ferricyanide treatment decreased the rate of *N*-demethylation in the control microsomes, the isoniazid inhibition of aminopyrine *N*-demethylation was reversed. The ferricyanide decrease in drug metabolism in control microsomes has been reported previously, but has not been characterized [8].

The results reported here for isoniazid differ from experimental findings reported for phenelzine (phenylethylhydrazine). In the presence of NADPH, phenelzine produced a concomitant decrease in the binding of CO to cytochrome P-450 and in the heme content of the microsomes [15]. The spectral characteristics of the isoniazid complex differ from those formed by the alkyl hydrazine derivatives reported by Hines and Prough [16]. Thus, the various hydrazines and hydrazides can interact differently with the cytochrome P-450 system.

In summary, ferricyanide reversal of the isoniazid inhibition of microsomal activity strongly suggests that isoniazid forms a metabolic intermediate (absorption maximum at 449 nm) in the presence of NADPH and oxygen which binds to cytochrome P-450. As with other metabolic intermediate complexes, a decrease in the functionally available cytochrome P-450 results and the rate of drug metabolism can, in turn, be diminished due to the decreased terminal oxidase.

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REFERENCES

1. H. Kutt, R. Brennan, H. Dehejia and K. Verebeley, *Am. Rev. resp. Dis.* **101**, 377 (1970).
2. G. Sutton and J. Kupferberg, *Neurology* **25**, 1179 (1975).
3. H. Kutt, K. Verebeley and F. McDowell, *Neurology* **18**, 706 (1968).
4. S. F. Muakkassah, W. R. Bidlack and W. C. T. Yang, *Biochem. Pharmac.* **30**, 1651 (1981).
5. M. R. Franklin, *Xenobiotica* **1**, 581 (1971).
6. P. V. Parke and H. Rahman, *Biochem. J.* **123**, 9 (1971).
7. J. B. Schenkman, B. J. Wilson and D. L. Cinti, *Biochem. Pharmac.* **21**, 2373 (1972).
8. M. K. Buening and M. R. Franklin, *Drug Metab. Dispos.* **4**, 244 (1976).
9. J. Werringloer and R. W. Estabrook, *Life Sci.* **13**, 1319 (1973).
10. D. G. Wolff and W. R. Bidlack, *Biochem. biophys. Res. Commun.* **73**, 850 (1976).
11. Y. Masuda and T. Murano, *Biochem. Pharmac.* **26**, 2275 (1977).
12. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
13. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
14. T. Nash, *Biochem. J.* **55**, 416 (1953).
15. S. F. Muakkassah and W. C. T. Yang, *Fedn Proc.* **40**, 719 (1981).
16. R. N. Hines and R. A. Prough, *J. Pharmac. exp. Ther.* **214**, 80 (1980).

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Retention of phosphate transport function of rat renal brush border membranes isolated from frozen cortex

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Extensive studies of the transport properties of isolated luminal brush border membrane (BBM) vesicles from the renal proximal tubule have led to identification of specific Na^+-dependent transport systems for glucose, amino acids, and various organic and inorganic ions including inorganic phosphate (P_i) [1, 2]. The P_i transport system in BBM represents a rate-limiting step in transtubular P_i reabsorption and is of particular interest because it is altered in response to several hormones and drugs administered *in vivo*. Examples of these agents are parathyroid hormone, growth hormone, 1,25-dihydroxyvitamin D_3, dibutyryl cyclic AMP, triamcinolone, nicotinamide, and diphosphonates [3, 4]. A combination of *in vivo* and *in vitro* methods provides an extremely useful approach [5–7] for elucidating the mode of action of agents which regulate renal P_i transport. The procedure is to study first with clearance techniques the effects of administered agents on renal function and on transtubular P_i transport. Then, at the end of these experiments, the kidneys are removed, BBM vesicles are prepared from the cortex, and P_i uptake by the vesicles is measured to provide direct determination of any changes in the P_i transport system in the BBM of the proximal tubule. A major disadvantage of this experimental design is that completion without interruption requires an extended period of time (about 15 hr). This problem would be avoided if, following the clearance studies, the renal cortex could be stored until the next day (or longer) before preparing a BBM vesicle fraction. Evidence is presented here that the P_i transport function of rat renal BBM is well preserved in renal cortical tissue stored frozen by liquid nitrogen. Further, differences in P_i transport induced *in vivo* by low P_i diet or nicotinamide are retained in BBM vesicles prepared from frozen cortex.

Rats were anesthetized with ether, kidneys were removed, decapsulated, and rinsed in ice-cold buffered saline (154 mM NaCl, 1 mM Tris–HEPES*, pH 7.5), and the cortex was dissected free from the medulla. The cortex

* HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

was either used immediately for preparation of a BBM vesicle fraction, or was snap frozen by clamping between aluminium tongs cooled in liquid nitrogen and was stored in a liquid nitrogen refrigerator (Union Carbide, Indianapolis, IN). Frozen cortex was thawed by immersion in ice-cold buffered saline for 20 min. BBM vesicles were isolated from renal cortex by the calcium precipitation method [3, 7–9]. The purity of the BBM preparations from fresh and frozen cortex, assessed by measuring appropriate "marker" enzymes for subcellular organelles, was not different and was comparable to that reported previously [9]. Uptake of [^{32}P]-phosphate, D-[^{3}H]-glucose, L-[^{3}H]-proline, and $^{22}Na^{+}$ by BBM vesicles was determined by a Millipore filtration technique used and described in detail elsewhere [7–11] and in the legends to the figures.

Low P_i diet (0.07% phosphorus) was obtained from ICN Nutritional Biochemicals, Cleveland, OH [9, 11]; rats were given low P_i diet for 3 days and were pair-fed and processed in parallel with control rats receiving normal P_i diet (Wayne Lab-Blox; Allied Mills, Inc., Chicago, IL) containing 0.93% phosphorus. Nicotinamide was dissolved in 0.6 ml of isotonic saline (pH adjusted to 7.0 with NaOH) and was administered as a single intraperitoneal injection (1 g/kg body weight) to rats fed normal P_i diet and the kidneys were removed 9 hr later [11]. Control animals received saline injections. Protein and enzyme activities were assayed exactly as described previously [9, 11]. Results were evaluated statistically by Student's *t*-test for group comparisons; values of $P > 0.05$ were considered not significant.

In the presence of a Na^{+} gradient (initially 100 mM extravesicular and 0 mM intravesicular), P_i uptake by BBM vesicles from fresh cortex increased rapidly, reached a peak within 1 to 2 min ("overshoot"), and thereafter declined to a much lower level at 120 min ("equilibrium point") (Fig. 1, left panel). Replacement of NaCl by 100 mM KCl (represents Na^{+}-independent uptake) in the incubation medium abolished the initial overshoot and reduced P_i uptake at 0.5 min to 1/60 of the uptake in the presence of NaCl. These characteristics of the P_i transport system agree closely with previous reports using fresh cortex [3, 7, 9, 12] and are shared by BBM vesicles prepared from cortex stored frozen for 8 days (Fig. 1, left panel). The principal effect due to freezing the tissue was a 40–50 per cent reduction in P_i uptake at all time points. A similar reduction in net P_i uptake was observed when using cortex frozen for only 24 hr, indicating that the reduction in uptake is probably independent of time of storage. The P_i uptake at the peak of the overshoot was about 5-fold greater than the uptake at equilibrium (120 min) for vesicles from both fresh and frozen cortex (Fig. 1, right panel). These results suggest that tissue freezing did not impair the capacity of the BBM transport system to accumulate P_i against its concentration gradient. In contrast to freezing cortex prior to preparing BBM vesicles, when the isolated vesicles were stored frozen for the same time (results not shown) the overshoot was completely abolished, and the Na^{+}-dependent uptake was reduced by 85 per cent compared to freshly prepared BBM. Preservation of the P_i transport function in frozen BBM vesicles may require the presence of glycerol as reported for the D-glucose transport system [13, 14].

Like the P_i transport system, the uptake of D-glucose and L-proline by BBM vesicles from fresh cortex (Fig. 2) was markedly stimulated by a Na^{+} gradient (extravesicular > intravesicular) and there was an initial overshoot relative to the uptake at the equilibrium point [8–10]. In marked contrast to the preservation of the P_i transport function, there was severe impairment of the D-glucose transport system in BBM vesicles from cortical tissue stored frozen for only 24 hr (Fig. 2, left panel). While D-glucose uptake by vesicles from stored cortex was stimulated at initial time points by a Na^{+} gradient, the uptake did not exceed the equilibrium value, i.e. there was no transient overshoot. L-Proline transport was preserved better; the uptake by BBM vesicles from stored cortex was stimulated by a Na^{+} gradient and initially exceeded the equilibrium uptake by 3-fold at 0.5 min (Fig. 2, right panel), suggesting retention of the capacity to transport L-proline against its concentration gradient. However, the size of the overshoot was diminished compared to Na^{+} gradient-stimulated uptake of L-proline by vesicles from fresh cortex. L-Proline transport may be a useful control to determine the specificity of any changes in P_i transport by BBM vesicles from frozen tissue.

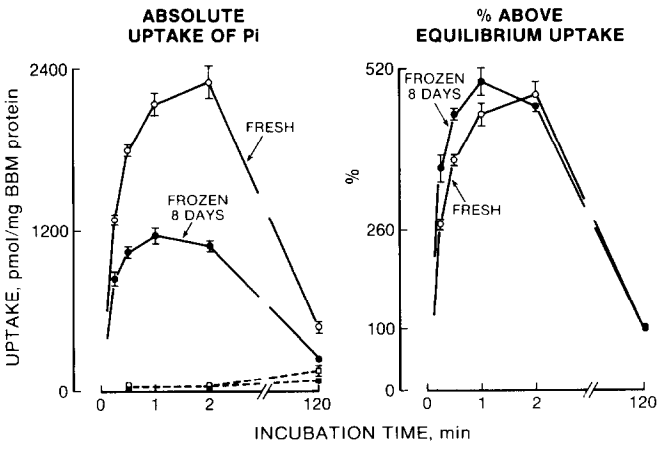

Fig. 1. Phosphate uptake by rat renal BBM vesicles prepared from fresh cortex (○) and from cortex previously stored frozen (●) for 8 days under liquid nitrogen. BBM vesicles in 300 mM mannitol were incubated at 20° in 100 mM NaCl, 100 mM mannitol, and 5 mM Tris–HEPES (pH 8.5) containing 0.1 mM $K_2H^{32}PO_4$. Uptake was stopped at various times by addition of ice-cold 135 mM NaCl, 10 mM arsenate and 5 mM Tris–HEPES (pH 8.5), and the vesicles were collected by filtration using a Millipore filter. Uptake of phosphate when NaCl in the incubation medium was replaced by KCl is also shown (□, fresh cortex; ■, frozen cortex). Fresh and frozen cortex were processed in parallel and the data are expressed both as absolute uptake (left panel) and as % increase over uptake at the equilibrium point at 120 min (right panel). Each point is the mean ± S.E. of measurements in triplicate using one preparation of BBM vesicles derived from the pooled renal cortices of three rats.

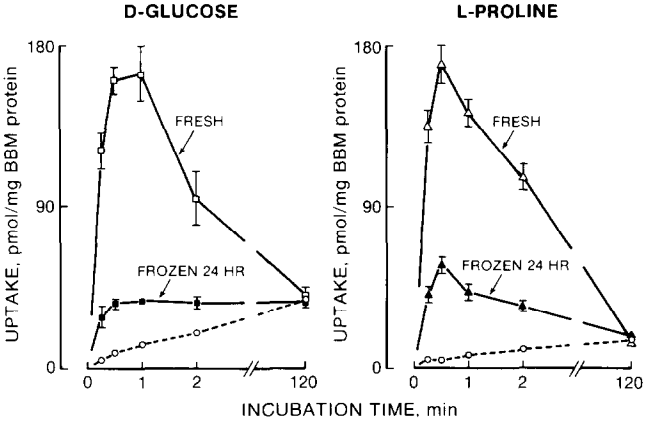

Fig. 2. Uptake of D-glucose (left panel) and L-proline (right panel) by rat renal BBM vesicles prepared from fresh cortex (□, △) and from cortex previously stored frozen (■, ▲) for 24 hr under liquid nitrogen. Uptake was determined as described in the legend to Fig. 1 except that either 0.05 mM D-[^{3}H]-glucose or 0.025 mM L-[^{3}H]-proline replaced $K_2H^{32}PO_4$ in the incubation medium. Fresh and frozen cortex were processed in parallel. BBM uptake when KCl replaced NaCl in the incubation medium was not different between fresh and frozen cortex, and only one set of points (○) is shown. Each point is the mean ± S.E. of measurements in triplicate using one preparation of BBM vesicles from the pooled renal cortices of three rats.

At the equilibrium point the uptake of D-glucose and of L-proline was not different between BBM vesicles from fresh and frozen cortex (Fig. 2), which indicates that the two populations of vesicles have the same intravesicular space. Thus, the reduced Na^+ gradient-dependent uptake of P_i by vesicles from frozen cortex (Fig. 1) is not due to decreased vesicle size.

The possibility that the changes in P_i transport were secondary to changes in Na^+ transport was eliminated by two types of experiments. First, direct measurement of $^{22}Na^+$ uptake at 0.25, 0.5, 1.0, and 120 min revealed no differences in Na^+ transport by BBM vesicles from fresh and frozen cortex. At 0.5 min the uptake (nmoles/mg BBM protein; mean ± S.E.) of $^{22}Na^+$ was 23 ± 4 for fresh cortex compared to 25 ± 4 for frozen cortex. Second, BBM uptake of P_i in the presence of Na^+ but without a transmembrane gradient (extravesicular $[Na^+]$ = intravesicular $[Na^+]$) was lower at all time points in vesicles from frozen cortex compared to fresh cortex (Table 1). These data suggest that the freezing step has a direct effect on the BBM transport system for P_i.

Kinetic analysis by double-reciprocal plots [7, 12] of the initial rate of Na^+ gradient-dependent P_i uptake by BBM vesicles incubated with various concentrations (range 0.05 to 1.0 mM) of P_i indicates that the major change is a decrease in the apparent V_{max} [pmoles · (mg protein)$^{-1}$ · 0.5 min^{-1}] for P_i uptake from 2729 ± 199 in vesicles from fresh cortex to 1309 ± 278 (mean ± S.E. from three different BBM preparations; $P < 0.025$, group t-test) in vesicles from frozen cortex. The apparent K_m for P_i transport was not significantly different between vesicles from fresh and frozen cortex (fresh, 46 ± 11 μM; frozen, 36 ± 5 μM; mean ± S.E.). The initial P_i uptake, measured at 0.5 min, was used for kinetic studies and at this part of the time course binding of P_i to vesicles accounts for less than 10 per cent of the total P_i uptake [7, 12]. Any decrease in P_i binding after freezing would have a negligible effect on the total uptake at this time and on determination of the kinetic parameters of transport. By similar reasoning it is unlikely that a decrease in P_i binding alone could explain the reduced P_i uptake at the equilibrium point at 120 min since, again, P_i binding at this time accounts for only a small proportion (about 15 per cent) of the total P_i uptake [15].

Two maneuvers which influence specifically, but with opposite effects, renal transport of P_i *in vivo* were used to determine whether the expected changes in P_i uptake by isolated BBM vesicles were still present following freezing and storage of renal cortex. Feeding low P_i diet to rats leads, within 3 days, to a marked increase in BBM transport of P_i as assessed by measuring P_i uptake by BBM vesicles isolated from fresh cortex [3, 9]. As shown in Fig. 3 (left panel), the increased uptake of P_i in response to low P_i diet was preserved in BBM vesicles from cortex stored frozen for 24 hr. The other maneuver was to inject rats with pharmacological doses of nicotinamide which causes dose-dependent inhibition of P_i uptake by subsequently isolated renal BBM vesicles [11]. Decreased P_i uptake due to nicotinamide treatment was clearly evident in BBM vesicles from cortex stored for 24 hr (Fig. 3, right panel). Thus, changes in the P_i transport system in BBM in response to certain stimuli are preserved in BBM vesicles from stored cortex.

Preliminary experiments suggest that the P_i transport function of BBM is preserved also in dog cortex stored frozen for up to 23 days. These findings indicate that preservation of the P_i transport capacity is not a species-specific phenomenon. Thus, another advantage of the freezing step would be to facilitate collection and storage of human renal cortex which is available usually only at short notice and with unpredictable frequency. The combination of these factors is probably the major reason why P_i transport by human renal BBM has not been characterized in either normal or diseased states.

Table 1. Phosphate uptake by BBM vesicles pre-equilibrated with Na^+*

Time (min)	Uptake (pmoles/mg BBM protein) Fresh cortex	Frozen cortex
0.25	83 ± 3	54 ± 4
2	103 ± 3	74 ± 4
15	132 ± 4	79 ± 4
120	135 ± 6	110 ± 6

* Values are means ± S.E. of triplicate measurements from one vesicle preparation. Vesicles were incubated for 2 hr at 20° in 50 mM NaSCN, 200 mM mannitol and 5 mM Tris–HEPES (pH 8.5) prior to measuring phosphate uptake in a similar incubation medium containing 0.1 mM $K_2H^{32}PO_4$.

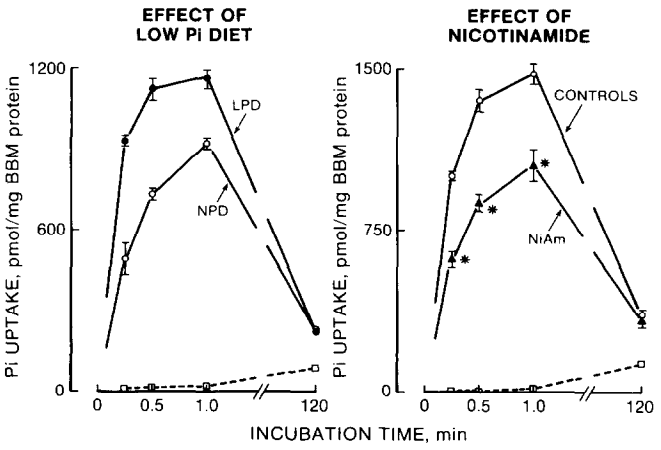

Fig. 3. Phosphate uptake by BBM vesicles prepared from rat renal cortex stored frozen for 24 hr under liquid nitrogen. Left panel: Effect of feeding rats on low P_i diet (LPD) for 3 days compared to control rats pair-fed normal P_i diet (NPD). NPD (○) and LPD (●) rats were processed in parallel. Each point is the mean ± S.E. of measurements in triplicate using one preparation of BBM vesicles from the pooled renal cortices of three rats. Right panel: Effect of injection of nicotinamide (NiAm) to rats fed NPD. Control rats (○) and NiAm rats (▲) were processed in parallel. Each point is the mean ± S.E. obtained from three different BBM preparations; each BBM preparation was derived from one rat. Key: (*) $P < 0.01$, compared to controls (group t-test). In both of these studies, BBM uptake of P_i was not different between groups when KCl replaced NaCl in the incubation medium and only one set of points (□) is shown.

In summary, while net uptake of P_i was reduced in BBM vesicles isolated from rat renal cortex stored frozen, the degree of stimulation of P_i uptake by a Na^+ gradient and the capacity to accumulate P_i in excess of the equilibrium uptake were comparable to that observed in BBM vesicles from fresh cortex. Stimulation of P_i uptake by feeding low P_i diet and inhibition of P_i uptake by nicotinamide injection also were preserved in BBM vesicles from stored cortex.

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REFERENCES

1. B. Sacktor, *Curr. Topics Bioenergetics* **6**, 39 (1977).
2. H. Murer and R. Kinne, *J. memb. Biol.* **55**, 81 (1980).
3. H. Murer, H. Stern, G. Burckhardt, C. Storelli and R. Kinne, *Adv. exp. Med. Biol.* **128**, 11 (1980).
4. T. P. Dousa and S. A. Kempson, *Miner. Electrolyte Metab.*, in press.
5. S. A. Kempson, T. Berndt, D. Zimmerman, F. G. Knox and T. P. Dousa, *Kidney Int.* **16**, 823 (1979).
6. T. E. Northrup, *Fedn Proc.* **40**, 462 (1981).
7. M. R. Hammerman, I. E. Karl and K. A. Hruska, *Biochim. biophys. Acta* **603**, 322 (1980).
8. J. C. Beck and B. Sacktor, *J. biol. Chem.* **253**, 5531 (1978).
9. S. A. Kempson, S. V. Shah, P. G. Werness, T. Berndt, P. H. Lee, L. H. Smith, F. G. Knox and T. P. Dousa, *Kidney Int.* **18**, 36 (1980).
10. M. R. Hammerman and B. Sacktor, *J. biol. Chem.* **252**, 591 (1977).
11. S. A. Kempson, G. Colon-Otero, S. Y. L. Ou, S. T. Turner and T. P. Dousa, *J. clin. Invest.* **67**, 1347 (1981).
12. L. Cheng and B. Sacktor, *J. biol. Chem.* **256**, 1556 (1981).
13. K. Hittelman, R. D. Mamelok and S. B. Prusiner, *Analyt. Biochem.* **89**, 324 (1978).
14. S. A. Hilden and B. Sacktor, *Kidney Int.* **14**, 279 (1978).
15. S. V. Shah, S. A. Kempson, T. E. Northrup and T. P. Dousa, *J. clin. Invest.* **64**, 955 (1979).