*Biochem. J.* (1982) **204**, 635–638
*Printed in Great Britain*



# Hydrolysis of nicotinamide–adenine dinucleotide by purified renal brush-border membranes

## Mechanism of $NAD^+$ inhibition of brush-border membrane phosphate-transport activity

Harriet S. TENENHOUSE and Yee L. CHU
*MRC Genetics Group, McGill University–Montreal Children's Hospital Research Institute, Montreal, Quebec H3H 1P3, Canada*

(*Received 13 October 1981/Accepted 11 March 1982*)

Purified rat renal brush-border membrane vesicles possess a heat-labile enzyme activity which hydrolyses $NAD^+$. A reciprocal relationship exists between the disappearance of $NAD^+$ and the appearance of adenosine; 2 mol of $P_i$ are liberated from each mol of $NAD^+$ incubated with brush-border membrane vesicles. Freezing and thawing brush-border membrane vesicles does not enhance the initial rate of $NAD^+$ hydrolysis. Preincubation of brush-border membrane vesicles with $NAD^+$ results in inhibition of $Na^+$-dependent $P_i$-transport activity, whereas $Na^+$-dependent glucose transport is not affected. EDTA, which prevents the release of $P_i$ from $NAD^+$ and which itself has no direct effect on brush-border membrane $P_i$ transport, reverses the $NAD^+$ inhibition of $Na^+$-dependent $P_i$ transport. These results suggest that it is the $P_i$ liberated from $NAD^+$ and not $NAD^+$ itself that inhibits $Na^+$-dependent $P_i$ transport.

The proximal tubule is the major site of $P_i$ reabsorption along the nephron (Knox *et al.*, 1973). Although various agents can influence the proximal-tubular handling of $P_i$ (Dennis *et al.*, 1979), the exact mechanism by which renal $P_i$ reabsorption is controlled is not yet elucidated. Kempson *et al.* (1981) have implicated $NAD^+$ as a possible intracellular regulator of renal $P_i$ transport in rat. They demonstrated that increasing renal cellular $NAD^+$ by hormonal administration (Berndt *et al.*, 1981) or by nicotinamide feeding (Kempson *et al.*, 1981) led to significant phosphaturia and that brush-border membrane vesicles prepared from treated rats had lower $P_i$-transport activity. Although the studies *in vivo* by Kempson *et al.* (1981) suggest a regulatory role of $NAD^+$, they do not provide any information on the mechanism whereby this effect is achieved.

Kempson *et al.* (1981) also demonstrated that $NAD^+$ specifically inhibited $Na^+$-dependent $P_i$ transport when added directly to purified renal brush-border membrane vesicles *in vitro*. Because neither nicotinamide nor adenosine alone influenced brush-border membrane uptake of $P_i$, they attributed the inhibitory effect to $NAD^+$. In the present study, we demonstrate that renal brush-border membrane vesicles possess a heat-labile enzyme activity which degrades $NAD^+$. Furthermore, we present evidence that the $P_i$ liberated from $NAD^+$ inhibits $Na^+$-dependent $P_i$ transport activity.

## Materials and methods

### *Materials*

Sephadex G-10 was purchased from Pharmacia Fine Chemicals, Montreal, Canada, and AG 1-X8 (100–200 mesh) from Bio-Rad Laboratories, Richmond, CA, U.S.A. $\beta$-$NAD^+$ was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. [*adenine*-2,8-$^3$H]$NAD^+$ (NET-443), $^{32}P_i$ (NEX-054), D-[1-$^3$H]glucose (NET-050) and Formula 963 scintillator were purchased from New England Nuclear (Canada), Montreal, Canada.

### *Preparation of brush-border membrane vesicles*

Brush-border membrane vesicles were prepared from rat renal cortex by the $Mg^{2+}$ precipitation procedure (Booth & Kenny, 1974) and purification was confirmed as described previously (Tenenhouse & Scriver, 1978). The brush-border membrane vesicles were washed and suspended in 300 mM-mannitol/5 mM-Tris/Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 8.5, as described by Kempson *et al.* (1981) to yield a protein concentration of 3–4 mg of protein/ml.



0306-3283/82/060635-04$01.50/1 

*Demonstration of NAD$^+$ hydrolysis by brush-border membrane vesicles and kidney homogenates*

Brush-border membranes or kidney homogenates were incubated with 300$\mu$M-NAD$^+$ in 300mM-mannitol/5mM-Tris/Hepes, pH8.5 at 20°C (0.5ml reaction mixture). Two controls in the same buffer were performed in each set of experiments. The first involved incubation of NAD$^+$ in the absence of brush-border membrane vesicles. The second consisted of incubating NAD$^+$ in the presence of brush-border membrane vesicles that had been boiled for 2min. After appropriate times of incubation, the reaction was stopped by addition of 0.1ml of ice-cold 3.6M-$HClO_4$. The brush-border membranes were pelleted by centrifugation (10000*g*, 10min, 0°C). The supernatants were neutralized with 0.1ml of 3.6M-KOH, and the resulting $KClO_4$ precipitates were removed by centrifugation as described above. The deproteinized and neutralized supernatants were frozen and processed as described below.

(i) $P_i$ liberated from NAD$^+$ in the neutralized incubation mixtures was assayed as described by Tenenhouse & Scriver (1975).

(ii) Liberation of [$^3$H]adenosine from [$^3$H]NAD$^+$ (0.15$\mu$Ci/0.5ml reaction mixture) was monitored by gel filtration on a Sephadex G-10 column (1cm × 26cm) equilibrated with 0.15M-NaCl/5mM-Hepes/0.02% $NaN_3$, pH7.0. Deproteinized and neutralized reaction mixtures (0.4ml, containing about 75000 c.p.m.) were applied to the column and fractions (1.2ml) were monitored for radioactivity. Recovery of radioactivity (c.p.m.) from the columns was 98%. NAD$^+$ was eluted in the exclusion volume.

(iii) The presence of 5'-[$^3$H]AMP in the deproteinized and neutralized reaction mixtures was assessed by chromatography on AG 1-X8 in the formate form. Standards of adenosine, NAD$^+$ and 5'-AMP were eluted in water, 0.05M-formic acid and 0.25M-formic acid respectively. Radioactivity in each fraction was monitored.

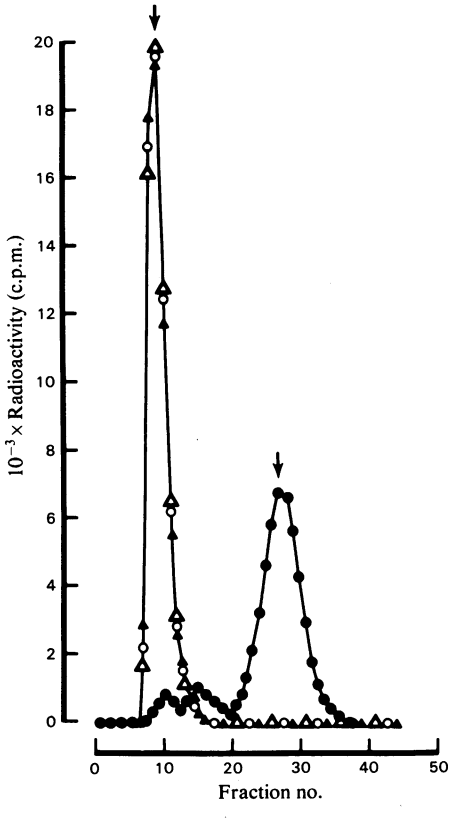


Fig. 1. *Sephadex G-10 elution profiles of reaction mixtures containing 300$\mu$M-NAD$^+$ and purified rat renal brush-border membrane vesicles*

Incubations were all for 30min at 20°C, and reactions were stopped as described in the Materials and methods section. Arrows indicate elution positions of standard NAD$^+$ and adenosine respectively. ●, NAD$^+$ + brush-border membrane vesicles; △, NAD$^+$ + brush-border membrane vesicles incubated in the presence of 1mM-EDTA; ▲, NAD$^+$ + brush-border membrane vesicles that had been boiled for 2min; ○, NAD$^+$ in the absence of brush-border membrane vesicles.

*Transport studies*

Transport of $^{32}$P and [$^3$H]glucose into purified brush-border membrane vesicles was studied by the rapid-filtration method as described previously (Tenenhouse & Scriver, 1978). Final concentrations of $P_i$ and glucose were 100$\mu$M and 10$\mu$M respectively. Where indicated, NAD$^+$ (300$\mu$M) and EDTA (1mM) were preincubated with brush-border membrane vesicles for 30min at 20°C as described by Kempson *et al.* (1981). The test compound was included in the incubation medium at the same concentration as that used in the preincubation.

## Results

*Hydrolysis of NAD$^+$*

Purified brush-border membrane vesicles were incubated with 300$\mu$M- NAD$^+$ for 30min at 20°C as described by Kempson *et al.* (1981). The reaction mixture was chromatographed on Sephadex G-10 (see the Materials and methods section). Fig. 1 shows that brush-border membranes degrade NAD$^+$ to completion in 30min; 91% of the radioactivity was recovered as adenosine, whereas some radioactivity was eluted in an intermediate position and may be 5'-AMP. Hydrolysis of NAD$^+$ or release of adenosine did not occur in the absence of brush-border membranes, in the presence of denatured brush-border membranes or of brush-border mem-

branes treated with 1mM-EDTA for 30min at 20°C (Fig. 1).

Characteristics of the reaction were examined by measuring the amount of $P_i$ released after various times of incubation. The reaction is linear with time up to 10min and approaches completion at about 30min. To determine the stoichiometry of the reaction, we measured the amount of $P_i$ released at reaction completion, which was 2mol of $P_i$/mol of $NAD^+$ in the reaction mixture. Incubation of brush-border membranes with 1mM-EDTA prevented the release of $P_i$ from $NAD^+$. Disappearance of $NAD^+$ and release of adenosine were dependent on the amount of brush-border membrane protein in the reaction mixture (Fig. 2). These results are based on 10min incubations at 20°C. The initial rate of reaction was also dependent on the concentration of $NAD^+$ in the mixture. Kinetic parameters could not be calculated, because more than one enzyme is presumably involved in the degradation of $NAD^+$ to adenosine, namely a phosphodiesterase and an alkaline phosphatase.

To determine whether the enzyme responsible for the degradation of $NAD^+$ is a brush-border-membrane-associated activity, we compared the initial rate of $NAD^+$ hydrolysis by purified brush-border membranes with that by kidney-cortex homogenates. Our results indicate that the initial rate of $P_i$ release by brush-border membranes is 3.5-fold greater than by the original homogenate. We found no significant difference in the amount of $P_i$ released from $NAD^+$ incubated with fresh and with freeze–thawed brush-border membrane vesicles [73.98 ± 3.75 and 74.22 ± 5.31 nmol of $P_i$/min per mg of protein respectively ($n = 5$; not significant)].

EDTA inhibits the degradation of $NAD^+$ by brush-border membranes (Fig. 1). To determine whether EDTA inhibited the phosphodiesterase activity or whether it acted exclusively on alkaline phosphatase, as reported previously (Tenenhouse *et al.*, 1980), we developed a method using AG 1-X8 to separate $NAD^+$ from 5'-AMP and adenosine (see the Materials and methods section). Neither 5'-AMP nor adenosine was produced when brush-border membranes were incubated with $NAD^+$ in the presence of 1mM-EDTA. These results indicate that EDTA inhibits the phosphodiesterase and the alkaline phosphatase activities in brush-border membranes.

*Transport studies*

We studied the effect of $NAD^+$ on the initial rate (15s) of $Na^+$-dependent $P_i$ and glucose transport by purified brush-border membrane vesicles (Table 1). Preincubation of brush-border membrane vesicles with 300μM-$NAD^+$ for 30min at 20°C resulted in a 43% inhibition of $Na^+$-dependent $P_i$ transport ($P < 0.001$), whereas no significant effect on glucose transport was evident. With no preincubation there was a slight (5%) inhibition of the initial rate of $P_i$ transport by $NAD^+$ that was not statistically

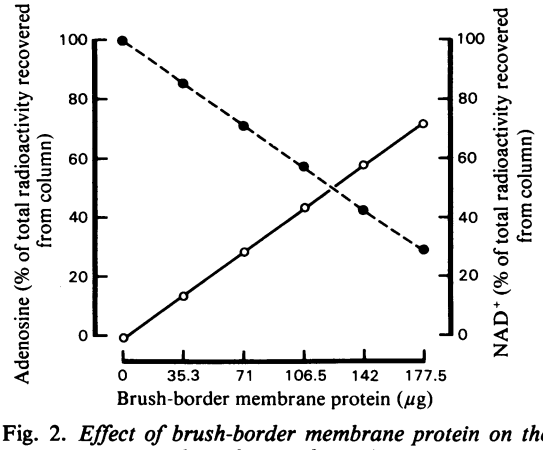


Fig. 2. *Effect of brush-border membrane protein on the degradation of NAD+*

Incubations were for 10min at 20°C. Disappearance of $NAD^+$ and liberation of adenosine in the reaction mixture were monitored by Sephadex G-10 chromatography as described in the Materials and methods section. ○, Adenosine; ●, $NAD^+$.

Table 1. *Effect of NAD+, EDTA and $P_i$ on initial rate of Na+-dependent $P_i$ and D-glucose transport into brush-border membrane vesicles*

The data shown represent the $Na^+$-dependent part of the total uptake, i.e. the diffusional uptake in the KCl gradient was subtracted from the total uptake in the NaCl gradient. The final concentrations in the incubation mixture were: $^{32}P$, 100μM; D-[$^3$H]-glucose, 10μM; EDTA, 1mM; $P_i$, 60μM, which is the contribution of $P_i$ that would be liberated during preincubation of brush-border membrane vesicles with 300μM-$NAD^+$ (0.01ml of brush-border membrane vesicles preincubated with 300μM-$NAD^+$ were added to 0.09ml of transport mixture). Where indicated, preincubations were performed for 30min at 0°C. Each value is the mean ± S.E.M. for 12 determinations (from two different brush-border membrane preparations). Significance was calculated by Student's *t* test; †$P < 0.001$.

| | Transport (pmol/15s per mg of protein) | |
|---|---|---|
| | $P_i$ | Glucose |
| Control | 1403 ± 63 | 144 ± 7 |
| $NAD^+$, preincubation | 799 ± 32† | 141 ± 6 |
| $NAD^+$, no preincubation | 1322 ± 44 | 131 ± 6 |
| EDTA, preincubation | 1459 ± 31 | 130 ± 9 |
| $NAD^+$ + EDTA, preincubation | 1357 ± 90 | 139 ± 5 |
| $P_i$ | 992 ± 27† | 124 ± 8 |

significant. With a longer period of incubation (2 min) and no preincubation, $NAD^+$ significantly inhibited by 15% ($P < 0.02$) $Na^+$-dependent $P_i$ transport (results not shown).

Preincubation with 1 mM-EDTA did not affect transport of $P_i$ or glucose (Table 1; Tenenhouse *et al.*, 1980). However, preincubation with EDTA in the presence of $NAD^+$ reversed the inhibitory effect of $NAD^+$ (Table 1), presumably by preventing $NAD^+$ hydrolysis and the subsequent release of $P_i$. In fact, the addition of $P_i$ to the brush-border membrane vesicles in an amount equivalent to that liberated from 300 $\mu$M-$NAD^+$ resulted in a significant decrease in $Na^+$-dependent $P_i$ transport (Table 1). It should be noted that the above manipulations altered neither the diffusional transport of $P_i$ and glucose measured in the presence of a KCl gradient nor the $Na^+$-dependent transport of glucose.

## Discussion

Kempson *et al.* (1981) provided evidence for a possible role of $NAD^+$ as an intracellular regulator of renal $P_i$ transport. Their theory implies that $NAD^+$ acts at the cytoplasmic surface of the brush-border membrane to inhibit $P_i$ transport. Therefore it is of interest that $NAD^+$ also inhibits $P_i$ transport when added to the luminal surface of brush-border membrane vesicles *in vitro* (Kempson *et al.*, 1981). These authors also showed that the inhibitory effect of $NAD^+$ *in vitro* was not specific: $NAD^+$ was as effective as NADH, and the biologically inactive stereoisomer of $NAD^+$, $\alpha$-$NAD^+$, was equally potent (Kempson *et al.*, 1981). Their results also indicated that, when $NAD^+$ was added to brush-border membrane vesicles without preincubation, the inhibition was present, but less extensive than with preincubation (Kempson *et al.*, 1981).

In the present study, we show that purified rat renal brush-border membrane vesicles hydrolyse $NAD^+$ (Fig. 1) and that 2 mol of $P_i$ are liberated from each mol of $NAD^+$ incubated with brush-border membrane vesicles. The $P_i$ liberated from $NAD^+$ competes for $Na^+$-dependent $P_i$-transport activity (Table 1). EDTA, which prevents the release of $P_i$ from $NAD^+$, and which itself has no direct effect on brush-border membrane $P_i$ transport, reverses the $NAD^+$ inhibition of $Na^+$-dependent $P_i$ transport (Table 1). We believe that the results *in vitro* of Kempson *et al.* (1981) require re-interpretation. Our data suggest that it is $P_i$ released from $NAD^+$, and not $NAD^+$ itself, that inhibits brush-border-membrane $Na^+$-dependent $P_i$ transport.

Phosphodiesterase activity is tightly associated with the renal brush-border membrane (Filburn & Sacktor, 1976). Our studies indicate that the initial rate of NAD hydrolysis by brush-border membranes is increased 3.5-fold over that by the original kidney homogenate. This increase in brush-border-membrane relative specific activity is not as high as the 10-fold and 9-fold enrichment in brush-border-membrane maltase and alkaline phosphatase activities respectively. These results are most probably due to the presence of phosphodiesterase activity in the soluble fraction of renal-cortical cells (Filburn & Sacktor, 1976).

Since freshly prepared brush-border membrane vesicles have a right-side-out orientation (Haase *et al.*, 1978), we suggest that an impermeant molecule such as $NAD^+$ would only be accessible to the enzyme if it resided on the luminal surface of the brush-border membrane. Freezing and thawing of brush-border membrane vesicles, a treatment which destroys $Na^+$-dependent $P_i$ transport activity (results not shown), did not enhance the initial rate of $NAD^+$ hydrolysis. These results are compatible with an external orientation of the brush-border membrane phosphodiesterase. Thus $NAD^+$ added to intact, freshly prepared brush-border membrane vesicles can be completely degraded and the $P_i$ liberated can decrease $^{32}P$ uptake by these vesicles.

We thank Dr. C. R. Scriver and Dr. A. Tenenhouse for their helpful suggestions, encouragement and valuable discussions, and also Lynne Merid and Huguette Ishmael for preparation of this manuscript. This work was supported by the M.R.C. of Canada (Genetics Group Award) and Natural Sciences and Engineering Research Council of Canada (summer student support to Y. L. C.). This is publication no. 82009 of the McGill University–Montreal Children's Hospital Research Institute.

## References

Berndt, T. J., Knox, F. G., Kempson, S. A. & Dousa, T. P. (1981) *Endocrinology* **108**, 2005–2007

Booth, A. G. & Kenny, A. J. (1974) *Biochem. J.* **142**, 575–581

Dennis, V. W., Stead, W. W. & Meyers, J. L. (1979) *Annu. Rev. Physiol.* **41**, 257–271

Filburn, C. R. & Sacktor, B. (1976) *Arch. Biochem. Biophys.* **174**, 249–261

Haase, W., Schafer, A., Murer, H. & Kinne, R. (1978) *Biochem. J.* **172**, 57–62

Kempson, S. A., Colon-Otero, G., Ou, S.-Y. L., Turner, S. T. & Dousa, T. P. (1981) *J. Clin. Invest.* **67**, 1347–1360

Knox, E. G., Schneider, E. G., Willis, L. R., Strandhoy, J. W. & Ott, C. E. (1973) *Kidney Int.* **3**, 346–353

Tenenhouse, H. S. & Scriver, C. R. (1975) *J. Clin. Invest.* **55**, 644–654

Tenenhouse, H. S. & Scriver, C. R. (1978) *Can. J. Biochem.* **56**, 640–646

Tenenhouse, H. S., Scriver, C. R. & Vizel, E. J. (1980) *Biochem. J.* **190**, 473–476