Phosphate Transport after Acute Changes in Total NAD Content in Renal Proximal Tubules (43031)

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Abstract. **Suspensions of proximal tubules were obtained by collagenase digestion of rat renal cortex followed by centrifugation on a percoll gradient. NAD content in tubules incubated at 37°C was decreased by 40–60% compared with tubules incubated at 4°C. This change occurred within 30 min and was maintained for up to 2 hr. Inhibitors of NAD hydrolysing enzymes prevented the depletion of cellular NAD at 37°C. Acute changes in proximal tubule NAD content at 37°C were not accompanied by changes in phosphate uptake by brush border membrane vesicles subsequently prepared from the same tubules. In contrast, incubation of tubules with parathyroid hormone (10^{-6} *M*) produced the expected inhibition (20%) of brush border membrane transport of phosphate. One implication of these findings is that acute changes in total NAD content of proximal tubules at 37°C may not influence the phosphate transport system in the renal brush border membrane. Other interpretations are discussed.** [P.S.E.B.M. 1990, Vol 193]

The proposed role of NAD as a regulator of Na^+-dependent phosphate transport across the renal brush border membrane (BBM) was based on the observations that an increase in NAD content of the renal proximal tubule was accompanied by marked and specific inhibition of the phosphate transport system in the BBM (1–3). Although still controversial, there is evidence that cytosolic NAD could be used for covalent modification of the phosphate transporter in the BBM by means of an ADP-ribosylation reaction (4, 5).

The involvement of NAD as a coenzyme in cellular redox reactions occurs without net changes in the total cellular pool of NAD and NADH. The activity of NAD hydrolysing enzymes, however, produces marked changes in cellular NAD content. The most important of these enzymes are a membrane-associated NAD glycohydrolase (6) and a chromatin-bound poly(ADP-ribose) polymerase (7). The activity of the latter has been implicated in the depletion of intracellular NAD in primary cultures of rat liver cells (8) and in Chinese hamster ovary cells (9).

We found that endogenous depletion of intracellular NAD also occurs in suspensions of isolated proximal tubules. This *in vitro* system allows studies of the effects of acute changes in total NAD content on phosphate transport, in contrast to all previous work which focused on chronic changes in NAD content (1, 2).

Materials and Methods

Preparation of Proximal Tubules. Adult male Sprague-Dawley rats (270–300 g) were used in all experiments and were given standard laboratory chow and water *ad libitum*. Kidneys surgically removed from three to six rats under ether anesthesia were used for the preparation of proximal tubule suspensions by a modification (10) of the procedure of Vinay *et al.* (11). Two major bands of material separate on the percoll gradient, one at the top and one near the bottom. These fractions were carefully recovered and washed twice by resuspension in ice-cold Krebs-Henseleit saline (KHS) and centrifugation at 1000 revolutions/min. The KHS was modified by addition of L-glutamate (5 m*M*), glucose (5.5 m*M*), and bovine serum albumin (0.5 g/liter). All centrifugations were at 4°C. The final pellets were resuspended in five to six volumes of modified KHS

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and kept at 4°C. Samples were taken for microscopy and for determination of hormone sensitivity and protein content.

Morphology. Aliquots of the cortical digest and percoll gradient fractions were examined routinely by phase contrast microscopy. The bottom fraction also was processed for electron microscopy by a standard procedure described previously (5).

Hormone Sensitivity. Samples (0.05 ml) of the bottom gradient fraction were incubated in modified KHS (containing 5 m*M* glutamate) at 37°C for 1 hr with and without either 10^{-6} *M* arginine vasopressin (synthetic acetate salt; Sigma) or parathyroid hormone (synthetic bovine 1-34 fragment; Sigma). All incubation solutions contained 0.5 m*M* 1-methyl-3-isobutylxanthine and the incubation tubes were sealed after gassing with O_2-CO_2. The incubations were terminated by addition of perchloric acid to a final concentration of 0.6 *M*. Precipitated protein was removed by centrifugation and the cyclic AMP content of the neutralized supernatants was determined by radioimmunoassay (1).

Protein, Enzymes, and ATP. Protein was determined according to the Lowry procedure (12). γ-Glutamyltranspeptidase was assayed as described previously (1, 2, 13) using γ-glutamyl-*p*-nitroanilide as substrate. The procedure of Bergmeyer and Bernt (14) was used to assay lactate dehydrogenase and tubule ATP content was determined by the hexokinase/glucose-6-phosphate dehydrogenase reaction (15).

Oxygen Consumption. Oxygen consumption rates of isolated tubule suspensions (100 μl) in modified KHS were measured at 30°C with a YSI oxygen meter (Yellow Springs, OH) and oxygen electrode as described previously (16). The electrode was calibrated with the modified KHS buffer solution that had been saturated with 50% oxygen. Oxygen consumption was recorded on a chart recorder and was linear for 2 min.

NAD Depletion Studies. Proximal tubule suspensions (0.5 ml) in modified KHS (containing 5 m*M* glutamate) were incubated at 37°C in a shaking water bath for various times up to 2 hr. Control tubules were kept at 4°C throughout the incubation periods. The incubations were stopped by chilling and immediate extraction of NAD with 0.6 *M* perchloric acid. The NAD content of the protein-free extracts was determined by the cycling method of Nisselbaum and Green (17).

BBM Transport of Phosphate. Isolated proximal tubules obtained from the pooled renal cortices of eight rats by collagenase digestion and percoll gradient centrifugation, as described above, were resuspended in 15 ml of ice-cold modified KHS solution (containing 5 m*M* glutamate) and divided into three aliquots. After incubation for 2 hr under various conditions (see Results), duplicate 0.05-ml samples were taken from each tubule suspension for NAD determination. The remainder of each of the tubule suspensions was processed immediately for preparation of a BBM vesicle fraction by the Mg^{2+} precipitation procedure (18, 19). Uptake of [^{32}P]phosphate by each of the three BBM vesicle fractions was determined on the same day by the rapid filtration technique used and described previously (1, 13). In order to confirm the results of these studies, another series of experiments was conducted in an identical way, except that the proximal tubules were incubated in a medium containing different gluconeogenic substrates, as described by Balaban et al. (20, 21). The incubation medium for these experiments contained 115 m*M* NaCl, 25 m*M* $NaHCO_3$, 4 m*M* Na_2HPO_4, 2.3 m*M* $CaCl_2$, 5 m*M* KCl, 0.1 m*M* $MgSO_4$, 5 m*M* glucose, 4 m*M* lactate, 1 m*M* alanine, and 6% dextran (M_r 40,000), gassed with O_2-CO_2 (19:1), pH 7.4. The incubation period was reduced to 1 hr to minimize the possibility of impairing tubule viability.

Results

Fractionation of collagenase-digested renal cortex on a gradient preformed from 37.5% percoll yielded two major fractions. The top fraction consisted of free cells, glomeruli, and a mixture of different tubular segments. The bottom fraction was composed primarily of numerous thick elongated proximal tubular segments and a few free cells (Fig. 1A). Light micrographs of sectioned specimens of the bottom fraction revealed good structural integrity and a brush border characteristic of proximal tubules (Fig. 1B). These observations were confirmed by electron micrographs (Fig. 1C). The morphologic quality of the proximal tubule enriched preparations is equivalent to that described by other laboratories (19, 20).

Basal cyclic AMP content of the proximal tubule preparation was 50 ± 15 (mean ± SE) pmol/mg protein and was increased to 143 ± 7 pmol/mg ($P < 0.005$, group *t* test, $n = 3$–4) by parathyroid hormone. In contrast, the cyclic AMP content after incubation with vasopressin (69 ± 8 pmol/mg) was not significantly different from the basal level. The activity of γ-glutamyltranspeptidase, an enzyme marker for the proximal tubule BBM, was increased 2 to 3-fold ($n = 2$) in the proximal tubule fraction compared with whole renal cortex. These observations support the morphologic evidence for the proximal tubular origin of the tubules in the bottom fraction. This fraction was used in all subsequent studies.

Oxygen consumption by the proximal tubule fraction was 21.3 nmol/min/mg protein and was reduced to 10.9 nmol/min/mg by the presence of ouabain (1 m*M*). The inhibition (49%) of oxygen consumption by ouabain is comparable to that reported elsewhere (16, 20–22) for rabbit and rat proximal tubules. This effect suggests that sodium transport is closely linked to oxy-

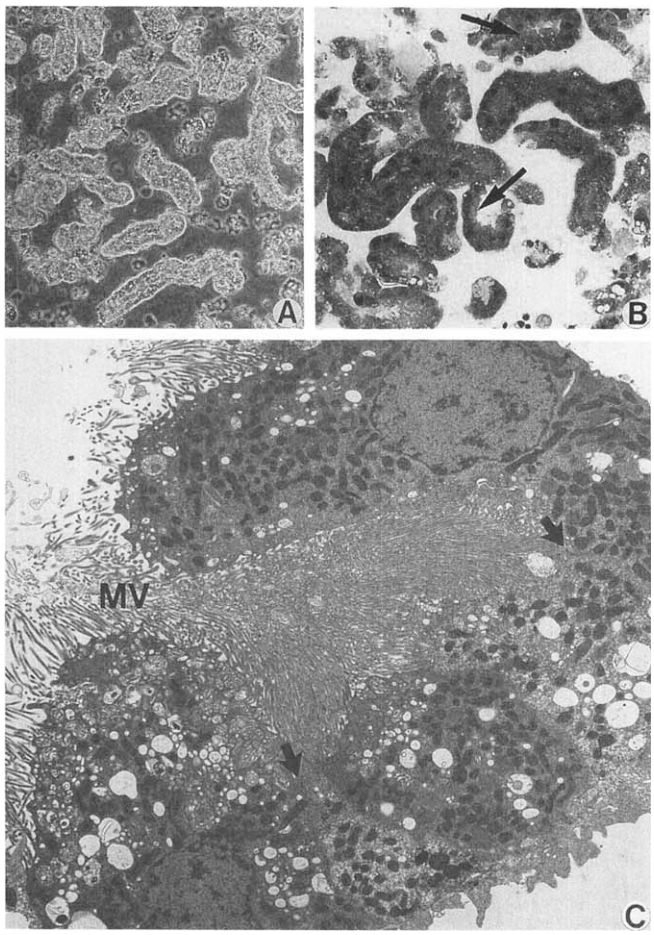

Figure 1. Morphology of isolated rat renal proximal tubule segments. Phase contrast light micrograph (A) of percoll gradient-enriched proximal tubules illustrates numerous elongated tubule segments (original magnification ×93). Light micrograph (B) of a tissue section shows numerous tubule segments that possess a distinct brush border (arrows) (original magnification ×256). Transmission electron micrograph (C) shows portions of several cells from an isolated proximal tubule segment. The cells possess apical junctions (arrows) and a prominent microvillus brush border (MV) (original magnification ×5320).

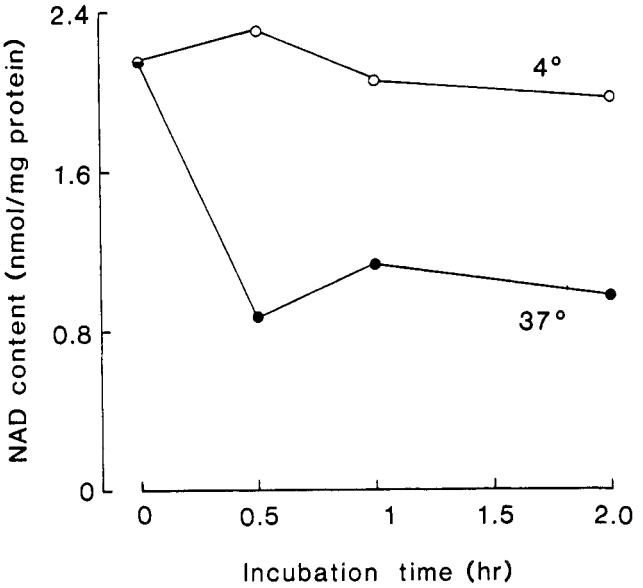

Figure 2. NAD content of proximal tubule suspensions incubated for various times at either 4°C or 37°C. The incubation medium was modified KHS containing 5 m*M* glutamate. Each point is the mean value from two separate experiments.

Table I. NAD and ATP Levels in Suspensions of Isolated Proximal Tubules[a]

Incubation conditions	NAD content (nmol/mg protein)	ATP content (nmol/mg protein)
2 hr at 4°C	2.49 ± 0.32 (6)	4.73 ± 2.10 (4)
2 hr at 37°C	1.25 ± 0.20[b] (6)	5.68 ± 0.90 (4)

[a] Values are mean ± SE of the number of preparations shown in parentheses.
[b] Significantly different ($P < 0.005$, paired t test) compared with tubules incubated at 4°C. The tubule incubation medium was modified KHS containing 5 m*M* glutamate.

gen uptake, as would be expected in a viable tubule preparation.

The NAD content of proximal tubule suspensions incubated at 4°C was maintained for up to 2 hr. In contrast, there was a rapid fall in the NAD content of tubules incubated at 37°C (Fig. 2). The decline in NAD occurred within 30 min and remained at a low level for the next 90 min (Fig. 2). Release of lactate dehydrogenase, a cytosolic enzyme, was not detectable during incubation of proximal tubules at either 4°C or 37°C for 2 hr. As shown in Table I, the depletion of NAD at 37°C was not accompanied by a change in ATP content of the same tubule preparations. The ATP content was within the range of values reported by others for proximal tubules obtained by collagenase digestion (19–23). Maintenance of normal ATP content and the absence of lactate dehydrogenase release strongly suggest that the tubule suspensions were viable during the incubations and that the fall in NAD content at 37°C was not due to leakage of NAD from the cells. Glutamate was present as a gluconeogenic substrate during these incubations (see Materials and Methods) so that normal metabolic activities would be maintained.

We determined next if NAD-glycohydrolase and poly(ADP-ribose)polymerase played a role in NAD depletion in isolated proximal tubules. 3-Aminobenzamide, 5-methylnicotinamde, thymidine, or picolinamide, specific inhibitors of these enzymes, were included in the incubation media (2.5 or 5.0 m*M* final concentrations) of tubules incubated at 37°C. In the absence of any inhibitor, the NAD content of proximal tubules was decreased by about 50% after incubation for 2 hr, as observed previously. The NAD content was significantly higher in all of the tubules incubated for 2 hr with one of the inhibitors at 5 m*M* (Fig. 3). Except for 3-aminobenzamide, each of the inhibitors also was effective at 2.5 m*M* and picolinamide appeared to be

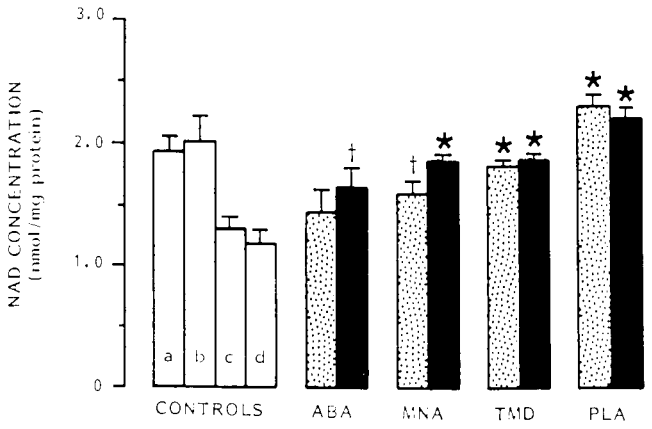

Figure 3. Effect of inhibitors on NAD depletion at 37°C. Controls are freshly prepared tubules (a), tubules incubated at 4°C for 2 hr (b), tubules incubated at 37°C for 1 hr (c) or 2 hr (d). Inhibitors of NAD catabolic enzymes were used at final concentrations of 2.5 m*M* (dotted columns) and 5 m*M* (shaded columns) and the tubules were incubated at 37°C for 2 hr. The basic incubation medium was modified KHS containing 5 m*M* glutamate. Values are mean ± SE of three experiments. *$P < 0.025$ and †$P < 0.05$ (group *t* test) compared with control Group d. ABA, 3-aminobenzamide; MNA, 5-methylnicotinamide; TMD, thymidine; PLA, picolanamide.

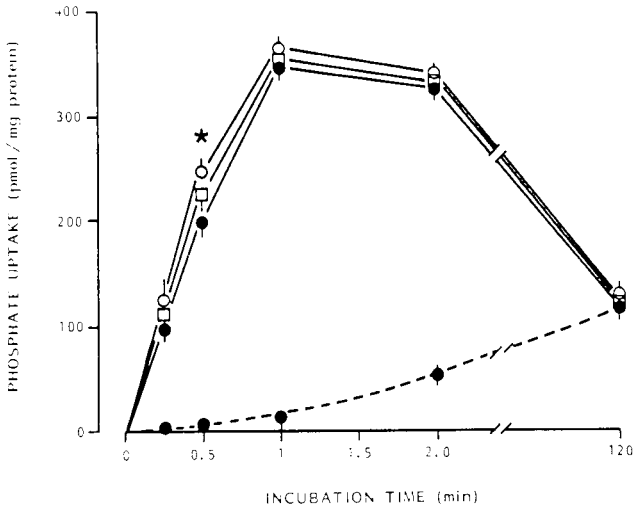

Figure 4. Time course of phosphate uptake by brush border membrane vesicles. The vesicles were prepared from isolated proximal tubule suspensions incubated for 2 hr at 4°C (solid circles) or 37°C (open circles) or in the presence of 2.5 m*M* picolinamide at 37°C (open squares). In all cases the basic incubation medium for tubules was modified KHS containing 5 m*M* glutamate. Phosphate uptake was determined in the presence of a Na^+ gradient (solid lines) and when NaCl in the incubation medium was replaced by KCl (broken line). Na^+-independent uptake was similar in the three groups and only one set of points is shown. Each point is the mean ± SE of three separate experiments, the three groups were always compared within the same experiment. * Phosphate uptake at 0.5 min by vesicles from tubules incubated alone at 37°C is significantly different ($P < 0.05$, group *t* test) compared with vesicles from tubules incubated at 4°C.

most effective. The NAD levels in tubules incubated with picolminamide, both 2.5 and 5.0 m*M*, were equivalent to those of freshly prepared tubules and tubules maintained at 4°C (Fig. 3).

In order to determine whether the decrease in NAD content was accompanied by a change in Na^+-dependent phosphate transport, BBM vesicles were prepared from proximal tubule suspensions which had been incubated under different conditions. The BBM vesicle fractions had 9-fold enrichment of alkaline phosphatase and 4- to 5-fold enrichment of γ-glutamyl transpeptidase and leucyl aminotranspeptidase when compared with proximal tubule homogenates. The enrichment of the latter two enzymes is comparable to the 5.6-fold enrichment of maltase reported by Hammerman *et al.* (19). The reason for the greater enrichment of alkaline phosphatase is not understood at the present time. The BBM vesicles retained the features of Na^+ gradient-dependent phosphate uptake (Fig. 4) that are characteristic of BBM vesicles prepared from renal cortical tissue by standard procedures (1, 2, 13).

In this series of experiments, three groups of tubules were used in each experiment and all groups were incubated for 2 hr. Although most of the NAD depletion occurs within the first 30 min (Fig. 2), a 2-hr incubation was used to allow time for any subsequent changes in P_i transport to become established. One group of tubules was incubated at 4°C, the second group was incubated at 37°C, and the third group was incubated at 37°C in the presence of 2.5 m*M* picolinamide to prevent the fall in NAD. At the end of the incubation period, the tubule NAD content was 2.2 ± 0.5 (mean ± SE, $n = 3$) in the first group, 1.2 ± 0.3 ($P < 0.005$) in the second group and 1.9 ± 0.3 ($P > 0.05$) nmol/mg protein in the third group.

Na^+-dependent phosphate uptake during the initial phase of uptake by BBM vesicles was not different at 15 sec or 1 min when vesicles from 4°C incubated tubules were compared with 37°C incubated tubules. The only significant difference was at 0.5 min when the uptake was increased by 20% in vesicles from 37°C incubated tubules (Fig. 4). The absence of any change in phosphate uptake at 15 sec, the time point which best represents the initial rate of phosphate uptake, strongly suggests that the acute 45% depletion of intracellular NAD by 37°C incubation leads to no marked or consistent change in BBM transport of phosphate.

Tubules incubated at 37°C may not be directly comparable to tubules incubated at 4°C. Chilling inhibits cell metabolism and this may interfere with proximal tubule cell functions (24). These concerns are avoided when tubules incubated at 37°C are compared with tubules incubated at 37°C in the presence of picolinamide. There were no differences in Na^+-dependent phosphate uptake at any time point by BBM vesicles prepared from these two groups (Fig. 4). Thus, the acute decrease (38%) in NAD content at 37°C produced no change in BBM transport of phosphate. These manipulations of NAD levels in proximal tubules also had no

effect on Na^+-independent phosphate uptake by BBM vesicles (Fig. 4).

Another series of experiments was carried out on tubules incubated in the presence of different gluconeogenic substrates, specifically lactate and alanine (see Materials and Methods). The incubation time was reduced to 1 hr, a time at which the fall in NAD was at a maximum (Fig. 2). At 37°C the NAD content of the tubules fell by 49% compared with tubules kept at 4°C (Table II), in agreement with the previous findings when glutamate was the substrate (Fig. 2). As before, the fall in NAD was prevented by the presence of picolinamide at 2.5 m*M*. The changes in NAD were not accompanied by any significant changes in Na^+-dependent phosphate uptake by isolated BBM vesicles (Table II), confirming the previous findings.

As a positive control, tubules were incubated with parathyroid hormone (10^{-6} *M*), a known inhibitor of the phosphate transport system in the proximal tubule BBM (4). The incubations were carried out for 1 hr at 37°C in the medium containing lactate and alanine as exogenous substrates. Na^+-dependent phosphate uptake by BBM vesicles prepared at the end of the incubation period was 529 ± 72 in controls compared with 442 ± 73 pmol/mg/min after parathyroid hormone treatment ($P < 0.025$, *t* test). This represents an inhibition of 20% due to parathyroid hormone treatment. In contrast, the uptake at equilibrium (120 min) was almost identical in the two groups, 384 ± 30 in controls compared with 359 ± 29 pmol/mg protein after parathyroid hormone. These are the mean values (±SE) from four separate experiments using different tubule preparations.

Discussion

The marked and rapid fall in NAD content during 37°C incubation (Fig. 2), in the absence of changes in ATP content (Table I), is most likely due to catabolism of intracellular NAD at 37°C rather than to loss of intact NAD from the cells. This is supported by the observation that NAD depletion at 37°C was prevented when the tubules were incubated in the presence of specific inhibitors of NAD-glycohydrolase and poly-(ADP-ribose) polymerase, the major intracellular NAD hydrolysing enzymes (Fig. 3). The relative importance of these two enzymes in depletion of NAD in renal proximal tubules cannot be assessed from the present studies. Poly(ADP-ribose) polymerase may have the major role because the fall in NAD content was blocked by both 3-aminobenzamide and thymidine, compounds which inhibit the polymerase but not the glycohydrolase (7, 8, 25, 26). It has been suggested (9) that depletion of intracellular NAD at 37°C compared with 4°C may represent a response to the stress produced by marked changes in environmental temperature. The NAD depletion may serve to slow down energy-requiring reactions until temperature fluctuations have ceased.

A chronic increase in NAD content of rat renal cortex, including the proximal tubule, was induced in previous *in vivo* studies by injection of nicotinamide (1, 3) and was accompanied by marked and specific inhibition of BBM transport of phosphate. The inhibition of Na^+-dependent phosphate transport was observed at all time points throughout the initial uphill phase of uptake. In the present *in vitro* studies, however, the acute changes in proximal tubule NAD content at 37°C were not accompanied by significant changes in phosphate transport. Incubation of isolated proximal tubules with parathyroid hormone produced the expected inhibition of Na^+-dependent phosphate transport, indicating that the experimental system is sensitive to a known physiologic regulator of BBM phosphate transport. Thus, acute changes in total NAD content of isolated proximal tubules at 37°C may not influence the phosphate transport system in the BBM.

Additional support for this conclusion is provided by recent studies with streptozotocin (Abraham and Kempson, unpublished data). Proximal tubule suspensions maintained at 37°C were treated with streptozotocin under conditions which produce a 40% fall in tubule NAD content within 1 hr, compared with controls which were incubated at 37°C in the absence of the drug (27). Na^+-dependent phosphate transport by

Table II. Tubule NAD Content and BBM Transport of Phosphate after Incubation of Tubules with Lactate and Alanine as Exogenous Substrates[a]

Tubule incubations	NAD content (nmol/mg protein)	Na^+-dependent phosphate transport (pmol/mg protein)		
		0.5 min	1.0 min	120 min
1 hr at 4°C	1.01 ± 0.25	489 ± 146	642 ± 70	244 ± 20
1 hr at 37°C	0.52 ± 0.20[b]	390 ± 60	447 ± 26	368 ± 51
1 hr at 37°C with 2.5 m*M* picolinamide	0.90 ± 0.22	409 ± 79	569 ± 149	393 ± 209

[a] Proximal tubule suspensions were incubated in buffer (see Materials and Methods) containing 5 m*M* glucose, 4 m*M* sodium lactate, 1 m*M* alanine, and 6% dextran. Phosphate transport was determined in BBM vesicles prepared at the end of the incubations. Each value is the mean ±SE of four to five independent experiments.

[b] Significantly different ($P < 0.05$, group *t* test) compared with controls incubated at 4°C.

BBM vesicles isolated from the two groups of tubules was not different.

The findings of the present study should be interpreted with caution for two reasons. First, the effect of acute NAD changes on phosphate transport may be rapidly reversed and not detected in isolated BBM vesicles, or additional important cytosolic factors may be lost during the homogenization and washing steps involved in BBM preparation. This may be important since previous studies on chronic changes in NAD content noted a change in the amount of NAD associated with isolated BBM vesicles (28). The NAD content of the final BBM fraction was not determined in the present acute study. Second, it is recognized that the measurements of tubule NAD content reflect the total amount of protein-bound NAD and free NAD. Only free cytosolic NAD is likely to be available for interaction with the BBM and it remains possible that this NAD pool may have been unchanged during the acute manipulations of total NAD content.

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