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Research Article

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Possible Role of Nicotinamide Adenine Dinucleotide as an Intracellular Regulator of Renal Transport of Phosphate in the Rat

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ABSTRACT In these experiments we investigated whether NAD could serve as an intracellular modulator of the brush border membrane (BBM) transport of inorganic phosphate (Pi). NAD, both oxidized (NAD^+) and reduced (NADH) form, inhibited the Na^+-dependent uptake of ^{32}Pi in the concentration range of 10–300 μM NAD when added in vitro to BBM vesicles isolated from rat kidney cortex, but did not inhibit BBM uptake of D-[^{3}H]glucose or BBM uptake of $^{22}Na^+$. Neither nicotinamide (NiAm) nor adenosine alone influenced BBM uptake of ^{32}Pi. NAD had a similar relative effect (percent inhibition) in BBM from rats stabilized on low Pi diet (0.07% Pi), high Pi diet (1.2% Pi), or normal Pi diet (0.7% Pi).

Subsequently, we examined the renal effects of changing the tissue NAD level in vivo. Rats stabilized on low Pi diet were injected intraperitoneally with NiAm (0.25–1.0 g/kg body wt); urinary excretions of Pi ($U_{Pi}V$), of fluid, and of other solutes were measured before and after NiAm injection, then renal cortical tissue nucleotide content was determined, and a BBM fraction was isolated for transport measurements. In BBM from NiAm-treated rats, the Na^+-dependent uptake of ^{32}Pi was decreased, but BBM uptake of D-[^{3}H]glucose and BBM uptake of $^{22}Na^+$ were not changed. NiAm injection elicited an increase in NAD^+ (maximum change, 290%), a lesser increase in NADH (maximum change, +45%), but no change in the content of ATP or cyclic AMP in the renal cortex. Na^+-dependent BBM uptake of ^{32}Pi was inversely correlated with NAD^+ content in renal cortex ($r = -0.77 \pm 0.1$; $P < 0.001$) and with $U_{Pi}V$ ($r = -0.67 \pm 0.13$; $P < 0.01$). NAD^+ in renal cortex was positively correlated with $U_{Pi}V$ ($r = 0.88 \pm 0.05$; $P < 0.001$). Injection of NiAm elicited a marked increase in $U_{Pi}V$, but no change in excretions of creatinine or K^+, or in urine flow; excretion of Na^+ and Ca declined. NiAm injection caused similar renal responses, in normal and in thyroparathyroidectomized rats, as well as in rats on normal Pi diet and low Pi diet.

We conclude that NAD can serve as an intracellular modulator (inhibitor) of Na^+-dependent transport of Pi across the renal luminal BBM and across the proximal tubular wall by its direct interaction with BBM. We propose that at least some hormonal and/or metabolic stimuli elicit phosphaturia by increasing NAD^+ in cytoplasm of proximal tubular cells.

INTRODUCTION

A Na^+-dependent Pi uptake by the luminal brush border membrane (BBM) and its regulation by various hormonal and metabolic stimuli (1–3) are initial and perhaps quantitatively determining cellular steps in proximal tubular reabsorption of phosphate (Pi). Parathyroid hormone (PTH) or vitamin D (1, 3) has been reported to decrease BBM uptake of Pi, whereas growth hormone reportedly has an opposite effect (4). In phosphaturia associated with starvation (5) or a high Pi diet (2), the capacity of the BBM to transport Pi is diminished; feeding with a diet selectively deficient in Pi, however, leads to a marked increase in BBM uptake of Pi (1–3, 5), which is apparently independent of the

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regulatory actions of PTH, calcitonin, thyroid hormones, or vitamin D (1–3).

Very little is known about the biochemical mechanisms by which the BBM is modulated to transport Pi. It is assumed that the effect of PTH on BBM uptake of Pi is mediated by cyclic (c)AMP, since the in vivo injection of N^6,O^2-dibutyryl cyclic AMP (DBcAMP) resulted in decreased BBM uptake of Pi (1). However, cAMP added directly to the BBM vesicles in vivo had no effect on the BBM uptake of Pi (1, Kempson and Dousa, unpublished observations). Prominent changes in the rate of BBM uptake of Pi caused by variation of the dietary Pi intake (1, 2) or by starvation (5), seem to occur independently of the regulatory influence of cAMP (1, 6, 7). Thus, although cAMP may indeed play a role in the actions of some phosphaturic hormones (e.g., PTH, calcitonin) (8), the intracellular factors which regulate the rate of Pi transport across BBM of proximal tubules in response to various pathophysiologic states remain to be identified (8).

Alkaline phosphatase (AlPase), localized in luminal BBM, was suggested to play some role, direct or indirect, in BBM transport of Pi (2). In many instances the AlPase activity in BBM changes in parallel with the rate of BBM transport of Pi (2), and in vivo administration of AlPase inhibitors was reported to reduce renal Pi reabsorption (9); however, the potential involvement of AlPase in BBM transport of Pi remains controversial (1, 2). A recent report (10) that NAD inhibits AlPase extracted from hog kidney deserves special attention. NAD is a naturally occurring nucleotide and serves as a coenzyme of diverse dehydrogenases (11) in numerous oxidation-reduction reactions at various steps of mammalian intermediary metabolism, though mainly in cytoplasm. In addition, NAD plays a role in biochemical processes unrelated to the redox reactions; e.g., intracellular NAD^+ (the oxidized form of NAD) is the source for ADP-ribosylation of plasma membrane proteins (12).

The NAD^+/NADH ratio depends on the metabolic status of the cell and is higher in cytoplasm than in mitochondria (13); the predominant form of NAD in the cytoplasm is NAD^+ (13). Moreover, NAD^+ is less tightly bound to dehydrogenases, compared with NADH (14); it is thus conceivable that free NAD^+ in the cytoplasm (cytosol) could interact with at least some proteins, e.g., AlPase, in the luminal BBM. Based on these premises, we considered and tested the hypothesis that NAD acts on renal luminal BBM and thereby regulates the rate of Pi transport across this membrane.

GLOSSARY OF TERMS

AlPase	Alkaline phosphatase
BBM	Brush border membrane
Cr	Creatinine
DBcAMP	N^6,O^2-dibutyryl cyclic AMP
GNG	Gluconeogenesis
HPD	High phosphate diet
LPD	Low phosphate diet
NiAm	Nicotinamide
NPD	Normal phosphate diet
Pi	Inorganic phosphate
PTH	Parathyroid hormone
P_X	Plasma levels of X
TPTX	Thyroparathyroidectomized
U_XV	Urinary excretion of X

METHODS

Experimental design. Adult male Sprague-Dawley rats weighing 200–250 g were used in all experiments and were allowed unlimited access to distilled water. Rats that had been surgically thyroparathyroidectomized (TPTX), as indicated in the text, were given water supplemented with calcium as described (15). For collection of urine, the rats were housed individually in metabolic cages (5, 15). Unless stated otherwise, all animals were fed regular rat diet (Purina Laboratory Chow, Ralston Purina Co., St. Louis, Mo.) containing 0.7% Pi. High phosphorus diet (HPD) contained 1.2% Pi and was prepared by Pi supplementation of commercial low phosphorus diet (LPD) containing 0.07% Pi (ICN Pharmaceuticals Inc., Life Sciences Group, Cleveland, Ohio), as described in detail (5, 16). HPD and LPD rats, when compared, were placed simultaneously on the different diets, were pair-fed, and were monitored, sacrificed, and analyzed simultaneously as described in detail (5, 15, 16).

Experiments with acute effects of nicotinamide. Nicotinamide (NiAm) dissolved in 0.6 ml of isotonic saline (pH adjusted to 7.0 with NaOH) was administered as a single intraperitoneal injection to rats kept in metabolic cages. Control animals were injected with the same volume of saline vehicle. In some experiments, urine was collected for at least 12 h prior to injection of NiAm or vehicle. After injection in all experiments, urine was collected for 9 h; then the rats were anesthetized with ethyl ether, venous blood was drawn, the kidneys were removed, and the renal cortex was dissected rapidly free from the medulla. Part of the renal cortical tissue was snap-frozen (within 30 s after removal of kidneys from the animal) by clamping between stainless steel tongs precooled in liquid nitrogen (17), and the frozen tissue was stored in a liquid nitrogen freezer until extraction and analysis. The remaining cortical tissue was placed immediately in ice-cold solution (154 mM NaCl, 1 mM Tris-Hepes; pH 7.5) and was used for preparation of a BBM fraction.

Preparation of a BBM fraction and transport measurements. BBM fractions were prepared from homogenized rat renal cortex by the calcium precipitation procedure described in detail in our previous studies on rats (5, 15). Verification of the identity and purity of the BBM vesicle fraction used for transport studies was described in full detail previously (5, 15). In experiments where BBM was prepared from kidneys of one rat, to obtain a sufficient amount of BBM for transport studies particular care was paid to recover all BBM material in the course of preparation. For studies in vitro of the effects of NAD and other tested compounds on transport, isolated BBM vesicles suspended in 300 mM mannitol, 5 mM Tris-Hepes (pH 8.5), were preincubated for 30 min at 20°C in the absence (controls) or in the presence of the test compound. In the subsequent period of actual measurement of BBM uptake, the test compound was included in the incubation medium at the same concentration as used for preincubation. Uptake of ^{32}P-phosphate and D-[^{3}H]glucose by isolated BBM vesicles was measured by the Millipore filtration tech-

nique (3, 5, 15) used and described in detail in our previous studies (5, 15, 16). Final concentrations of ^{32}Pi and D-[^{3}H]-glucose were 0.1 and 0.05 mM, respectively. Uptake was terminated by rapid addition of ice-cold stopping solution (135 mM NaCl, 10 mM sodium arsenate, 5 mM Tris-Hepes, pH 8.5) followed by filtration (5, 15). Uptake of $^{22}Na^+$ was determined (in the absence of Pi or D-glucose) by an identical procedure; the incubation medium contained buffered 100 mM mannitol (pH 8.5), 100 mM $^{22}NaCl$ (~4×10^5 cpm/tube). Uptake of $^{22}Na^+$ had fast onset (within 5 min it reached 103 nmol $^{22}Na^+$/mg protein), followed by slower increase (154 nmol $^{22}Na^+$/mg protein at 90 min). The stopping solution for $^{22}Na^+$ uptake was 150 mM $MgSO_4$, 5 mM Tris-Hepes (pH 8.5) (18). In each BBM preparation the uptake was measured in triplicate at each time period. All solutions used for the preparation of BBM fractions and for transport measurements were filtered through a 0.45 μm Millipore filter on the day of use (5, 15).

Determination of nucleotides in renal tissue. Acidic and alkaline extracts of snap-frozen cortical tissue were prepared by the methods described by Klingenberg (19). Briefly, ~100–200 mg of the tissue was powdered while still frozen in liquid nitrogen and extracted with either 0.6 N $HClO_4$ (acid extract) at a ratio of 5:1 ($HClO_4$:tissue; wt/wt) or with alcoholic 0.5 N KOH (alkaline extract) at a ratio of 10:1 (KOH:tissue; wt/wt). After neutralization followed by centrifugation to remove proteins, the clear extracts were assayed immediately for content of NAD^+, ATP (acid extract), and NADH (alkaline extract). A sample of frozen renal cortex (100 mg) was deproteinized with 5 ml of ice-cold 5% TCA and was processed for determination of the cAMP level by the procedure for renal cortical tissue described in recent studies from this laboratory (20).

Fractionation of renal cortical tissue. In studies on the subcellular distribution of NAD^+ in rat renal cortex a 10% (wt/vol) homogenate of cortical tissue was prepared using five passes of a motor-driven glass-teflon homogenizer. The homogenization medium was 0.25 M sucrose, 5 mM Tris-HCl (pH 7.4), containing 50 mM NiAm to inhibit enzymatic degradation of NAD^+ (21, 22). Part of the homogenate was extracted immediately with TCA (22), while the remainder was centrifuged for 60 min at $10^5 g$. The resulting supernate (cytosol fraction) and pellet (particulate fraction) were separated and extracted immediately with TCA (22). After centrifuging the extracts to remove denatured protein, the TCA was removed by several washes with 3 vol of water-saturated ether (22). The pH of the washed extracts was adjusted to neutrality and the NAD^+ content was determined the same day. All steps in the fractionation and extraction procedure were performed at 0°–4°C.

Assays. cAMP was assayed by radioimmunoassay as described in our previous study (20); recoveries of cAMP ranged between 80 and 90%.

Assays for NAD^+ and NADH were based on the interconversion of lactate and pyruvate catalyzed by lactate dehydrogenase and requiring NAD (19). The final volume of the reaction mixtures was 2.0 ml, and the reactions were started by addition of 0.005 ml of lactate dehydrogenase (3,000 U/ml). The changes in fluorescence were measured on an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, Md.) with an excitation wavelength of 340 nm and emission wavelength of 470 nm.

The reaction mixture for NAD^+ assay contained 0.025 mM lactate, 5 mM hydrazine sulfate, 0.1 M glycine-NaOH (pH 10.0), and up to 0.1 ml of the acid extract or standard NAD^+ solution. The increase in fluorescence, after incubation for 30 min at 37°C, was linear in the concentration range of 1–20 nmol of NAD^+.

The reaction medium for NADH measurement contained 0.15 mM pyruvate, 0.1 M sodium phosphate buffer (pH 7.5), and up to 0.1 ml of alkaline extract or standard NADH solution. After incubation for 17 min at 37°C, the decrease in fluorescence was linear in the range of 1–10 nmol of NADH.

ATP was determined from fluorimetric measurement of NADPH using the hexokinase and glucose 6-phosphate dehydrogenase method (23). The final reaction mixture (total volume 2.0 ml) contained 10 mM D-glucose, 5 μM NADP, 1.5 mM $MgCl_2$, 0.6 mM EDTA, 12.5 mM triethanolamine HCl (pH 8.0), and up to 0.1 ml of the acid extract or standard ATP solution. The reaction was started by addition of 0.01 ml of glucose 6-phosphate dehydrogenase (125 U/ml) and 0.03 ml of hexokinase (132 U/ml). After 15 min at 37°C, the increase in fluorescence (excitation at 365 nm, emission at 460 nm) was proportional to the amount of ATP in the range of 0–15 nmol. If nucleotide content is expressed per tissue water, concentrations would be higher; however, tissue levels of NAD and ATP are expressed per wet weight as in most other reports (19, 22).

AlPase was assayed in principle as described previously (15, 16), at 20°C, pH 8.5, and using *p*-nitrophenyl phosphate as substrate in a medium identical to that used for the Pi transport studies (16). Plasma and urine content of Pi was measured by the method of Chen et al. (24), and plasma and urine creatinine (Cr) were determined colorimetrically (25). calcium (Ca) in plasma and urine samples was measured by an atomic absorption spectrophotometer (Perkin Elmer Corp., Norwalk, Conn., model 303), and sodium (NA^+) and potassium (K^+) were determined by flame photometry (Instrumentation Laboratory Inc., Lexington, Mass., model 143) as in previous studies (15, 26). Protein was determined by the method of Lowry et al. (27) after solubilization of the samples in 1% sodium lauryl sulfate, as in our previous studies (5, 15).

In preliminary experiments, we determined that neither NiAm or nicotinic acid interfered with assays of metabolites, colorimetric measurement of Pi and Cr, or radioimmunoassay of cAMP.

All analyses were run in duplicates or triplicates, and all enzyme assays were carried out under conditions in which the reaction rate was linear with respect to both time and protein content. These conditions were established in preliminary experiments. Animals on different diets or treated with drugs were always processed simultaneously with controls. Likewise, all analyses, assays, and transport measurements were done simultaneously on control and experimental material.

Basically, in experiments examining the effect of the addition of NAD and other agents in vitro (time-course, concentration-dependence), replicate assays were done on BBM prepared from pooled renal cortex of several kidneys. Therefore, Tables I–III and Fig. 1 represent data from different experiments; each of these experiments used on BBM preparation from the pooled renal cortices of 5–6 rats, and in each experiment the measurements were made in triplicate; experiments were done more than once. All data presented in Tables II, IV, V, and VII, and in Fig. 2 are the mean ± SE of results obtained from different animals. Thus, in these experiments n denotes for statistical purposes the number of animals; each animal was processed separately and all measurements (urinary values, nucleotide content, and transport measurements) on each animal were made in duplicate or triplicate. Controls and NiAm-treated animals were processed in parallel on the same day. The results were evaluated statistically by Student's t test for group comparisons. Values of $P > 0.05$ were considered not significant.

Materials. ^{32}P-phosphate and $^{22}NaCl$ were purchased from

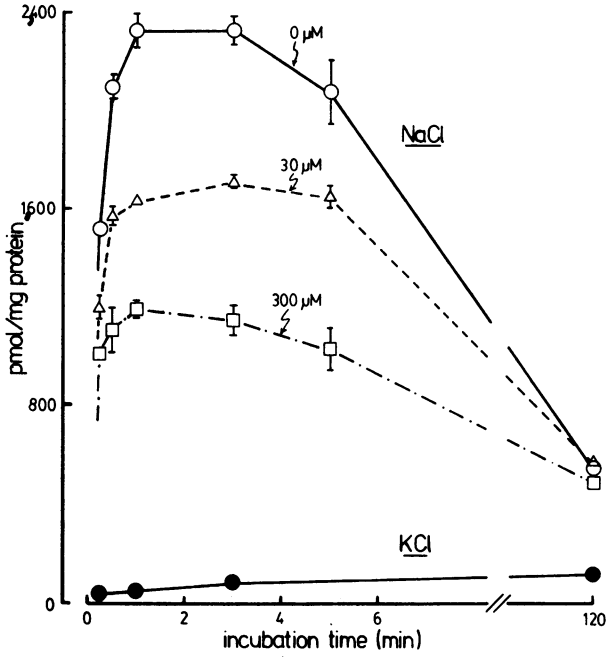

FIGURE 1 Time-course of ^{32}P-phosphate uptake by rat renal BBM vesicles: effect of NAD$^+$ in vitro. Uptake was determined as described under Methods by incubating the BBM vesicles in buffered 100 mM mannitol containing 100 mM NaCl; the final concentration of ^{32}Pi was 0.1 mM: BBM vesicles were incubated either without NAD$^+$ (○ —— ○), or with NAD$^+$ at 30 μM (△ — — △) or at 300 μM (□ -·- □). Closed symbols (● —— ●) represent the ^{32}Pi uptake by BBM when NaCl in the incubation medium was replaced by 100 mM KCl (Na$^+$-independent Pi uptake). Na$^+$-independent uptake of ^{32}Pi in the absence of NAD$^+$, or measured in the presence of 30 or 300 μM NAD$^+$, did not differ significantly and falls into the same points and line. Values are the mean ±SE of triplicate determinations.

New England Nuclear, Boston, Mass.; and 6-D-[^{3}H]glucose was from Amersham Corp. Arlington Heights, Ill. Radioimmunoassay kits for cAMP measurements including antibodies and ^{125}I-labeled antigens, were purchased from B-D Immunodiagnostics Becton, Dickinson & Co., Orangeburg, N. Y. Lactate dehydrogenase was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and all other biochemicals, of the highest purity grades, were purchased from Sigma Chemical Co., St. Louis, Mo. Unless specified otherwise, stereoisomer β-NAD was used in our experiments.

RESULTS

The potential effect of NAD on BBM transport of Pi was studied both in vitro by examining the direct action of NAD on isolated renal BBM, and in vivo by maneuvers that change the intracellular level of NAD in renal tissue.

In vitro studies. The major portion of ^{32}Pi uptake by renal cortical BBM vesicles was dependent on the presence of a transmembrane Na$^+$ gradient (1, 2, 5, 15), and the uptake reached a maximum within 1–2 min (Fig. 1), the "overshoot" phase (1, 5, 15). Addition of 30 μM NAD$^+$ to isolated BBM vesicles caused a marked decrease in Na$^+$-dependent ^{32}Pi uptake in the whole "overshoot" phase, and this effect was even more pronounced with 300 μM NAD$^+$ (Fig. 1). In subsequent experiments, ^{32}Pi uptake was measured in the initial "uphill" phase, at time intervals <1 min. Inhibition of Na$^+$-dependent BBM uptake of ^{32}Pi by NAD$^+$ was dose dependent; in the concentration range 10–300 μM NAD$^+$, there was a progressive decrease in the initial ^{32}Pi uptake, and at 300 μM NAD$^+$ the inhibition reached was ~50% (Table I). Increasing the NAD$^+$ concentration above 300 μM produced no further inhibition of ^{32}Pi uptake. NADH, the reduced form of NAD,

TABLE I
Dose-dependent Effect of NAD (NAD$^+$ and NADH) on Initial Uptake of ^{32}P-Phosphate and D-[^{3}H]Glucose by BBM Vesicles In Vitro

Concentration of NAD$^+$ or NADH added	^{32}P-Phosphate uptake		D-[^{3}H]Glucose uptake	
	NAD$^+$	NADH	NAD$^+$	NADH
μM	pmol/mg protein		pmol/mg protein	
0 (Control)	1,792±53*		138±6	
10	1,616±40	1,789±24	—	—
30	1,291±22‡	1,363±62‡	142±13	158±23
100	1,295±14‡	1,227±44‡	—	—
300	903±65‡	949±83‡	154±3	133±6

Initial uptake was measured in the presence of a Na$^+$ gradient at 30 s for ^{32}Pi, and at 15 s for D-glucose; see Methods for details. Na$^+$-independent uptake (when NaCl in the incubation medium was replaced by KCl) of ^{32}Pi and D-[^{3}H]glucose was 17±2 and 13±1 pmol/mg protein, respectively, and was not affected by either NAD$^+$ or NADH at concentrations 10–300 μM.

* Mean±SEM of triplicate determinations.

‡ Values significantly different ($P < 0.01$ or higher level of significance; group t test) compared with controls (the uptake in the absence of NAD$^+$ or NADH).

TABLE II

Effects of In Vitro Addition of NAD (as NADH), Arsenate, and Phlorizin on Initial Uptake of ^{32}P-Phosphate and D-[^{3}H]Glucose by BBM Vesicles Prepared from Rats Fed Either LPD or HPD for 4 d

Conditions	^{32}P-Phosphate uptake		D-[^{3}H]Glucose uptake	
	LPD	HPD	LPD	HPD
	pmol/mg protein		*pmol/mg protein*	
No additions	3,130±31*	1,134±22§	221±12	278±16§
NADH (10 μM)	3,222±167	1,069±26§	—	—
NADH (30 μM)	2,299±168‡	843±16§	—	—
NADH (100 μM)	1,974±59‡	778±43†§	—	—
NADH (300 μM)	1,647±117‡	482±6†§	188±12	248±9§
Arsenate (1 mM)	647±32‡	281±18†§	—	—
Phlorizin (250 μM)	—	—	9±2‡	8±2‡

Initial uptake was measured in the presence of a Na^+ gradient at 30 (for ^{32}Pi) or 15 s (for D-[^{3}H]glucose); for details see Methods. Na^+-independent uptake was not altered by the dietary Pi intake (data not shown, see also references 3 and 5). BBM preparations from both dietary groups were prepared and tested on the same day.

* Mean±SEM of triplicate measurements.

‡ Values significantly ($P < 0.01$ or higher level of significance; group t test) different from control (no additions).

§ Values significantly different ($P < 0.05$ or higher level of significance; group t test) compared with uptake at same NADH (or arsenate) concentration in LPD group.

caused inhibition of BBM transport of ^{32}Pi in a similar manner to NAD^+ (Table I). In the absence of Na^+ (when NaCl in medium was replaced by KCl), no inhibitory effect of NAD on ^{32}Pi uptake by BBM was observed (Fig. 1, Table I). When NAD was added without preincubation, the inhibition was present, but less extensive than with preincubation. Likewise, NAD effect appeared to be reversible by removing nucleotide from incubation medium (preliminary observations).

To determine whether this effect of NAD^+ was specific for BBM transport of Pi, uptake of D-glucose was measured in the same BBM preparations in parallel with that of Pi. Unlike the ^{32}Pi transport system, NAD had no inhibitory effect upon Na^+-dependent BBM uptake of D-[^{3}H]glucose (Tables I and II). To ascertain further that the inhibitory effect of BBM transport of Pi was not due to dissipation of the Na^+ gradient across BBM, the effect of NAD on uptake of $^{22}Na^+$ by BBM vesicles was examined as described in Methods. NAD^+ or NADH in the concentration 300 μM, which inhibited maximally BBM transport of ^{32}Pi, had no significant effect on $^{22}Na^+$ uptake by BBM vesicles at any point in the course (0.5–15 min) of $^{22}Na^+$ uptake by BBM (data not shown).

NiAm and adenosine are major structural components of NAD, and because NiAm was used in subsequent experiments in vivo to increase the level of NAD in renal tissue, we tested NiAm and adenosine in vitro for direct effects on ^{32}Pi uptake by BBM. Whereas NAD^+ and NADH inhibited Pi transport as in other experiments (Tables I and II), NiAm or adenosine in equimolar concentrations (30 or 300 μM) had no effect on the rate of initial Na^+-dependent transport of ^{32}Pi across BBM vesicle isolated from the renal cortex (data not shown). Effect of NAD stereoisomer, α-NAD (28), was compared with that of β-NAD under the same conditions and in the same concentrations (30 and 300 μM). The Na^+-dependent BBM uptake of ^{32}Pi was inhibited to similar extents by equimolar concentrations of α-NAD^+ and natural nucleotide β-NAD (data not shown).

The inhibitory effect of NAD Pi transport was examined in the BBM preparation from kidneys of rats fed either LPD or HPD, which differ markedly in the rate of BBM uptake of Pi (16). In BBM prepared from both LPD rats and HPD rats, NADH elicited a similar dose-dependent inhibition of BBM uptake of ^{32}Pi, but had no effect on uptake of D-[^{3}H]glucose (Table II). Although the relative degree of ^{32}Pi transport inhibition by NAD, compared with that of controls, was similar in BBM vesicles from both LPD and HPD groups, reaching a maximum inhibition of about 50% (Table II), the net decrease in the rate of ^{32}Pi uptake caused by equimolar NAD concentrations was markedly greater in BBM vesicles with a high rate of ^{32}Pi transport prepared from LPD rats (Table II). Inhibitory potency of NAD is, on a molar basis, comparable to inhibition of ^{32}Pi uptake by arsenate (Table III), a known inhibitor of BBM transport of Pi (1, 16). Lack of inhibition of D-[^{3}H]-glucose transport across BBM (Table I) from either

TABLE III

Comparison of Effects of Various Doses of NAD (as NADH) and Arsenate on Initial Uptake of ^{32}P-Phosphate by BBM Vesicles In Vitro

Additions	^{32}P-Phosphate uptake
	pmol/mg protein
None (control)	1,378±26*
NADH (30 μM)	1,021±23‡
NADH (100 μM)	973±3‡
NADH (300 μM)	755±22‡
Arsenate (30 μM)	1,130±22‡
Arsenate (100 μM)	1,098±20‡
Arsenate (300 μM)	756±10‡

Initial uptake was measured in the presence of Na^+ gradient at 0.5 min, for details see Methods.

* Mean±SEM of triplicate measurements.

‡ Values significantly ($P < 0.01$ or higher level of significance; group t test) different from control (no additions).

LPD or HPD rats (Table II) is not due to some unusual insensitivity of the tested BBM preparations to inhibitors, since phlorizin, a specific inhibitor of D-glucose transport (29) elicited an expected inhibitory effect (Table II).

These in vitro results suggested that the inhibitory effect of NAD was specific for the Na^+-dependent component of the Pi transport system in renal BBM, and that the effect was due to direct interaction of NAD with the isolated BBM.

In vivo studies. Administration of large doses of NiAm, an NAD precursor (21, 22, 28), resulted in an increased level of NAD (mainly NAD^+) in the kidney (21, 30) and other tissues (21, 22, 28). Since NiAm added in vitro did not affect Pi uptake, injection of a large dose of NiAm was employed to increase the level of NAD^+ in renal tissue in vivo.

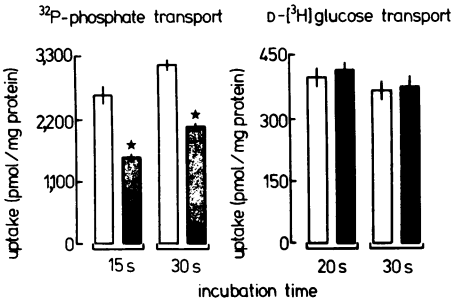

FIGURE 2 Uptake of ^{32}P-phosphate (left panel) and of D-[^{3}H]-glucose (right panel) by brush border membrane (BBM) vesicles from control rats (open columns) and rats injected with 1 g/kg body wt of NiAm (shaded columns). The BBM vesicles were prepared (as described under Methods) from the renal cortex of the same animals in which $U_{Pi}V$ and renal NAD^+ levels were determined (results given in Table IV). Each bar represents mean±SE of the same animals as in Table IV. The uptake was measured in the presence of a Na^+ gradient in the "overshoot" phase (time intervals 15–30 s) of the uptake; final concentration of ^{32}Pi was 0.1 mM, and that of D-[^{3}H]glucose, 0.05 mM (see Methods). *, Values significantly ($P < 0.001$; t test, $n = 3$) different from controls at the same time interval.

Rats were stabilized on LPD and were kept in metabolic cages for collection of urine samples from control and post-NiAm injection periods, as described in Methods. After the control period, the rats were given an intraperitoneal injection of NiAm (1 g/kg body wt), and the urine was collected for an additional 9 h. In rats injected with NiAm, the content of NAD^+ in the renal cortex markedly increased (Table IV), and the rate of ^{32}Pi uptake by BBM vesicles prepared from kidneys of the same NiAm-treated rats was mark-

TABLE IV

Effect of NiAm on $U_{Pi}V$ and on Tissue Level of NAD^+ in the Renal Cortex

	n	$U_{Pi}V$		NAD^+ content in cortex
		Control period	After injection	
		μmol/l h/100 g body wt		*nmol/g wet wt*
Controls	4	0.062±0.004*	0.033±0.012	426±28
NiAm-injected	4	0.054±0.003	6.871±1.192‡	1573±23‡

After a control period of 14 h rats were given an intraperitoneal injection of either NiAm (1 g/kg body wt) or vehicle solution. Urine was collected 9 h after injection, then the rats were killed. Part of the renal cortex was taken for determination of NAD^+; the rest of the renal cortex was used for the BBM preparation employed in transport measurements, the results of which are portrayed in Fig. 2. All animals were stabilized on LPD before the start of the control period (for further details see text). n, number of animals.

* Mean±SE.

‡ Values significantly different from control rats and, in case of $U_{Pi}V$, also different from the value before NiAm injection ($P < 0.005$; group t test).

TABLE V

Reversibility of Phosphaturic Effect of NiAm in Rats Maintained on LPD

Parameter	Day 1	Day 2	Day 3	Day 4
Phosphate excretion ($U_{Pi}V$), *μmol/24 h/100 g body wt*				
Control	16.7±9.6*	5.0±3.2	1.0±1	1.3±0.4
Experimental	8.7±6.7	228.0±77.9§	10.7±5.1	1.7±0.4
P‡	NS	<0.05	NS	NS
Urine flow, *ml/24 h*				
Control	13.2±1.7	12.3±1.1	12.2±2.1	13.0±3.9
Experimental	16.3±1.9	37.4±6.5	21.6±4.2	20.8±3.0
P‡	NS	NS	NS	NS
Creatinine excretion ($U_{Cr}V$), *mg/24 h/100 g body wt*				
Control	3.40±0.72	3.39±0.12	3.69±0.15	3.18±0.27
Experimental	3.12±0.18	3.40±0.11	3.19±0.55	3.86±0.07
P‡	NS	NS	NS	NS
Sodium excretion ($U_{Na}V$), *μeq/24 h/100 g body wt*				
Control	1,244±106	1,384±107	906±62	675±107
Experimental	1,065±78	628±129	670±72	778±69
P‡	NS	<0.005	NS	NS
Potassium excretion ($U_K V$), *μeq/24 h/100 g body wt*				
Control	1,191±78	1,506±78	1,028±48	1,067±460
Experimental	1,066±31	1,230±117	830±106	810±100
P‡	NS	NS	NS	NS
Calcium excretion ($U_{Ca}V$), *mg/24 h/100 g body wt*				
Control	9.1±2.6	15.5±4	14.9±2.6	7.8±1.6
Experimental	11.8±2.0	2.5±0.9	9.1±3.0	10.9±2.4
P‡	NS	<0.02	NS	NS

All animals were stabilized on LPD prior to the start day 1. At the start of day 2, experimental rats were injected intraperitoneally with NiAm (1 g NiAm/kg body wt). Rats in control and experimental groups (4 rats/group) were pair-fed and age and sex matched.

* Mean±SEM.

‡ For significance of difference between values of control and experimental groups on the same day (group *t* test).

§ Significantly different from values for the same group on day 1 and day 3 ($P < 0.05$; group *t* test).

edly lower than in controls (Fig. 2), but uptake of D-[^{3}H]glucose was not different between NiAm-injected and control rats (Fig. 2). In addition, injection of NiAm caused a dramatic 100–200-fold increase in urinary excretion of Pi, compared with the control period or with the vehicle-injected rats (Table IV).

Specificity in terms of electrolyte excretion and reversibility of the phosphaturic effect of NiAm were explored in the following experiment. Rats were stabilized on LPD, urine was collected at 24-h periods, and urinary excretion of Pi ($U_{Pi}V$), sodium ($U_{Na}V$), potassium ($U_K V$), calcium ($U_{Ca}V$) and creatinine ($U_{Cr}V$) was monitored daily (Table V). Injection of NiAm caused a huge increase in $U_{Pi}V$ and some increase in urine flow; there were no differences between control and NiAm-injected rats in $U_{Cr}V$ and $U_K V$; on the other hand, $U_{Ca}V$ and $U_{Na}V$ decreased (Table V). NiAm injection did not cause glycosuria. On the day after NiAm injection (day 3), $U_{Pi}V$ returned to a level that was not significantly different either from $U_{Pi}V$ in the same rats before NiAm injection (day 1) or from $U_{Pi}V$ in the control rats on day 3 (Table V). In a control experiment using the same design as that described in Table IV, the effect of NiAm on plasma creatinine (P_{Cr}), plasma Pi (P_{Pi}), and plasma calcium (P_{Ca}) was determined. NiAm-injected rats with marked phosphaturia ($U_{Pi}V$ was more than 40 times higher than in controls) did not differ significantly from vehicle-treated controls in P_{Pi}

TABLE VI

Effects of Various Doses of NiAm Injected In Vivo on BBM Transport, Tissue Levels of Nucleotides, and Pi Excretion

	Dose of NiAm (g/kg body wt)			
	0	0.25	0.50	1.00
Transport in BBM				
Phosphate uptake, *pmol/mg protein at 30 s*				
NaCl in medium	2,740±92	2,673±289	1,828±165*	1,672±134*
KCl in medium	68±18	53±20	95±23	43±13
Glucose uptake, *pmol/mg protein at 15 s*				
NaCl in medium	325±16	324±5	341±12	373±44
KCl in medium	37±6	43±3	43±5	32±2
$^{22}Na^+$ uptake, *nmol/mg protein at 1 min*	61±4	61±2	58±5	50±4
n	6	3	5	4
Content of nucleotides in cortex				
NAD^+, *nmol/g tissue*	527±44	969±58*	1,715±252*	2,056±150*
NADH, *nmol/g tissue*	223±17	217±24	313±21*	324±10*
NAD^+/NADH ratio	2.42±0.25	4.58±0.65*	5.44±0.56*	6.36±0.43*
ATP, *nmol/g tissue*	641±84	668±20	781±45	669±144
cAMP, *pmol/mg protein*	7.62±0.64	7.44±0.58	7.46±0.36	7.45±0.65
n	6	3	5	4
Urine values				
$U_{Pi}V$, *μmol Pi/l h/100 g body wt*	0.021±0.006	0.097±0.008	2.606±1.146*	4.066±0.938*
$U_{Cr}V$, *mg/l h/100 g body wt*	0.17±0.01	0.15±0.02	0.19±0.03	0.13±0.02
n	6	3	5	4

Rats were stabilized on LPD and TPTX 2 d prior to the experiment. Urine was collected for 9 h after injection of NiAm or vehicle solution. At the end of the collection period the animals were killed, and renal cortical tissue was taken for preparation of BBM vesicles for transport studies and for analysis of nucleotides. Content of NAD^+, NADH, and ATP is expressed relative to the wet weight of tissue. For further details, see Methods. *n*, number of animals; each rat was processed and analyzed separately. All values are mean±SE.

* Values significantly different from controls (no NiAm injected); $P < 0.05$ or higher degree of significance (group *t* test).

or P_{Cr} determined in plasma obtained at the end of the urine collection period; P_{Ca} was significantly lower than in controls (data not shown). NiAm injection did not cause glycosuria.

To determine the relationships among NiAm-induced changes in transport processes in the BBM, changes in the content of NAD in kidney cortical tissue, and changes in $U_{Pi}V$, various doses (0.25–1.0 g/kg body wt) of NiAm were injected into rats stabilized on LPD and TPTX 2 d before NiAm injection. The design of the experiment was analogous to that described in Table IV. The rate of Na^+-dependent ^{32}Pi transport in BBM isolated from the kidney cortex of NiAm-injected animals declined, and the extent of this decrease was more pronounced with higher doses of NiAm (Table VI). In contrast, there were no differences between control and NiAm-treated animals in BBM uptake of ^{32}Pi in the absence of a Na^+ gradient (when NaCl in the incubation medium was replaced by KCl) measured in the same BBM preparation in which Na^+-dependent ^{32}Pi uptake was decreased. In BBM vesicles from NiAm-injected rats, the Na^+-dependent and Na^+-independent (KCl medium) uptake of D-[^{3}H]glucose and the uptake of $^{22}Na^+$ did not differ significantly from the controls (Table VI). Injection of NiAm also caused elevation of the tissue NAD^+ level, and the extent of the NAD^+ increase was basically proportional to the dose of injected NiAm (Table VI). Renal cortical NADH content increased after injection of 0.5 or 1.0 g/kg NiAm but to a much lesser extent than the increase in NAD^+. This increase in NADH, although statistically significant, was relatively minor (maximum change, 45%) compared with the increase in NAD^+ (maximum change, 290%). Consequently, the tissue NAD^+/NADH ratio progressively increased from 2.4 to 6.4 in response to NiAm injection (Table VI). Tissue levels of ATP and of cAMP were determined in aliquots of the same cortical tissue samples in which NAD^+ and NADH were measured, and showed no difference between control rats and rats injected with NiAm (Table VI).

The rate of the Na^+-dependent uptake of ^{32}Pi by BBM vesicles was inversely proportional to both the increase in renal cortical content of NAD^+ ($r = -0.77 \pm 0.10$;

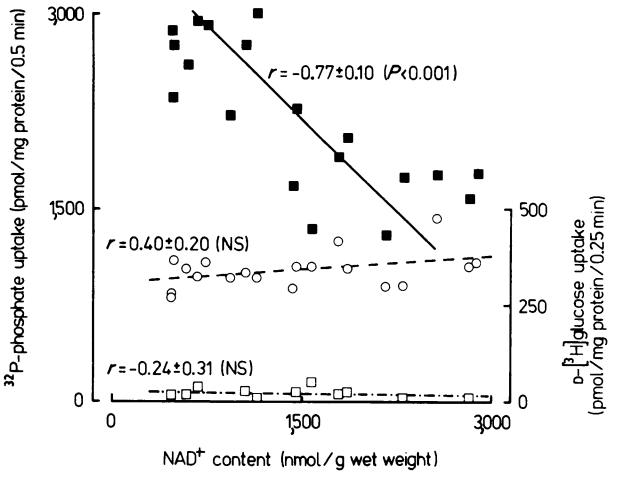

FIGURE 3 Depiction of relationship between NAD^+ content of rat renal cortex and rate of ^{32}P-phosphate and D-[^3H]glucose uptake by BBM vesicles. Separate portions of the renal cortex from one rat were used for measurement of tissue NAD^+ and for preparation of BBM. Cortical tissue was taken from control rats and from rats given various doses of NiAm 9 h previously (see Methods and Table VI). Each point is the value from one rat, and the symbols represent the initial Na^+-dependent BBM uptake of ^{32}Pi (■ — — ■) measured at 30 s (n = 19), Na^+-dependent BBM uptake of D-[^3H]glucose (○ — — ○) measured at 15 s (n = 19), and BBM uptake of ^{32}Pi independent of Na^+ (KCl replaced NaCl in medium) (□ -·- □) at 30 s (n = 11). $P > 0.05$, NS.

$P < 0.001, n = 19$; see Fig. 3) and to the increase in the NAD^+/NADH ratio ($r = -0.72 \pm 0.11$; $P < 0.001$, $n = 19$), but the Na^+-independent uptake of ^{32}Pi and the uptake of D-[^3H]glucose were not significantly correlated with changes in NAD^+ or the NAD^+/NADH ratio (Fig. 3).

As before (Table V), NiAm treatment led to increased $U_{Pi}V$ but caused no significant change in $U_{Cr}V$ (Table VI). When $U_{Pi}V$ was related to biochemical and transport parameters, $U_{Pi}V$ was inversely correlated with the Na^+-dependent ^{32}Pi uptake ($r = -0.67 \pm 0.13$; $P < 0.01$, $n = 19$) and positively correlated with both renal cortical NAD^+ ($r = +0.88 \pm 0.05$; $P < 0.001$, $n = 19$) and the NAD^+/NADH ratio ($r = +0.80 \pm 0.08$; $P < 0.001, n = 19$). On the other hand, $U_{Pi}V$ was not significantly correlated with the Na^+-independent uptake of ^{32}Pi, and the rather loose positive correlation between $U_{Pi}V$ and the Na^+-dependent uptake of D-[^3H]glucose ($r = +0.49 \pm 0.17$; $P < 0.05$, $n = 19$) was opposite to the close inverse correlation between $U_{Pi}V$ and ^{32}Pi uptake.

In the next experiment, rats were kept on normal phosphate diet (NPD), surgically TPTX 2 d before the experiment, and injected with NiAm in a dose 1 g/kg, with a similar time schedule to those in previous experiments (in which animals were stabilized on LPD). Before NiAm injection, $U_{Pi}V$ in TPTX animals fed NPD (Table VII) was significantly ($P < 0.05$) higher than in TPTX rats kept on LPD (Table VI). However, even in animals fed NPD, NiAm injection caused a striking phosphaturia, with no change in P_{Pi} (Table VII). Injection of NiAm to NPD animals was also associated with an increase in NAD^+, and to a lesser degree, an increase in NADH. As in the previous experiment (Table VI), no changes in the tissue levels of cAMP or ATP were observed after NiAm treatment (Table VII).

Renal cortical homogenate was fractionated, as de-

TABLE VII
Phosphaturic Effect of NiAm in TPTX Rats Fed NPD (0.7% Pi)

	Control	Experimental	*P*-value*
Plasma and urine values			
$U_{Pi}V$, *μmol/l h/100 g body wt*			
A	0.94±0.48	1.25±0.45	NS
B	0.40±0.17	6.04±1.21	<0.005
Plasma, Pi, *mM*	3.6±0.4	3.1±0.4	NS
Nucleotide content in renal cortex			
NAD^+, *nmol/g tissue*	702±114	2,534±697	<0.005
NADH, *nmol/g tissue*	138±23	340±46	<0.01
ATP, *nmol/g tissue*	525±43	592±25	NS
Cyclic AMP, *pmol/mg protein*	4.35±0.52	2.92±0.34	NS

Timing and experimental design were analogous to that described in the legends to Tables IV and VI; for details see text. Experimental animals (n = 4) received 1 g/kg i.p. of NiAm and controls (n = 4) received vehicle. $U_{Pi}V$ was determined prior to A and after B injection of NiAm or vehicle solution. At the end of the experiment, plasma and renal cortex were taken for analyses; for details see Methods. All data are mean±SE.

* Significance of difference between controls and experimental values (group t test).

scribed in detail in Methods, to cytosolic fraction (100,000-*g* supernate) and total particulate fraction (100,000-*g* pellet), and NAD^+ content was determined. The majority of NAD^+ (>75%) was found in cytosol (data not shown).

DISCUSSION

Studies in vitro show that NAD added directly to isolated rat renal BBM vesicles inhibits dose-dependently the Na^+-dependent ^{32}Pi uptake, and its specificity is underscored by the observation that NAD does not influence D-glucose transport or Na^+-independent Pi transport (Fig. 1, Table I). The lack of an effect of NAD on $^{22}Na^+$ uptake further indicates that inhibition of ^{32}Pi transport is not due to dissipation of the Na^+ gradient. NAD inhibits Na^+-dependent ^{32}Pi uptake by BBM with similar relative (percent inhibition) effectiveness regardless of whether the rate of Pi transport was enhanced by LPD or lowered by HPD (16) (Table II). Since NiAm and adenosine added in vitro were without effect on BBM uptake of ^{32}Pi, this observation suggests that NiAm administered in vivo (Fig. 2, Table VI) did not cause changes in BBM transport of ^{32}Pi by direct action on the BBM.

The similarity of in vitro effects of NAD^+ and NADH suggests that the action of NAD on BBM uptake of ^{32}Pi may not depend on the redox status of the NAD^+/NADH system. However, it is not precluded that one form of NAD is converted to the other at the BBM, prior to its action on the intramembranous site of the ^{32}Pi uptake mechanism in BBM. Findings that β-NAD and α-NAD had a similar effect, suggest that NAD action on BBM is not stereospecific (28), as are some other effects of NAD (11, 12). Our preliminary experiments show that NAD action does not follow a pattern of competitive inhibition, and that binding of radiolabeled NAD onto BBM is independent of the presence of Na^+ gradient (31), suggesting that NAD interaction with BBM may be complex (Kempson and Dousa, unpublished results). Detailed characteristics of NAD interaction with BBM remain to be explored in future systematic studies. Although it is not precluded that nucleotides and coenzymes other than NAD might possibly have a similar effect on BBM transport of Pi when added to BBM in vitro, there presently is no indication—by the nature of their localization in the cell, concentration in the cell, etc.—that other coenzymes play in vivo a similar role to that proposed for NAD.

Most BBM vesicles are oriented rightside-out (32). Although the intra-membranous site of NAD action on BBM will require systematic examination, some considerations suggest indirectly that NAD acts on the inner portion of the BBM membrane. Sidedness of BBM and its components is presently not amenable to examination in a direct way (32), but our finding that NAD inhibits AlPase activity located in isolated BBM in a similar way[1] to NAD inhibition of the purified enzyme isolated from the BBM structure (10), suggests that NAD could penetrate deep into the BBM structure when added in vitro.

The proposition that NAD can act from cell interior on BBM on (or close to) the cytoplasmic side is in basic agreement with observations in vivo (Table VI, Figs. 2 and 3), which show that BBM capacity for ^{32}Pi uptake changes in parallel with tissue NAD levels. Also, NAD inhibited BBM transport of Pi in vitro in a concentration range which is, in general, close to tissue levels of NAD found in renal cortex (Tables IV, VI, and VII) (30). It should be realized that such comparisons are approximate; actual concentrations of NAD^+ (or NADH) in cytoplasm in the vicinity of BBM are not yet known. To determine whether NAD inhibits ^{32}P transport across BBM also in vivo, we used parenteral administration of NiAm, which elevates tissue NAD both by increasing de novo biosynthesis of NAD (28, 34) and by inhibiting NAD breakdown (21, 28).

Injection of NiAm in vivo, which increased renal cortical NAD^+ (Tables IV, VI, and VII), had effects on BBM virtually identical to the effects of direct NAD addition to BBM in vitro. Na^+-dependent BBM uptake of ^{32}Pi was inhibited, but BBM uptake of D-[^{3}H]glucose, BBM uptake of $^{22}Na^+$, and the Na^+-independent BBM uptake of ^{32}Pi were not changed. The most plausible interpretation of these findings is that the Na^+-dependent BBM uptake of ^{32}Pi was inhibited by increased intracellular NAD, namely NAD^+, in the cytoplasm of tubular cells. Although the effect of NAD added in vitro on BBM transport resembles effects of changing NAD in vivo, the exact biochemical mechanism of these interactions remains to be determined, and in particular, the causal relationship between changes in NAD^+ content and BBM transport of Pi needs to be established.

Some limitations in these experiments should be

[1] To ascertain whether NAD inhibits AlPase activity located in the core of BBM (33), as reported for AlPase extracted and purified from pig kidney (10), the effect of NAD on AlPase activity in freshly prepared rat BBM vesicles was tested under conditions similar to those used for measurement of Pi uptake by BBM. Indeed, AlPase activity in BBM in the presence of 300 μM NAD^+ (11.9±0.9 μmol/h per mg protein was significantly ($P < 0.05$; $n = 3$) lower than the activity when no NAD was added (14.9±0.5 μmol/h per mg protein). NAD not only inhibited AlPase in BBM, but the pattern of the inhibitions in BBM was similar to the reported inhibition of purified isolated AlPase preparations as well (10). For example, NAD^+ at 300 μM inhibited AlPase in BBM by 20.1% (see above), less than did NADH (46.9% inhibition) in the same concentration; NADH was also a more potent inhibitor than NAD^+ when tested on the purified enzyme (10).

acknowledged. NAD^+ and other nucleotides were measured in the whole kidney cortex, which contains structures other than proximal tubules. However, since the proximal tubules account for the majority of the renal cortical mass (35), present measurements likely approximate NAD content in proximal tubules. Predominant cytoplasmic localization of NAD has been demonstrated in other tissues (21, 22, 28) and confirmed here for kidney cortex. It is thus likely that our measurements of cortical tissue contents of NAD^+ in response to NiAm reflect mostly changes in NAD^+ in the cytoplasm of proximal tubular cells.

NiAm administration had no effect on the levels of ATP[2] or cAMP in the renal cortex (Tables VI and VII), two other cellular factors which are currently believed to be involved in proximal tubular transport and its regulation (8, 36). Although it is realized that ATP levels alone provide limited information about active transport, these results suggest that changes in renal tubular handling of Pi elicited by NiAm administration (Tables IV, V, and VI) are not due to an altered supply of metabolic energy (in the form of ATP) for transport of Pi and are not due to the regulatory effect of intracellular cAMP (37).

The experiments on rats fed NPD indicate (Table VII) that feeding with LPD is not a requisite for the NiAm effect on $U_{Pi}V$ and on the tissue level of NAD^+. Since phosphaturia was elicited by NiAm in animals stabilized on LPD (Tables IV–VI), it is unlikely attributable to extracellular volume expansion or acute acidosis, since LPD rats are resistant to these nonhormonal phosphaturic stimuli (8). Rats stabilized on LPD are resistant to the phosphaturic effect of PTH (8). Observations that NiAm causes phosphaturia in TPTX rats fed either NPD (Table VII) or LPD (Table VI) argue against the possibility that NiAm acted through release of hormones from parathyroid and thyroid tissues, or by sensitizing kidney to endogenous PTH (39).

The phosphaturic effect of NiAm is very specific for Pi, since no other excretory parameter increased except $U_{Pi}V$ (Table V). GFR was assessed only indirectly in our metabolic studies by measuring $U_{Cr}V$ or P_{Cr} (5, 15), but inasmuch as NiAm administration did not change $U_{Cr}V$ (Tables V, VI) or P_{Cr}, and had no significant influence on P_{Pi} (Table VII), these observations suggest that the increase in $U_{Pi}V$ was due to decreased Pi reabsorption by tubules, as also indicated by clearance studies, when the fractional excretion of Pi increased after NiAm (39). Close correlation between the decrease in Pi uptake by BBM and the increase in $U_{Pi}V$, consequent to NiAm treatment (Fig. 3), suggests that tubular Pi reabsorption decreases mainly, if not exclusively, in the proximal tubule, the only segment having luminal BBM. However, the present data do not exclude the possibility that NiAm changes Pi reabsorption also in more distal nephron segments. Reversibility of the phosphaturic effect of single injection of NiAm (Table V) seems to be consistent with the expected biochemical mechanism of NiAm action, since a single injection of NiAm was shown to cause transient elevation of the intracellular NAD^+ in nonrenal tissues (21, 22, 28), but the exact time-course of renal effects of NiAm remains to be established. Also, the source of excreted Pi remains to be determined. Since P_{Pi} did not decrease significantly and $U_{Ca}V$ declined (Table V), these features may suggest indirectly that Pi was mobilized from tissues other than skeleton.

In evaluation of the present findings, it should be realized that correlations and associations between BBM transport parameters, tissue nucleotides, and excretory parameters do not necessarily imply cause-effect relationships; causality in these relationships has to be tested in future studies.

The question arises whether NAD^+ could serve in the physiologic control of renal tubular Pi transport. First, it should be emphasized that cytoplasmic pool NAD, rather than mitochondrial NAD, is more likely to regulate BBM uptake of ^{32}Pi in vivo, since the cytoplasmic NAD is accessible for direct contact with BBM. Moreover, NAD^+ rather than NADH is likely available for interaction with BBM in vivo. The ratio of free NAD^+/free NADH in cytoplasm of renal tissue was indirectly estimated to be more than 100:1 (40), a value much higher than NAD^+/NADH ratio in mitochondria (40) or than the NAD^+/NADH ratio measured in the whole tissue (30) (Table VI). NAD^+ is much less (about 100 times less) tightly bound to cellular proteins (11, 13, 14) than NADH, which is specifically bound on NADH-dependent cytosolic dehydrogenases (14). Hence, NAD^+ is more available for intracellular shifts and for interaction with BBM.

In considering the potential physiologic role of cytoplasmic NAD^+ in regulation of Pi transport, one should recall the following points (Fig. 4). Some major phosphaturic stimuli, including PTH (acting via cAMP), stimulate gluconeogenesis (GNG) in renal cortex (42–44), a metabolic process expected to lead to increased

[2] Tissue content of ATP measured in the experiment portrayed in Tables VI and VII is lower than that reported for rat cortex obtained by instant *in situ* freezing (6, 38), and this feature is likely due to the short delay in freezing the tissue (38). However, since the timing in tissue sampling was the same for experimental and control animals, the results do indicate that no major differences in ATP levels were caused by NiAm, an observation analogous to findings on hepatic tissue (21) following NiAm administration. In the control experiment, renal cortical tissue for determination of ATP was obtained *in situ* by clamping the kidney in a stainless steel clamp precooled in liquid N_2 and immersing it into N_2. Content of ATP in renal cortex of control rats (1,167.7 ±67.8 nmol/g, mean±SEM, n = 3) and in NiAm-treated rats (1,165.2±41.4 nmol/g, mean±SEM, n = 3) was not different.

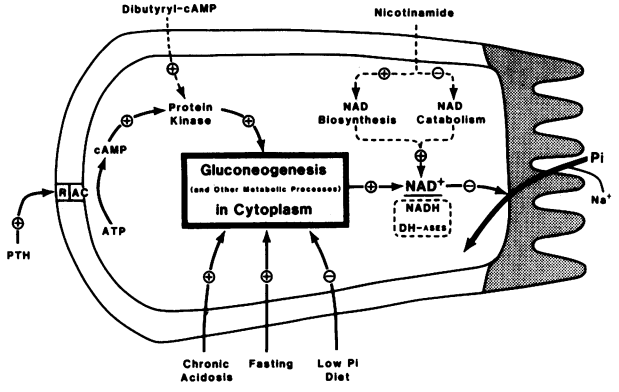

FIGURE 4 Schematic outline of hypothetical role of cytoplasmic NAD^+ in regulation of Pi transport across the proximal tubular cell in response to various phosphaturic stimuli and in response to NiAm and DBcAMP administration. R, receptor for PTH (and/or other hormones acting via cAMP); AC, adenylate cyclase; DH-ases, cytoplasmic dehydrogeneases using NAD^+/NADH as coenzyme system. —⊕→; positive effect (increase, stimulation); —⊖→; negative effect (decrease, inhibition). Under normal conditions the prevailing form of cytoplasmic NAD is NAD^+ (because less NADH is present), and, moreover, NADH is bound more tightly to DH-ases (denoted by interrupted circle) compared with NAD^+. Increased cytoplasmic gluconeogenesis causes an increase in NAD^+ (46), and consequent inhibition of Na^+-dependent Pi uptake across the BBM from the tubular lumen. GNG may be enhanced by metabolic stimuli which include fasting (45, 48) and chronic acidosis by NH_4Cl loading (41). In contrast, GNG may be depressed by feeding LPD (49). PTH (and other phosphaturic hormones) also enhances the rate of GNG (42, 44) and NAD^+ generation via an increase in cAMP, after stimulation of AC, and perhaps by activation of protein kinase (8, 37). Administration of DBcAMP bypasses hormone-dependent cAMP formation. NiAm administration increases NAD^+ both by increasing NAD biosynthesis (22, 28, 34) and by inhibiting NAD^+ breakdown (28, 21) by NAD glycohydrolase. Since NiAm bypasses all previous cellular steps that influence the NAD^+ level, its effect to increase NAD^+ inhibits Pi transport across BBM regardless of the preceeding status of cell metabolic and regulatory systems, e.g., depressed GNG in LPD rats (49).

generation of cytoplasmic NAD^+ from NADH at the glyceraldehyde 3-phosphate dehydrogenase step (45). The rate of GNG might be due to increased availability of substrates (43) or induction of key enzymes (45). GNG from pyruvate (47) and several key GNG enzymes are localized only in proximal tubules (48); activities of these GNG enzymes are enhanced in acidosis and starvation (48). This suggests that the potential to enhance NAD^+ levels via GNG (46) is limited to proximal tubules. In tubules isolated from LPD rats, the GNG rate was found to be decreased (49). It is tempting to speculate that lack of a response to PTH (or to DBcAMP) in animals fed LPD (7, 8) may be due to suppressed GNG (49), and consequently to insufficient generation of cytoplasmic NAD^+ needed to inhibit BBM uptake to Pi, in spite of an adequate supply of intracellular or extracellular cAMP. Such an explanation seems to be in agreement with our recent finding (39) which showed that infusion of PTH to rats maintained on NPD caused both phosphaturia and an increase in NAD^+/NADH ratio, whereas in rats on LPD, both $U_{Pi}V$ and NAD^+/NADH ratio failed to increase in the response to PTH (39). Pretreatment of LPD rats with NiAm, which increased renal cortical NAD^+, restored the phosphaturic response to PTH and increase in NAD^+/NADH ratio (39).

In conclusion, based on these multifaceted considerations we propose the hypothesis that NAD, specifically NAD^+, serves in the cytoplasm of proximal tubular cells as the final intracellular mediator of some, albeit not necessarily all hormonal and metabolic stimuli that regulate proximal tubular Pi reabsorption, by adjusting the rate of Pi uptake by the luminal BBM as schematically outlined in Fig. 4. We acknowledge that it remains an open question whether changes in other coenzymes and metabolites linked to NAD (14, 50) play a similar or contributory role in response to the phosphaturic stimuli or in the response to NiAm injection.

Finally, the unique phosphaturic effects of NiAm administration, even in the situation when the kidney is resistant to other phosphaturic stimuli, could be employed in experimental and clinical settings for testing the renal responsiveness, or for restoration of sensitivity (39) to those phosphaturic stimuli, e.g., PTH, which are presumably acting via NAD^+.

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REFERENCES

1. Murer, H., H. Stern, G. Burckhardt, C. Storelli, and R. Kinne. 1980. Sodium-dependent transport of inorganic phosphate across the renal brush border membrane. *Adv. Exp. Med. Biol.* **128**: 11–23.
2. Dousa, T. P., S. A. Kempson, and S. V. Shah. 1980. Adaptive changes in renal cortical brush border membrane. *Adv. Exp. Med. Biol.* **128**: 69–76.
3. Stoll, R., R. Kinne, H. Murer, H. Fleisch, and J-P. Bonjour. 1979. Phosphate transport by rat renal brush border membrane vesicles: influence of dietary phosphate, thyroparathyroidectomy, and 1,25-dihydroxyvitamin D_3. *Pfluegers Arch. Eur. J. Physiol.* **380**: 47–52.
4. Hammerman, M. R., and K. A. Hruska. 1980. Growth

hormone regulation of Na^+ gradient linked transport of phosphate in dog renal brush border membrane vesicles. *Clin. Res.* **28:** 349A. (Abstr.)
5. Kempson, S. A., S. V. Shah, P. G. Werness, T. Berndt, P. H. Lee, L. H. Smith, F. G. Knox, and T. P. Dousa. 1980. Renal brush border membrane adaptation to phosphorus deprivation: effects of fasting versus low-phosphorus diet. *Kidney Int.* **18:** 36–47.
6. Kreusser, W. G., K. Kurokawa, E. Aznar, and S. G. Massry. 1978. Phosphate depletion. Effect on renal inorganic phosphorus and adenine nucleotides, urinary phosphate and calcium and calcium balance. *Miner. Electrolyte Metab.* **5:** 30–42.
7. Beck, N., S. K. Webster, and H. J. Reineck. 1979. Effect of fasting on tubular phosphorus reabsorption. *Am. J. Physiol.* **237:** F241–F246.
8. Knox, F. G., A. Hoppe, S. A. Kempson, S. V. Shah, and T. P. Dousa. 1980. Cellular mechanisms of phosphate transport. *In* The Renal Handling of Phosphate. H. Fleisch and S. G. Massry, editors. Plenum Publishing Co., New York. 79–114.
9. Plante, G. E., C. Petitclerc, T. Nawar, and R. Erian. 1979. Increased phosphaturia during inhibition of renal alkaline phosphatase. *Miner. Electrolyte Metab.* **2:** 258–259. (Abstr.)
10. Ramasamy, I., and P. J. Butterworth. 1973. The inhibition of pig kidney alkaline phosphatase by oxidized or reduced nicotinamide-adenine dinucleotide and related compounds. *Biochem. J.* **131:** 359–367.
11. Kaplan, N. O., and R. H. Sarma. 1970. The structure of pyridine coenzymes as related to binding. *In* Pyridine Nucleotide-Dependent Dehydrogenases. H. Sund, editor. Springer Verlag, New York. 39–56.
12. Brady, R. O., and P. H. Fishman. 1979. Biotransducers of membrane-mediated information. *Adv. Enzymol. Relat. Areas Mol. Biol.* **50:** 303–320.
13. Williamson, D. H., P. Lund, and H. A. Krebs. 1967. The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver. *Biochem. J.* **103:** 514–527.
14. Bucher, T., and H. Sies. 1976. Mitochondrial and cytosolic redox states in perfused rat liver: methods and problems in metabolic compartmentation. *In* Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies. J. M. Tager, H. D. Soling, and J. R. Williamson, editors. North Holland Publishing Co., Amsterdam. 41–64.
15. Shah, S. V., S. A. Kempson, T. E. Northrup, and T. P. Dousa. 1979. Renal adaptation to a low phosphate diet in rats; blockade by actinomycin D. *J. Clin. Invest.* **64:** 955–966.
16. Kempson, S. A., and T. P. Dousa. 1979. Phosphate transport across renal cortical brush border membrane vesicles from rats stabilized on a normal, high or low phosphate diet. *Life Sci.* **24:** 881–888.
17. Burch, H. B., O. H. Lowry, and P. Von Dippe. 1963. The stability of triphosphopyridine nucleotide and its reduced form in rat liver. *J. Biol.Chem.* **238:** 2838–2842.
18. Aronson, P. S., J. P. Hayslett, and M. Kashgarian. 1979. Dissociation of proximal tubular glucose and Na^+ reabsorption by amphotericin B. *Am. J. Physiol.* **236:** F392–F397.
19. Klingenberg, M. 1974. Nicotinamide-adenine dinucleotides (NAD, NADP, NADH, NADPH); spectrophotometric and fluorimetric methods. *In* Methods of Enzymatic Analysis. H. U. Bergmeyer, editor. Academic Press, Inc., New York. 2nd edition. **4:** 2045–2059.
20. Northrup, T. E., P. A Krezowski, P. G. Palumbo, J. K. Kim, Y. S. F. Hui, and T. P. Dousa. 1979. Insulin inhibition of hormone-stimulated protein kinase systems of rat renal cortex. *Am. J. Physiol.* **236:** E649–E654.
21. Blake, R. L., and E. Kun. 1971. Methods of enzyme induction by nicotinamide. *Methods Enzymol.* **18:** 113–123.
22. Greengard, P., G. P. Quinn, and M. B. Reid. 1964. Pituitary influence on pyridine nucleotide metabolism of rat liver. *J. Biol. Chem.* **239:** 1887–1892.
23. Greengard, P. 1963. Determination by fluorimetry. *In* Methods of Enzymatic Analysis. H. U. Bergmeyer, editor. Academic Press, Inc., New York. 551–558.
24. Chen, P. S., T. Y. Toribara, and H. Warner, 1956. Microdetermination of phosphorus. *Anal. Chem.* **28:** 1756–1758.
25. Levinson, S. A., and R. P. MacFate. 1969. Clinical Laboratory Diagnosis. Lea and Febiger, Philadelphia, Pa. 7th edition. 413–415.
26. Dousa, T. P., C. G. Duarte, and F. G. Knox. 1976. Effect of colchicine on urinary phosphate and regulation by parathyroid hormone. *Am. J. Physiol.* **231:** 61–65.
27. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
28. Kaplan, N. O. 1960. The pyridine coenzymes. *In* The Enzymes. P. D. Boyer, H. Lardy, and K. Myrback, editors. Academic Press, Inc., New York. 2nd edition. **3:** 105–169.
29. Sacktor, B. 1977. Transport in membrane vesicles isolated from the mammalian kidney and intestine. *Curr. Top. Bioenerg.* **6:** 39–81.
30. Preuss, H. G. 1968. Pyridine nucleotides in renal ammonia metabolism. *J. Lab. Clin. Med.* **72:** 370–382.
31. Kempson, S. A., S-Y. L. Ou, and T. P. Dousa. 1981. Nicotinamide-adenine dinucleotide (NAD) as a cellular regulator of renal phosphate (Pi) transport. *Kidney Int.* **19:** 112.
32. Haase, W., A. Schafer, H. Murer, and R. Kinne. 1978. Studies on the orientation of brush-border membrane vesicles. *Biochem. J.* **172:** 57–62.
33. Booth, A. G., and A. J. Kenny. 1976. Proteins of the kidney microvillus membrane. Identification of subunits after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. *Biochem. J.* **159:** 395–407.
34. Lin, L-F. H., and L. M. Henderson. 1972. Pyridinium precursors of pyridine nucleotides in perfused rat kidney and in the testis. *J. Biol. Chem.* **247:** 8023–8030.
35. Pfaller, W., and M. Rittinger. 1980. Quantitative morphology of the rat kidney. *Int. J. Biochem.* **12:** 17–22.
36. Kinne, R. 1978. Current concepts of renal proximal tubular function. *Contrib. Nephrol.* **14:** 14–24.
37. Dousa, T. P. 1979. Cyclic nucleotides in renal pathophysiology. *In* Hormonal Function and the Kidney. B. M. Brenner and J. H. Stein, editors. Churchill Livingstone, New York. 251–285.
38. Jones, N. F., and L. G. Welt. 1967. Adenosine triphosphate in rat renal papilla: effects of vasopressin and of ischemia. *Am. J. Physiol.* **212:** 939–944.
39. Berndt, T. J., F. G. Knox, and T. P. Dousa. 1981. Role of nicotinamide-adenine dinucleotide (NAD) in the phosphaturic response to parathyroid hormone (PTH). *Kidney Int.* **19:** 108.
40. Kirsten, R., and E. Kirsten. 1972. Redox state of pyridine nucleotides in renal response to aldosterone. *Am. J. Physiol.* **223:** 229–235.
41. Lau, K., and B. Eby. 1981. Tubular mechanisms for the

phosphaturic effects of NH_4Cl administration. *Kidney Int.* **19:** 109.

42. Rasmussen, H., D. B. P. Goodman, N. Friedmann, N. E. Allen, and K. Kurokawa. 1976. Ionic control of metabolism. *In* Handbook of Physiology. G. D. Aurbach, editor. American Physiological Society, Washington, D. C. **7:** 225–264.
43. Roobol, A., and G. A. O. Alleyne. 1973. Regulation of renal gluconeogenesis by calcium ions, hormones and adenosine 3′:5′-cyclic monophosphate. *Biochem. J.* **134:** 157–165.
44. Morrison, A. R., J. Yates, and S. Klahr. 1976. Effect of prostaglandin E_1 on the adenyl cyclase-cyclic AMP system and gluconeogenesis in rat renal cortical slices. *Biochim. Biophys. Acta.* **421:** 203–209.
45. Pogson, C. I., I. D. Longshaw, A. Roobol, S. A. Smith, and G. A. O. Alleyne. 1976. Phosphoenolpyruvate carboxykinase and renal gluconeogenesis. *In* Gluconeogenesis: Its Regulation in Mammalian Species. R. W. Hanson and M. A. Mehlman, editors. John Wiley & Sons, New York. 335–368.
46. Ou, S-Y. L., S. A. Kempson, and T. P. Dousa. 1980. Relationship between gluconeogenesis (GNG) and content of oxidized nicotinamide adenine dinucleotide (NAD^+) in renal cortical tissue. *Clin. Res.* **28:** 750A.
47. Maleque, A., H. Endou, C. Koseki, and F. Sakai. 1980. Nephron heterogeneity: gluconeogenesis from pyruvate in rabbit nephron. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **16:** 154–156.
48. Burch, H. B., R. G. Narins, C. Chu, S. Fagioli, S. Choi, W. McCarthy, and O. H. Lowry. 1978. Distribution along the rat nephron of three enzymes of gluconeogenesis in acidosis and starvation. *Am. J. Physiol.* **235:** F246–F253.
49. Kreusser, W. J., C. Descoeudres, Y. Oda, S. G. Massry, and K. Kurokawa. 1980. Effect of phosphate depletion on renal gluconeogenesis. *Miner. Electrolyte Metab.* **3:** 312–323.
50. Veech, R. L., J. W. R. Lawson, N. W. Cornell, and H. A. Krebs. 1979. Cytosolic phosphorylation potential. *J. Biol. Chem.* **254:** 6538–6547.