein located in the membrane. Metabolic trapping can be discounted, since no metabolites were detected after incubation of [^{14}C]pantothenic acid with vesicles. The high T/P ratio of the perfused kidney at the 2 μM concentration also indicated that an active transport process must be maintaining these high levels,

since no metabolic trapping could be detected.

The results of inhibition experiments using vesicles (Table II) also supported the involvement of a specific transport protein in the membrane. At 0.5 mM phosphopantetheine inhibited moderately and phosphopantothenate rather strongly. The latter observation suggests that the carboxyl group is involved in the recognition by the transport protein. Inhibition by 4'-phosphopantetheine but not by pantetheine was a surprising result. Transport of pantothenic acid by *Escherichia coli* K-12 [17] and *Lactobacillus plantarum* [18] have been shown to be active processes. Transport in *E. coli* was inhibited by pantetheine, dephospho-CoA and CoA. The latter two would appear to be unlikely inhibitors due to their size and the shielding of the pantothenate moiety by the remainder of the molecules. The inhibition observed [18] may reflect hydrolysis during the 10 min period that transport was studied. In the kidney vesicles appreciable hydrolysis would not be expected in the 30 s exposure of the vesicles to the test compounds.

The transport studies of Sugarman and Munro [19] showed that pantothenic acid accumulation in isolated adipocytes was energy and temperature dependent and saturable. They postulated an active transport mechanism, but these results cannot be evaluated, since metabolite formation was not excluded.

Metabolism of pantothenic acid in the perfused kidney was detected only when the concentration of pantothenic acid in the perfusate was low and the specific activity was high. As the amount of unlabeled pantothenic acid added to the perfusate was increased, no metabolites could be found in the kidney due to lack of sensitivity of the detection methods. The kidney does not appear to release labelled metabolites of pantothenic acid into the urine or perfusate, even though they are present in the kidney tissue. Free pantothenic acid was the major labelled compound present in the kidney, although CoA, and several of its metabolites accumulated during the course of the perfusion.

Two processes appear to regulate excretion of pantothenic acid in the rat kidney. Tubular reabsorption and tubular secretion both operated and possibly simultaneously. Penicillin inhibited secretion of pantothenic acid; therefore both competed for the same secretory carrier. Caronamide has been shown to inhibit tubular secretion in humans of pantothenic acid [2] and penicillin [20]. These compounds are all carboxylic acids and it appears that this structural feature determines if the compound will be secreted. Since human kidney tubules appear to exhibit both reabsorption and secretion of pantothenic acid [2], as does the rat kidney, the rat may be a good model for the study of human urinary secretion.

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