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BINDING OF NICOTINAMIDE ADENINE DINUCLEOTIDE BY THE RENAL BRUSH BORDER MEMBRANE FROM RAT KIDNEY CORTEX

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The characteristics of nicotinamide adenine dinucleotide (NAD) binding on brush border membranes prepared from rat renal cortex were investigated with the use of radioactively labelled NAD, [*adenine*-2,8-^{3}H]NAD$^+$, as a ligand. (1) We found that NAD binds on brush border membrane and that the extent of NAD binding is linearly proportional to the brush border membrane protein, and progressively increases with concentration of NAD in the medium. (2) The rate of NAD binding was dependent on temperature. At 20°C, the equilibrium binding was obtained at 15 min, while NAD binding at 0°C was slower, but the final level of binding reached at 120 min was similar to that plateau of binding observed at 20°C. Brush border membrane inactivated by heating at 95°C for 3 min did not bind NAD. Binding of NAD on brush border membranes was reversed by simple dilution or by the addition of unlabelled NAD. Both α-NAD and β-NAD stereoisomers displaced bound [^{3}H]NAD. Reduced NAD (NADH) caused less displacement of bound NAD than oxidized NAD$^+$. Adenine, nicotinamide, pyrophosphate, of 5′-AMP did not displace bound NAD. (3) The NAD binding to brush border membranes was nearly saturable, approximating saturation at 10^{-4} M NAD. Kinetic analysis by Scatchard plot indicates two sets of NAD binding sites in brush border membranes: a high-affinity binding site ($K_d = 1.9 \cdot 10^{-5}$ M) and a low-affinity binding site ($K_d = 2.2 \cdot 10^{-3}$ M). (4) Unlike concentrative uptake of D-[^{14}C]glucose by brush border membrane vesicles, binding of NAD was not dependent on the presence of an outside-in sodium gradient [$Na_o^+ > Na_i^+$], nor was it abolished by repeated freezing and thawing of brush border membranes. Unlike D-[^{14}C]glucose uptake, NAD binding by brush border membranes did not change upon decrease of intravesicular volume in hypertonic media. These observations indicate that NAD association with brush border membranes is true binding rather than intravesicular uptake of this compound. (5) The presence of specific binding sites in renal brush border membrane capable of binding of NAD with a high degree of affinity suggests that such sites may be involved in previously observed (Kempson, S.A., Colon-Otero, G., Ou, S.L., Turner, S.T. and Dousa, T.P. (1981) J. Clin. Invest. 67, 1347) modulatory effect of NAD on sodium-gradient-dependent uptake of phosphate across luminal brush border membrane of proximal tubules.

Introduction

Recent studies from our laboratory have shown that the addition of NAD to microvillar brush border membranes isolated from rat kidney cortex inhibits Na^+-gradient [$Na_o^+ > Na_i^+$] dependent up-

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Abbreviations: [^{3}H]NAD, [*adenine*-2,8-^{3}H]NAD$^+$; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; K_d, dissociation constant.

take of phosphate (P_i) [1]. Moreover, increase in renal cortical tissue NAD in response to parenteral administration of NAD precursor, nicotinamide, in vivo was associated with a specific and dose-dependent decrease in Na^+-gradient-dependent transport of P_i across renal brush border membranes [1]. Based on these findings, we proposed that cytoplasmic NAD may act as an intracellular modulator of P_i uptake across luminal brush border membranes and consequently, of transepithelial secondary active P_i reabsorption from the lumen of proximal tubules [2]. The biochemical mechanism, by which NAD causes the inhibition of Na^+-gradient-dependent transport of P_i across brush border membranes is not known. Therefore, as the first step in exploring the newly proposed function of NAD in regulating brush border membranes transport of P_i, we now studied the interaction of radioactively labelled NAD ([^{3}H]NAD) with brush border membranes prepared from rat kidney cortex in vitro. The results show that the brush border membranes fraction isolated from rat kidney cortex contains sites capable of binding NAD with a high degree of affinity and specificity.

Materials and Methods

Methods

Adult Sprague-Dawley male rats weighing 200–250 g were used in all experiments. The animals were allowed to eat a normal diet (Purina Rat Chow) containing a standard (0.7%) amount of phosphate and to drink water ad libitum [1].

For each experiment, 4–8 rats were killed and the kidneys were quickly removed and chilled in an ice-cold buffer comprising 154 mM NaCl/1 mM Tris-Hepes (pH 7.5). The renal cortex was dissected free of the capsule and medullary tissue and the brush border membranes fraction was prepared by the calcium-precipitation method as described in detail in our previous studies [1,3,4]. After the final centrifugation step, the brush border membranes preparation was suspended in 300 mM mannitol/5 mM Tris-Hepes (pH 8.5). Brush border membranes were used fresh for the binding studies, or in some experiments as indicated in the Results, brush border membranes were quickly frozen in solid CO_2 and kept at −80°C. An enzymatic characterization of brush border membranes fraction prepared from rat kidney with the use of calcium precipitation was described in detail previously [5,6]. The protein content of brush border membranes was determined as in our previous studies [1,3,4,6] by the method of Lowry et al. [7].

Determination of NAD^+ binding

The binding of NAD was measured with the use of the rapid filtration technique essentially analogous to the methods used previously by others for studies of binding of cyclic-3′,5′-AMP [8] or binding of folate [9] on renal brush border membranes preparations. The freshly prepared or frozen brush border membranes preparation, as indicated in the Results (80–150 μg protein per tube, unless stated otherwise), was incubated with [^{3}H]NAD in molar concentration(s) specified in individual experiments. The incubation mixture contained (final concentrations) 100 mM mannitol, 100 mM NaCl, 5 mM Hepes-Tris (pH 8.5) and brush border membranes preparation, in a final volume of 45 μl. Unless stated otherwise in the text, $1 \cdot 10^{-4}$ M [^{3}H]NAD (approx. 300000 cpm/tube) was used as a ligand. Additions or deletions of ingredients from this incubation mixture are all described in the Results section. Likewise, times and temperatures of the incubation are also indicated in the text of the Results. The incubations were stopped by the addition of 3 ml of ice-cold buffer (154 mM NaCl/5 mM Tris-Hepes, pH 8.5) and then immediately filtered over pre-wetted 0.65 μm cellulose ester (Millipore filter DAWP 02500) filter discs. The filters were washed with 12 ml of the ice-cold buffer; the whole procedure from the first dilution to the end of the washing on filters took 20 s or less. Retained radioactivity was determined by liquid scintillation counting. Incubations without added brush border membranes served as blanks and were substracted from measured values.

Transport measurements

The Na^+-gradient-dependent uptake of D-glucose by brush border membranes vesicles was measured with the rapid filtration technique described thoroughly in our previous communications [1,3,4]; 0.05 mM D-[6-^{3}H]glucose (approx. 10^6 cpm/tube) was used as the tracer. Incubations

without added brush border membranes preparation served as blanks [1,3,4]. The stop solution was the same as that used for NAD-binding assay.

All solutions used for preparation of brush border membrane fraction and for NAD binding or transport assays were filtered through a 0.45 μm Millipore filter on the day of use to exclude microbial contamination. Binding and transport parameters were measured in replicate samples. Each value (points in graphs) represents mean ± S.E. of replicate (at least triplicate) samples. Each experiment was conducted on at least three separate brush border membranes preparations. Unless stated otherwise, results are expressed as mean ± S.E.

Materials

[*adenine*-2,8-^{3}H]NAD$^+$ (specific radioactivity 20 μCi/mmol) was purchased from New England Nuclear, Boston, MA. D-[6-^{3}H]Glucose was purchased from Amersham-Searle, Arlington Heights, IL. NAD was purchased from P.L. Biochemicals, Milwaukee, WI. Other nonradioactive biochemicals, all of highest purity grades available, were purchased from the Sigma Company, St. Louis, MO.

Results

The binding of [^{3}H]NAD to isolated brush border membranes (30 min incubation; 0.1 mM [^{3}H]NAD) was linearly proportional ($r = 0.99$) to the brush border membranes protein at least up to 150 μg per tube. The effects of NAD on Na^+-gradient-dependent transport of phosphate, transport of D-glucose and other substances in our past studies [1,3,4], were measured in the presence of NaCl in the incubation medium; therefore, [^{3}H]NAD binding in the presence of NaCl was compared to binding when it was replaced by equimolar KCl. The results show (Fig. 1) that replacement of NaCl by KCl did not change the binding of [^{3}H]NAD on brush border membranes. In a separate series of experiments, time- and temperature-dependency of NAD binding on brush border membranes was examined at two concentrations (10^{-6} M and 10^{-4} M) of [^{3}H]NAD as ligand. At the concentration of 10^{-4} M NAD and at 20°C, the maximum binding was reached between 5 and 15 min of incubation time and remained constant, at least up to 60 min (Fig. 2). A period of 30 min was chosen for further experiments, as it is well on the plateau of maximum binding. Similar incubation at 0°C showed considerably slower binding, which only gradually approximated the maximum binding reached at 20°C in about 120 min (Fig. 2). All subsequent experiments were carried out at 20°C. At lower concentration ($1 \cdot 10^{-6}$ M) of [^{3}H]NAD, the course of

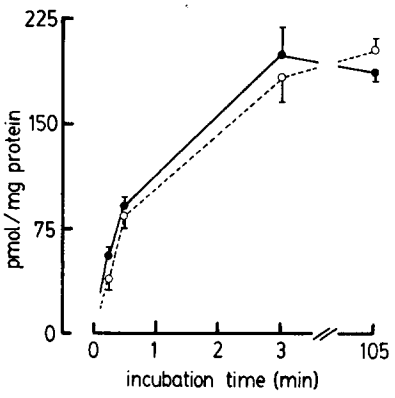

Fig. 1. NAD binding on brush border membranes in the presence of 154 mM NaCl (●———●) or of 154 KCl (○---○) onto brush border membranes. [^{3}H]NAD concentration was $1 \cdot 10^{-4}$ M.

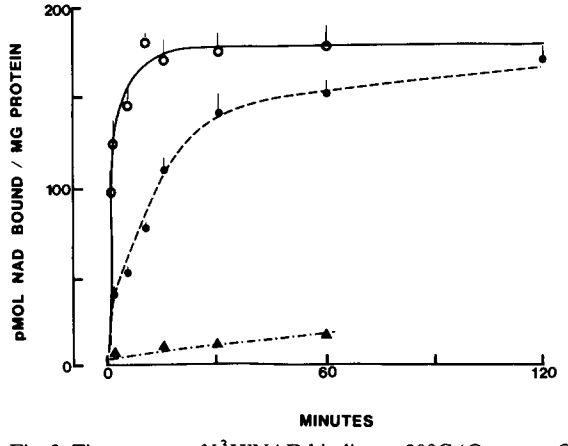

Fig. 2. Time-course of [^{3}H]NAD binding at 20°C (○———○) and 0°C (●---●). ▲·–·–·▲ denotes [^{3}H]NAD binding to heat-inactivated (boiled) membranes. NAD concentration was $1 \cdot 10^{-4}$ M; protein content 50–120 μg brush border membranes per tube. Mean ± S.E. of three experiments. Ordinate: [^{3}H]NAD bound (pmol/brush border membranes protein). Abscissa: time of incubation (mins).

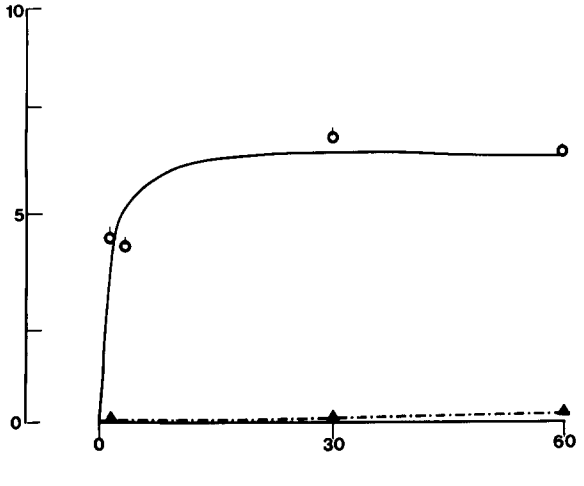

Fig. 3. Time-course of [^{3}H]NAD binding at $1 \cdot 10^{-6}$ M [^{3}H]NAD concentration in the incubation mixture. Ordinate: [^{3}H]NAD bound (pmol/mg of brush border membranes protein). Abscissa: time (min). Incubation temperature was 20°C; protein concentration 80–150 μg of brush border membranes protein per tube. (○———○) binding of [^{3}H]NAD on fresh brush border membranes; (▲·–·–·▲) binding on heat-activated membranes. Mean ± S.E. of two experiments. (See also the legend to Fig. 2).

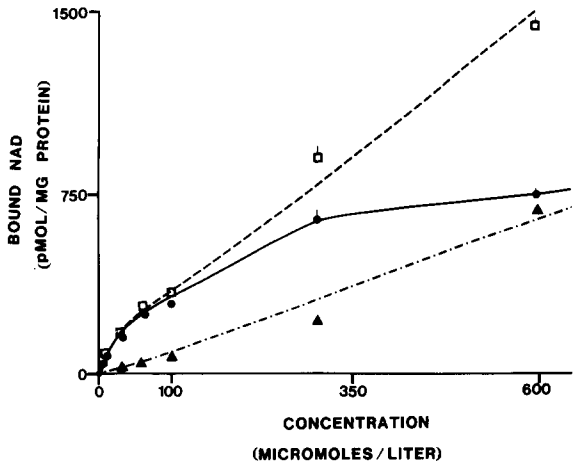

Fig. 4. Total, specific and nonspecific binding of [^{3}H]NAD on brush border membranes. Brush border membranes content was 50–150 μg per tube, incubation time 60 min at 20°C. Nonspecific binding represents binding in the presence of 1000-fold excess of nonlabelled NAD. Specific binding is total binding minus nonspecific binding. □-□, total binding, ▲·–·–·▲, nonspecific binding, ●———●, specific binding. Ordinate: Amount of NAD bound in pmol/mg brush border membrane protein. Abscissa: concentration of [^{3}H]NAD in the incubation mixture (μM). Mean ± S.E. of three experiments.

binding was similar to that at $1 \cdot 10^{-4}$ NAD (Fig. 3). At both concentrations of [^{3}H]NAD, the brush border membranes preparation which was inactivated before assay by heating in a boiling water bath for 3 min bound little or no NAD (Fig. 2, 3).

Next, we examined the relationship between the [^{3}H]NAD concentration in the incubation medium and the amount of the nucleotide bound on brush border membranes. Detailed conditions are specified in the legend to Fig. 4. With increasing concentration of [^{3}H]NAD, the extent of binding increases progressively, but no maximum binding or plateau was reached (Fig. 4). This apparent lack of saturability could be due to 'nonspecific' adsorption of [^{3}H]NAD on brush border membranes. The 'nonspecific' binding was assessed by measurement of [^{3}H]NAD binding in the presence of 1000-fold excess of unlabeled NAD in the incubation mixture. The 'nonspecific' binding progressed linearly with increasing concentration of [^{3}H]NAD (Fig. 4). Values obtained by substraction of 'nonspecific' binding from total binding are referred to as 'specific binding'. Up to 10^{-4} M of [^{3}H]NAD, nonspecific binding represents less than 15% of total binding, but at $0.6 \cdot 10^{-3}$ M [^{3}H]NAD, nonspecific binding reaches 50% (Fig. 4). The extent of specific [^{3}H]NAD binding onto brush border membranes begins to level off at [^{3}H]NAD concentrations of $5 \cdot 10^{-5}$ M to $1 \cdot 10^{-4}$ M, indicating that specific NAD^+ binding sites are at least partially saturable.

Some kinetic parameters of NAD binding on brush border membranes were examined at a wide range of concentrations of NAD and evaluated by Scatchard plot [10]. This graphic analysis (Fig. 5) indicates the presence of two binding sites, one site with higher affinity for NAD determined after substraction of nonspecific low-affinity binding (dissociation constant, $K_d \approx 1.9 \cdot 10^{-5}$ M NAD) and another site with lower affinity ($K_d \approx 2.2 \cdot 10^{-3}$ M NAD).

Next, we explored the reversibility of [^{3}H]NAD binding on brush border membranes. The brush border membranes preparation was first incubated

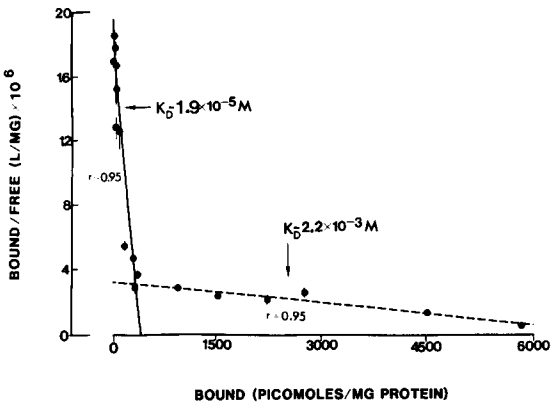

Fig. 5. Analysis of [^{3}H]NAD binding on brush border membranes by Scatchard plot. Dissociation constants (K_d) were graphically determined with the use of linear regression analysis. Representative experment, each point is mean ± S.E. of 3–4 replicate samples. r = correlation coefficient. Number of high affinity binding sites ≈ 372 pmol/mg protein; low affinity binding sites ≈ 7230 pmol/mg protein.

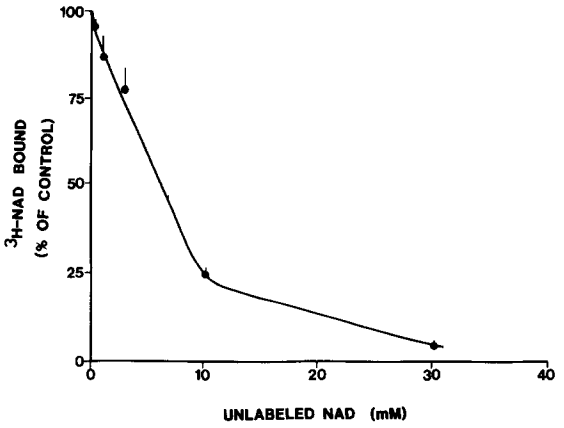

Fig. 6. Reversibility og [^{3}H]NAD binding by radioactive dilution with nonlabelled NAD. Brush border membranes were preincubated with 10^{-4} M [^{3}H]NAD for 30 min at 20°C, then various amounts of unlabelled NAD were added and incubation continued for another 30 min at 20°C. Abscissa: Concentration (mM) of unlabelled NAD added to incubation mixture at the beginning of the second phase of the incubation. Ordinate: Relative (%) decrease of [^{3}H]NAD binding upon addition of unlabelled NAD. Bindng of [^{3}H]NAD with no addition of unlabelled NAD in second phase of incubation was taken as 100%. Representative experiment, each point denotes mean ± S.E. of 3–4 replicate samples.

with $1 \cdot 10^{-4}$ M [^{3}H]NAD for 30 min at 20°C, then various amounts of unlabelled NAD^+ were added, and incubation was continued for an additional 30 min. The amount of [^{3}H]NAD bound on brush border membranes diminished with increasing concentration of added unlabelled NAD until at 30 mM NAD more than 85% of bound [^{3}H]NAD was displaced (Fig. 6).

Displacement of bound [^{3}H]NAD by unlabelled NAD was time- and temperature-dependent. Binding of $1 \cdot 10^{-6}$ M [^{3}H]NAD was reversible at both 20°C and 0°C. After incubation of brush border membranes with $1 \cdot 10^{-6}$ M [^{3}H]NAD for 30 min (20°C) 1000-fold excess of unlabelled NAD was added and the incubation continued for 10 min (88.03 ± 0.95% displaced) or 20 min (86.9 ± 3.1% displaced), when more than 85% of [^{3}H]NAD was displaced at both times.

Incubation of $1 \cdot 10^{-6}$ M [^{3}H]NAD at 0°C for 30 min then the addition of 1000-fold excess of unlabelled NAD and continuing the incubation for 30 min showed a similar degree of displacement (84.9 ± 3.46%). Therefore the interval of 30 min after the addition of unlabelled NAD was chosen as appropriate.

Binding of [^{3}H]NAD on brush border membranes was also reversed by decreasing [^{3}H]NAD concentration in the incubation mixture by simple dilution with incubation medium. After incubating brush border membranes with $1 \cdot 10^{-4}$ M [^{3}H]NAD for 30 min, the incubation mixture (45 µl) was diluted (67-times) by 3 ml of medium (100 mM mannitol/100 mM NaCl/5 mM Tris-Hepes; pH 8.5; 20°C) and the incubation was then continued. After dilution, the amount of [^{3}H]NAD bound on brush border membranes decreased by 74.4 ± 8.5% after 30 min of further incubation, and by 89.4 ± 9.3% after 60 min of further incubation.

The brush border membranes of proximal tubules contain a number of Na^+-gradient-dependent transport systems which are specific for a variety of ions and organic molecules [11,23]. Therefore, it was necessary to examine whether the observed association of [^{3}H]NAD with brush border membranes, as determined by the rapid filtration technique, reflects true binding, or the Na^+-gradient-dependent uptake of NAD from medium to intravesicular space of brush border membrane vesicles.

One aliquot of freshly prepared brush border membrane vesicles was kept at 0°C and another

TABLE I

COMPARISON OF THE EFFECT OF BRUSH BORDER MEMBRANES (BBM) TREATMENT BY REPEATED FREEZING AND THAWING UPON UPTAKE OF 0.05 mM D-[^{3}H]GLUCOSE OR [^{3}H]NAD BINDING

Uptake and binding on fresh and freeze-thawed aliquots of brush border membranes preparations were measured simultaneously. For further details see text. Each value is mean ± S.E. from two experiments. n = replicates.

D-[^{3}H]Glucose uptake (pmol/mg protein)			[^{3}H]NAD binding (pmol/mg protein)		
Time	Fresh BBM	Frozen-thawed BBM	Time	Fresh BBM	Frozen-thawed BBM
15 s	358.04 ± 6.59 (n = 7)	101.06 [a] ± 21.93 (n = 7)	30 s	144.00 ± 7.28 (n = 3)	128.71 ± 9.48 (n = 3)
60 s	405.92 ± 12.03 (n = 4)	143.78 [a] ± 14.20 (n = 4)	60 s	155.69 ± 16.32 (n = 7)	116.30 ± 14.46 (n = 5)
60 min	91.57 ± 7.43 (n = 3)	64.13 [a] ± 6.07 (n = 3)	60 min	249.58 ± 25.60 (n = 4)	195.00 ± 10.41 (n = 4)
120 min	12.91 ± 2.61 (n = 3)	18.13 ± 7.41 (n = 3)			

[a] Signifcantly different from fresh brush border membranes ($P < 0.05$, t-test).

aliquot was repeatedly frozen and thawed (six times) by placing the brush border membranes preparation in contact with solid CO_2 and then by equilibrating to 0°C at room temperature. Fresh brush border membranes and aliquots of brush border membranes subjected to repeated freeze-thaw cycles were tested simultaneously for the ability to bind [^{3}H]NAD and take up D-[^{3}H]glucose in the presence of Na^+ gradient [$Na_o^+ > Na_i^+$]. The results summarized in Table I show that repeatedly frozen-thawed brush border membranes preparation retained the ability to bind [^{3}H]NAD to the extent comparable to [^{3}H]NAD binding of fresh brush border membranes preparation. On the other hand, the uptake of D-[^{3}H]glucose measured simultaneously under exactly the same conditions was markedly decreased by repeating freezing and thawing.

The question of NAD binding vs. uptake of NAD by brush border membranes was further explored by examining the effect of hyperosmotic treatment of brush border membranes vesicles on [^{3}H]NAD binding and on D-[^{3}H]glucose uptake [11]. Brush border membranes vesicles were suspended in media of increasig osmolality by the addition of mannitol (300–700 mosmolal) and incubated with either D-[^{3}H]glucose or [^{3}H]NAD [3,4]. While the D-glucose uptake at equilibration time point (120 min) was inversely related to osmolality of incubation medium ($r = 0.9$; $P < 0.005$; t-test), the extent of [^{3}H]NAD binding did not change with osmolarity of the incubation en-

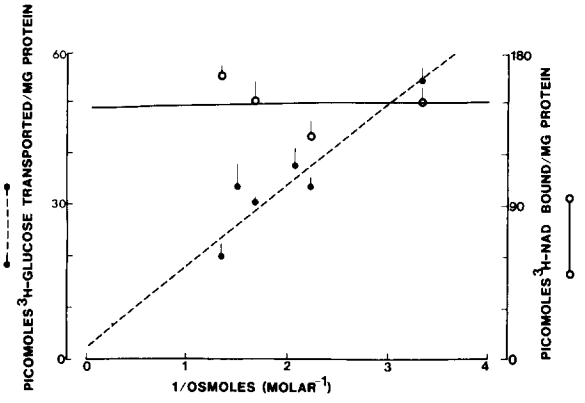

Fig. 7. Brush border membranes binding and/or uptake of [^{3}H]NAD and D-[^{3}H]glucose in media with increasing osmolality. Ordinates: Left: amount of D-[^{3}H]glucose taken up by brush border membranes after 120 min; right: amount of [^{3}H]NAD bound by brush border membranes under the same condition. Abscissa: Reciprocal value of osmolality (1/osmol) of incubation medium. D-Glucose uptake showed a significant positive correlation ($r = 0.967$; $P < 0.005$; t-test) with 1/osmol, but there was no correlation between [^{3}H]NAD binding and 1/osmol ($r = -0.301$). (●- - -●) D-[^{3}H]glucose uptake, (○———○) [^{3}H]NAD binding. For details, see text. Representative experiment, each point is mean ± S.E. of 3–4 replicate samples.

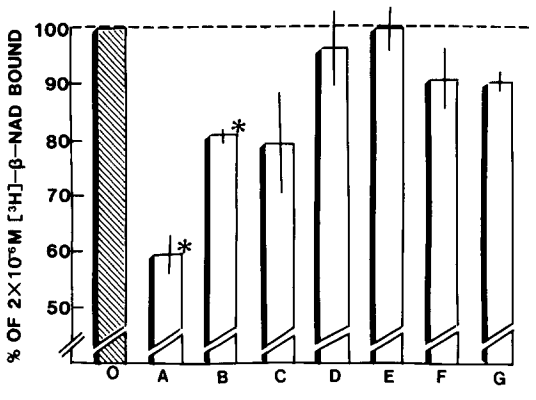

Fig. 8. Specificity of NAD binding. The brush border membranes were incubated first 20°C for 30 min with $2 \cdot 10^{-6}$ M [^{3}H]NAD, then various compounds (A–G) or none (control, O) were added, and the incubation was continued for a second 30 min. At the end of the second incubation the reaction was stopped by rapid filtration and washing (for details see Methods). Each bar is mean ± S.E. of three or two independent experiments done in replicate. Basal binding of [^{3}H]NAD, without added unlabelled compound in the second 30 min period (13.3 ± 3.9 pmol [^{3}H]NAD/mg protein), was taken as 100% (O, first bar, hatched). Incubations with various compounds (all in concentration $1 \cdot 10^{-4}$ M), added at the beginning of the second 30 min, are depicted by open bars: A, β-NAD; B, α-NAD; C, NADH; D, nicotinamide; E, pyrophosphate; F, 5′-AMP; G, adenosine. * Denotes values significantly ($P < 0.05$ or better; t-test) different from basal (control) binding.

vironment (Fig. 7). This finding indicates that, unlike the amount of D-[^{3}H]glucose taken up inside brush border membranes vesicles, which decreases with shrinking intravesicular volume [11], binding of [^{3}H]NAD on brush border membranes does not differ between media of various osmolality (Fig. 7).

Finally, we tested specificity of [^{3}H]NAD binding on brush border membranes with use of molecular components of NAD and some compound close to NAD in structure (Fig. 8). Besides naturally occurring β-NAD, stereoisomer α-NAD and the reduced form of β-NAD (NADH) also displaced [^{3}H]NAD bound on brush border membranes. In contrast, nicotinamide, adenosine, pyrophosphate or 5′-AMP did not displace appreciably [^{3}H]NAD bound on brush border membranes (Fig. 8).

Discussion

In the past, NAD binding on macromolecules has been studied mainly in relation to its role as a coenzyme of dehydrogenases [12–14]. In several studies it was found that cytoplasmic dehydrogenases bind preferentially the reduced form of NAD (NADH), rather than oxidized NAD [13–15]. The affinity for NADH binding on cytoplasic dehydrogenases was about 100-times higher than for oxidized NAD (NAD^+). The question of whether NAD can bind specifically on brush border membranes arose when we found that this nucleotide inhibits selectively Na^+-gradient-dependent uptake of P_i across brush border membranes [1].

The results of the present study show that brush border membrane is capable of binding the NAD in vitro, that this binding is time-dependent and temperature-dependent and also depends on the NAD concentration in the incubation medium. Brush border membrane inactivated by heating does not bind NAD, which suggests that the ability of brush border membranes to bind NAD depends on intact conformation of the membrane. Apparently, the binding of NAD by brush border membranes is not completely saturable at a higher concentration (over $1 \cdot 10^{-4}$ M) of the ligand, but nearly saturable up to $1 \cdot 10^{-4}$ M NAD (Fig. 4). Analysis of binding using a Scatchard plot [10] strongly suggests the presence of two populations of binding sites with two distinct affinities. The high-affinity binding sites ($K_d \approx 1.9 \cdot 10^{-5}$ M NAD) are capable of binding NAD in a concentration range prevalent in renal cortical tissue [1], renal isolated tubules and in isolated brush border membrane vesicles (Yusufi, Turner, Kusano, Dousa, unpublished data). Lack of effect of freeze-thawing cycles or an osmotic treatment on [^{3}H]NAD binding compared to demonstrated effects on D-glucose transport (Fig. 7) provides indication that [^{3}H]NAD is not taken up, but rather binds on brush border membranes. These features, along with the independence of [^{3}H]NAD binding on Na^+ [$Na_o^+ > Na_i^+$] gradient (Fig. 1) suggests that, as in the case of cAMP [8], the observed association between [^{3}H]NAD and brush border membranes is true binding rather than concentrative intravesicular uptake via Na^+-de-

pendent transport system.

While the present observations demonstrated that brush border membrane is capable of NAD binding with relatively high affinity and specificity, many questions related to NAD interactions with brush border membranes remain to be explored. Unresolved remains the question of at which site(s) NAD binds under in vitro and in vivo conditions. According to some studies, the majority of brush border membrane vesicles prepared with the use of the Ca^{2+} precipitation method [1,3,4,6,19,20] are oriented right-side-out [18]. However, our observation that NAD binding is not abolished by repeated freezing and thawing, a maneuver which renders brush border membrane vesicles incapable of concentrative Na^{+}-dependent uptake of solutes [19,20], suggests that NAD may have access to binding sites in brush border membranes from either side of the membrane. The concentration of NAD in plasma is very low (about $2 \cdot 10^{-6}$ M) compared to that in tissues [16]. Even if all the NAD were filtered in glomeruli, renal tissue NAD concentrations [1,2] would be much higher (10^{-4}–10^{-3} M); however, binding of NAD in intact cells may be modified by intracellular factors [17]. We would surmise that intracellular NAD from a cytoplasmic pool, rather than NAD in tubular fluid, is relevant for binding on brush border membranes [1,2,7], further interactions with brush border membranes and perhaps ultimately for modulation of brush border membrane transport of P_i [1,2,7].

While only stereoisomer β-NAD serves as a coenzyme of cytoplasmic dehydrogenases in virtually all mammalian tissues [12], stereospecificity is not a requirement for NAD binding on brush border membranes (Fig. 8). The reduced form of NAD, NADH, binds on cytoplasmic dehydrogenases [13–15] with about 100-times greater affinity, but the displacement study (Fig. 8) suggests that NADH has the same or even less affinity of binding on brush border membranes (Fig. 8). These observations indicate that binding of NAD on brush border membranes has characteristics different from NAD binding as a coenzyme on cytosolic proteins [13–15]. This consideration also argues against the possibility that binding of [^{3}H]NAD on brush border membranes represents NAD interactions with remnants of soluble dehydrogenases, traces of which might remain in purified brush border membranes preparations [5].

The major components of the NAD molecule, nicotinamide, adenosine and pyrophosphate, or 5′-AMP alone apparently do not interact with the NAD-binding sites on brush border membranes (Fig. 8). This finding indicates that only the integral molecule of NAD binds on brush border membranes with high affinity. Moreover, these observations also suggest that NAD proper, rather than its catabolites [12], is capable of interacting with brush border membranes. NAD binds even at 0°C (Fig. 2), under conditions where enzymatic transformation of NAD can be expected to be minimal.

Functional significance of NAD binding on brush border membranes, namely its relation to the proposed modulatory action of NAD on transport of P_i [1,2] remains to be defined. In order to inhibit P_i transport, NAD may possibly require covalent association with brush border membranes, either via ADPribosylation catalyzed by ADPribosyltransferase [17,21], or by indirect glycosylation – first by conversion of NAD to ADP-ribose (via NAD glycohydrolase) and subsequent Schiff base (aldimine) formation with amino groups of brush border membranes proteins [17,22]. Regardless of what the ultimate interaction of NAD with brush border membranes turns out to be, the binding of NAD on brush border membranes sites with high affinity observed in the present study may represent the initial step – attachment of NAD on brush border membranes – which may be then followed by enzymatically catalyzed formation of NAD-brush border membranes covalent complexes [17,22].

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