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Pantothenic Acid Uptake and Metabolism by the Red Blood Cell in the Rat

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PANTOTHENIC ACID UPTAKE AND METABOLISM BY THE RED BLOOD CELL IN THE RAT

By

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ABSTRACT

PANTOTHENIC ACID UPTAKE AND METABOLISM
BY THE RED BLOOD CELL
IN THE RAT

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Red blood cells (RBCs) contain bound forms of pantothenic acid (PA) which have never been characterized. Objectives of this research included determining the uptake and release mechanism for PA, and the PA derivatives, in the RBCs. Uptake of PA was studied by incubating RBCs with [^{14}C]PA, at varied specific activities, and varied sodium, glucose or pH levels. Release of PA was studied by incubating RBCs containing [^{14}C]PA, at three specific activities, in media. Uptake and release of PA by RBCs were nonsaturable, and uptake was unaffected by sodium, glucose or pH. PA derivatives were determined by enzymatic hydrolysis of RBCs followed by radioimmunoassay for PA, and paper chromatography. Radioimmunoassay quantitated PA, 4'-phosphopantothenic acid (4'-PPA) and pantetheine (PE). Peaks indicating the presence of PA and 4'-PPA appeared on chromatograms. We concluded that PA passively diffuses into and out of the RBC, and the RBC contains PA, 4'-PPA and PE.

To ALL my teachers.

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ABBREVIATIONS

ACP	acyl carrier protein
CoA	coenzyme A
dpCoA	dephospho-coenzyme A
HPLC	high performance liquid chromatography
PE	pantetheine
PA	pantothenic acid
PBS	phosphate buffered saline
4'-PPE	4'- phosphopantetheine
4'-PPA	4'- phosphopantothenic acid
4'-PPAcys	4'- phosphopantothenoylcysteine
RBC	red blood cell
RIA	radioimmunoassay

INTRODUCTION

Pantothenic acid (PA) is an essential B-vitamin needed to synthesize coenzyme A (CoA) and acyl carrier protein (ACP) of fatty acid synthase. CoA is necessary in the metabolism of carbohydrates, amino acids and lipids, and ACP is essential for fatty acid biosynthesis in all nucleated mammalian cells.

The estimated safe and adequate intake for PA has been set at 4 - 7 mg/day by the Food and Nutrition Board (1989) for adolescents and adults, and a higher unspecified intake is recommended for pregnant and lactating women. Although PA is ubiquitous in a normal diet, there are certain populations who may be at risk of attaining a marginal deficiency due to various physiological stresses: alcoholics, pregnant and lactating women, and adolescents. Alcoholics generally get most of their calories from alcohol and consequently suffer from B complex vitamin deficiencies (Olson 1973). Tao and Fox (1976) measured urinary excretion of PA in patients in an alcoholism rehabilitation program and found that the excretion decreased from 6.6 to 2.7 mg/day with increasing time in the program. They postulated that this was because the body was better able to utilize PA as it became healthier.

Pregnant and lactating women may not be able to consume enough food to provide the additional PA required. Cohenour and Calloway (1972) found that pregnant teenagers who had the same intake of PA as nonpregnant controls, had a lower blood bound PA concentration than controls and consequently advised supplementation of 5 - 10 mg/day during pregnancy. Song et al (1985) reported that pregnant and lactating women, whose PA intake was the same as controls, had a depressed blood concentration of PA.

Adolescents may become marginally deficient in PA due to poor diet choices. After determining PA intake in adolescents, Eissenstat et al. (1986) reported that 49% of female and 15% of male adolescents had intakes below the minimum suggested.

Although PA deficiency per se has not been reported in a free-living human population, there is no evidence that the deficiency does not exist or has not been identified because of the lack of sensitive and specific biochemical parameters to assess PA nutriture in an organism. The extent of health impacts of marginal or advanced deficiency of PA is yet unknown.

Total PA consists of the PA derivatives (4'-phosphopantothenic acid (4'-PPA), 4'-phosphopantothenoylcysteine (4'-PPAcys), 4'-phosphopantetheine (4'-PPE), dephospho-coenzyme A (dpCoA), CoA and pantetheine (PE)) as well as free PA. PA derivatives are also called bound forms of PA, referring to

the additional phosphate, cysteamine and adenosine groups attached to PA to make the derivative. Current biochemical methods to evaluate PA nutritional status include the measurements of total and free forms of PA in urine, serum and whole blood. It is not clear, however, which of these parameters, or combinations of them, predict true PA status of tissue concentrations or functional capacities: urinary PA concentration (mg/day) appears to reflect short-term dietary PA intakes rather than tissue stores; in serum which contains only free PA as delineated by Song et al. (1984, 1985), PA concentration is controlled by the gastrointestinal and renal systems and reflects transport of the vitamin between organs and tissues dependent on metabolic and hormonal states. Although whole blood PA concentration has been determined by several investigators in an attempt to assess PA nutriture, total PA concentration of whole blood varies widely in the published literature (Song et al. 1985).

Bound and free forms of PA have been quantitated in various blood components (Bates and Song 1987) and the erythrocyte contains most of the PA in the blood, in bound forms. However, the chemical forms of PA in the RBC, uptake mechanisms of PA by erythrocyte, and the interrelation between PA metabolism in erythrocytes and those in tissue and organ cells have never been reported. Unlike the biological fluids or usual dietary intake, organ and tissue samples are inaccessible to relate to nutritional status.

Information on PA uptake by the erythrocytes can be useful in relation to nutritional requirement for the vitamin; RBCs are easily accessible cells and metabolize PA as evidenced by containment of bound forms of the nutrient, which have never been identified or quantitated.

Our overall hypothesis, which encompasses the present study plus additional studies, is that the RBC mimics, in a limited way, organ or tissue cells in PA uptake or metabolism, and will serve as a reliable and sensitive measure of PA nutriture on the following grounds: 1) the first step of the synthetic pathway of PA to two important functional coenzyme forms, i.e., CoA and 4'-PPE of fatty acid synthase, is the rate limiting step modulated by pantothenate kinase. In order to possess bound forms of PA, RBCs must carry out the first and most important control step in the synthesis of CoA; 2) of the easily accessible biological fluids (e.g. urine and blood), only blood cells contain bound forms of PA. All nucleated tissue cells are known to contain CoA, as well as other bound forms of PA; 3) the abundance of the RBCs in blood, compared to other blood cells, will make it possible to use small amounts of blood samples for assay by existing methods.

Therefore, the objective of this research, which is the first step in proving our overall hypothesis, was to study the metabolism of PA by the RBC. Specific objectives in studying the metabolism of PA by the RBC were: 1) to determine the uptake and efflux mechanism of PA by the RBC,

and 2) to identify the PA derivatives in the RBC. We hypothesize that: 1) the uptake and efflux mechanism of PA by the RBC may be the same as for other tissues which have been studied, sodium dependent active transport, and 2) the RBC does not sythesize CoA, based on the cellular organization and metabolism and a few preliminary evidences that CoA could not be detected in the blood. The RBC would then maintain a relatively simple model for PA metabolism while retaining the critical control pathway to CoA.

REVIEW OF LITERATURE

PANTOTHENIC ACID DEFICIENCY

Although PA deficiency symptoms have not been reported in free-living humans, they can be induced with the metabolic antagonist omega-methyl PA added to a PA deficient diet. Studies using this antagonist have reported the following symptoms in humans after about 4 weeks: fatigue, anorexia, constipation, epigastric distress, numbness and tingling in the hands and feet, susceptibility to infection, personality changes and depression. Biochemical changes include an impaired ability to acetylate p-amino benzoic acid, increased sensitivity to insulin, adrenal cortical hypofunction and decreased plasma cholesterol (Bean et al. 1955, Hodges et al. 1958). In all the studies utilizing the antagonist omega-methyl PA, PA was never measured directly in whole blood or serum, because the antagonist interferes with measurement of PA in biological tissues (Fry et al. 1976). Instead, measurements of PA deficiency included insulin tolerance tests, acetylation of p-amino benzoic acid, excretion of 17 ketosteroids and eosinopenic response to ACTH. However, when young men were fed a purified, partly synthetic PA deficient diet without an antagonist, PA concentrations of biological fluids could be measured (Fry

et al. 1976). Urinary PA excretion decreased from 3.05 to 0.79 mg/day and total blood PA also decreased in the PA deficient group. Although, it was 12 weeks before the men became ill, and they never developed clinical symptoms of PA deficiency.

Rats develop the following signs of PA deficiency after 2 - 3 weeks on a PA deficient diet: decreased growth rate, discoloration of fur, anorexia, gastrointestinal disorders, reduced antibody production, impaired adrenal function as measured by depletion of sudanophilic and ketosteroid substances from the adrenals, diarrhea and muscle weakness (Barboriak et al. 1956). Reproductive problems reported in PA deficiency also include failure of implantation, resorption or defective litters such as low birth weight and low litter count (Nelson and Evans 1946). Since the rat develops PA deficiency with comparable signs to those seen in the human, the rat has often been used as a model to study pantothenic acid metabolism. Low serum PA concentrations have been measured in rats on PA deficient diets showing these deficiency signs. Therefore we can assume that the symptoms in humans are also accompanied by decreased serum PA, even though it could not be measured due to the interference by the antagonist. Also, very little is known about the mechanisms of the reported signs/symptoms or biochemical changes resulting from PA deficiency.

METHODS TO EVALUATE PANTOTHENIC ACID STATUS

The biochemical parameters that have commonly been used in assessment of PA status in humans include: total and free PA in whole blood and plasma, and urinary excretion of PA (mg/day). However, there is controversy on which, if any, is the best measure. Although physiologically related or interdependent, each of these biological fluids contains different forms of the vitamin in differing amounts. Plasma and urine contain only free PA at approximately 100 - 150 ng/ml and 3 - 5 μg/ml (computed with 1 liter urine per day), respectively (Song et al. 1985). Whole blood contains free and bound forms of PA at 400 - 600 ng/ml, although reported total PA levels have varied in healthy individuals from 130 - 2622 ng/ml in whole blood (Song et al. 1985). The bound form of PA in whole blood has never been identified, but is often assumed to be CoA (Friedrich 1988; Combs 1992; Machlin 1990). The weakness of using dietary intake of PA as a valid measure of the vitamins status lies in the variability of intake, accuracy of reporting, bioavailability of foods and physiological state of the host.

Several studies have reported correlations among the different parameters. Fox and Linkswiler (1961) reported a high correlation (r = 0.805) between urinary excretion of PA (mg/day) and dietary intake of PA, in a population of young adult women who were put on various diets containing different amounts of PA. In a study on adolescents (Kathman

and Kies 1984), a strong correlation was found between serum PA and dietary PA (r = 0.891), but no correlation was found between dietary PA and urinary PA or between serum PA and urinary PA. In contrast, the same study found in adults that dietary PA correlated well (r = 0.831) with urinary PA, and dietary PA was not correlated with serum PA concentration. Another study of adolescents (Eissenstat et al. 1986), reported a correlation between dietary PA and urinary PA (r = 0.60), and moderate correlations between dietary PA and whole blood PA (r = 0.38) and between dietary PA and RBC PA (r = 0.38). No correlation was reported between urinary PA and whole blood or RBC PA. Song et al. (1985) found similar results in free-living pregnant and lactating women. Dietary PA correlated with urinary PA (r = 0.5) and whole blood (r = 0.2), but whole blood PA and dietary PA did not correlate with plasma PA (r = -0.02).

The disagreement in the findings might have been partly attributed to inherent problems or potential errors associated with surveys with human subjects. For example, reported dietary intake data are not without significant errors on the part of subjects and interviewers, and also vary between subjects and among days. Biological specimens were also collected on the same day for the dietary data, although their interaction may not occur immediately. The conflicting results also infer that the metabolism of PA in each of the parameters is controlled by different mechanisms. Plasma PA is controlled by the

gastrointestinal and renal systems and reflects transport of the vitamin between organs and tissues dependent upon hormonal and metabolic states, while urinary PA reflects dietary PA intakes rather than tissue stores. Whole blood contains both plasma and RBCs, so it reflects the same controls as plasma plus the metabolism of PA in the RBC, since we know that the RBC contains mainly bound forms of PA. Each measurement then reflects a different stage of PA nutriture or metabolic state.

FUNCTION OF PANTOTHENIC ACID IN THE RBC

Figure 1 shows the metabolic pathway of PA to and from CoA and ACP, the active forms of PA in all nucleated mammalian cells. The first three enzymes, pantothenate kinase, phosphopantothenoylcysteine synthetase and phosphopantothenoylcysteine decarboxylase are located in the cytosol, while dephospho-CoA pyrophosphorylase and dephospho-CoA kinase complex are in the mitochondria as well as in the cytosol (Skrede and Halvorsen 1979).

Many reactions which require CoA in mitochondria include: conversion of pyruvate to acetyl-CoA, transfer of acetyl groups to oxaloacetate and conversion of ∝-ketoglutarate to succinyl CoA in the TCA cycle; formation of heme from succinyl CoA which condenses with glycine to form δ-aminolevulinic acid, the source for all nitrogen and carbon atoms of the porphyrin molecule of heme (Shemin et al. 1955); activation and oxidation of fatty acids via

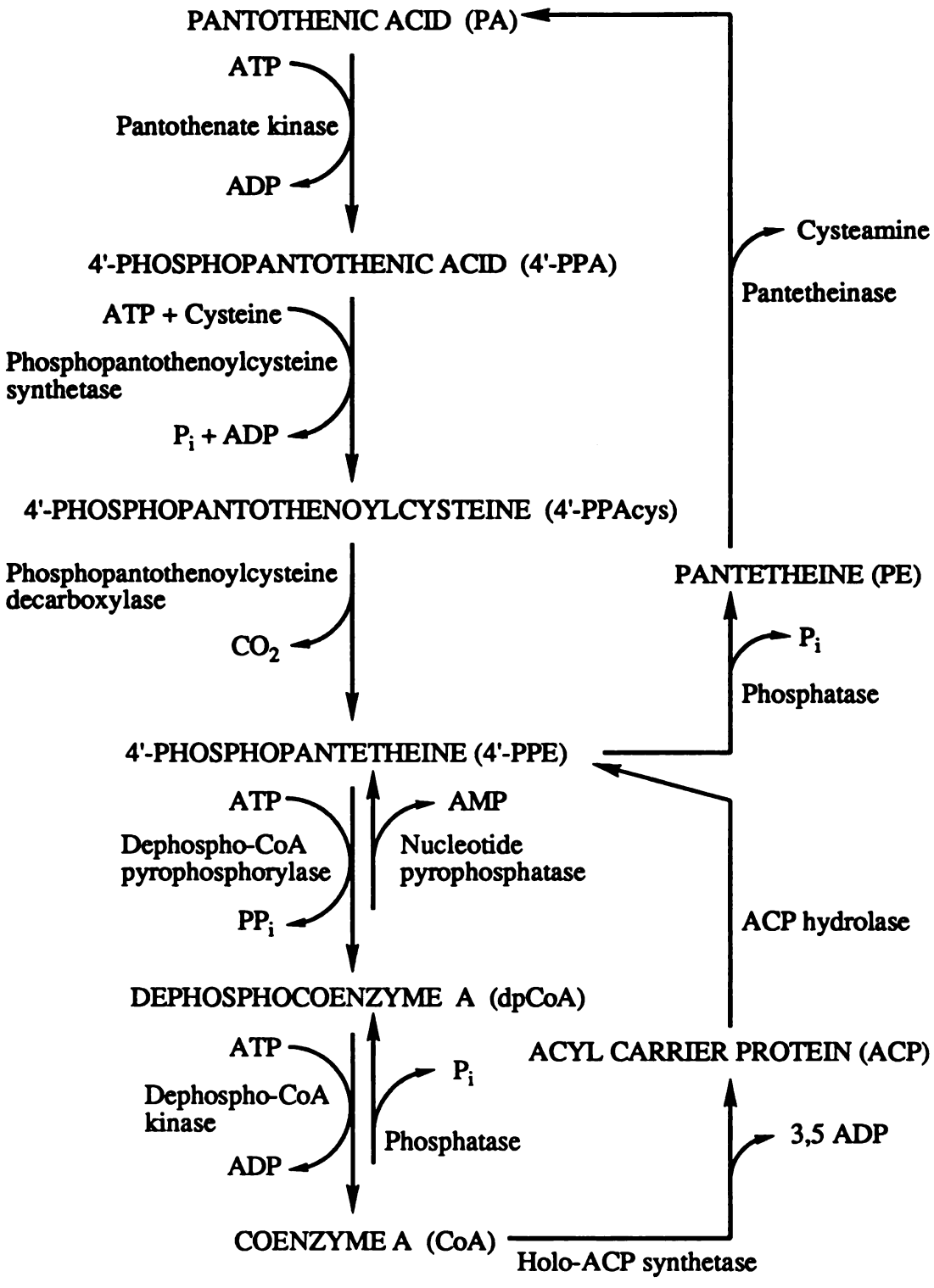


Figure 1. Metabolism of Pantothenic Acid.

β-oxidation; activation and metabolism of ∝-keto acids from branched chain amino acids; and synthesis of cholesterol, steroid hormones and prostaglandins. 4'-PPE of ACP, the prosthetic group of fatty acid synthase serves as a functional arm in fatty acid synthesis (Stryer 1975).

The reticulocyte has the metabolic capability of nucleated cells with mitochondria to perform these reactions. As the reticulocyte matures to the RBC, the mitochondria is lost as well as the enzyme acetyl CoA carboxylase (Pittman and Martin 1966), which is required for conversion of acetyl CoA to malonyl CoA in the pathway of fatty acid biosynthesis. Since the mature RBC does not need CoA or ACP, PA would not have to be converted to either of these. Instead it may metabolize PA to one of the other intermediates upon entering the RBC.

METHODS TO QUANTITATE PANTOTHENIC ACID AND ITS DERIVATIVES

Free PA is often quantitated in biological tissues by a microbiological assay (Skeggs and Wright 1944, Hatano 1962, Yoshioka et al. 1968), a radioimmunoassay (RIA; Wyse et al. 1979), an indirect enzyme linked immunosorbant assay (Song et al. 1990) or a semi automated radiometric-microbiological assay (Guilarte 1989). Quantitation of total PA by these assays requires the enzymatic hydrolysis of bound forms of PA (which include CoA and intermediates) in samples with alkaline phosphatase and pantetheinase prior to applying the assay procedures for free PA (Hoagland and Novelli 1954).

CoA can be quantitated by the phosphotransacetylase end point method (Michael and Bergmeyer 1974), the ∝-ketoglutarate dehydrogenase reaction (Garland et al. 1965), a radioisotopic assay (Knights and Drew 1988) or by high performance liquid chromatography (HPLC; Robishaw et al. 1982; DeBuysere and Olson 1983).

Acyl-CoAs can be quantitated by hydrolysis of acyl groups with 3 mol KOH/L at pH 11 and heating for 15 minutes at 55°C, then assaying for free CoA. Alternatively, acyl-CoAs can be directly quantitated with the HPLC (DeBuysere and Olson 1983). ACP can be quantitated by the same alkaline hydrolysis procedure as for acyl-CoAs. In this case, 4'-PPE is hydrolyzed and then assayed as for total PA.

Nakamura and Tamura (1972) separated qualitatively PA, 4'-PPA, pantethine, 4'-phosphopantetheine-S-sulfonic acid and pantetheine-S-sulfonic acid, in biological specimens, by a combination of paper chromatography and electrophoresis. Other methods, namely, paper chromatography (Michelson 1964) and ion exchange chromatography (Nakamura et al. 1972) separated the PA derivatives partially, with an overlap occurring between 4'-PPA, 4'-PPAcys and 4'-PPE (Skrede and Halvorsen 1979). A modification of Nakamura's ion exchange column procedure (Smith 1978), which used DEAE cellulose paper and formic acid (0.5 mol/L) as solvent, separated PA, dpCoA and CoA standards, which were purchased, and 4'-PPE which must be synthesized (Halvorsen and Skrede 1980).

These PA derivatives were quantitated in liver from the rat injected with [^{14}C]PA. The resulting paper chromatogram was cut into sample columns, each sample column was cut into ¼ - ½ inch segments and counted for radioactivity.

Halvorsen and Skrede (1980) presented several solvent systems for use on the HPLC to separate standards of PA, 4'-PPA, 4'-PPAcys, 4'-PPE, dpCoA and CoA. All standards were radiolabeled, except dpCoA. The eluate from the column was collected and counted for radioactivity and simultaneously monitored for uv absorption at 254 nm. DpCoA was quantitated by using CoA as an internal standard, all other PA derivatives could be quantitated by counting for radioactivity. In 1982, Robishaw et al. quantitated PA, 4'-PPA and CoA, from rat heart tissue, using a gradient system on the HPLC. The retention times of the standards, determined by absorbance at 205 nm, were 25, 9.5, 29.5, 45.5 and 38 minutes for PA, 4'-PPA, 4'-PPE, dpCoA and CoA, respectively. Hearts were perfused with radiolabeled PA, prepared and injected into the HPLC. Fractions were collected from the HPLC for each corresponding peak of the standards and counted for radioactivity. The PA derivatives in the tissue samples were quantitated by the radioactivity rather than by the absorbance because many other compounds in biological samples absorb at 205 nm. 4'-PPE and dpCoA could not be recovered because isotopically labeled standards were not available commercially and could not be synthesized.

Of the methods described, paper chromatography gives qualitative information on PA derivatives present and has the advantages of being easy, quick and economical. When combined with RIA for total and free PA, paper chromatography can provide much information in little time. However, if quantitation of all PA derivatives is required, the HPLC is the most efficient. The compromise for HPLC would be time and money for setting up initial conditions and separation of standards. If acyl-CoAs need to be identified and quantitated, the HPLC technique requires a separate set of initial conditions.

PANTOTHENIC ACID TRANSPORT MECHANISMS

Transport of PA has been studied in many cell or tissue systems. Many terms are used in the literature pertaining to transport. Uptake, influx and accumulation are interchangeable terms which encompass transport and part of metabolism. To measure only transport of a compound, the metabolism of the compound inside the cell must be made inoperative, with the implication that the metabolism is already known. Uptake studies do not require a disconnection of transport from metabolism in the cell. In fact, the metabolic continuity is maintained in order to learn about the metabolism. Efflux and release are also interchangeable terms indicating the amount or rate of the compound coming out of the cell.

Sugarman and Munro (1980) studied, in vitro, the

accumulation of radiolabeled PA by adult rat adipocytes. Inhibitors of oxidative metabolism (i.e. cyanide, 2,4-dinitrophenol) added to the incubation medium resulted in a decreased uptake of PA. Other water soluble vitamins did not affect the uptake of radiolabeled PA. Whereas, unlabeled PA and decreased temperature caused decreased uptake of radiolabeled PA suggesting that PA uptake was energy and temperature dependent, and the uptake process was specific for PA. Albumin, which was necessary in the media system to bind free fatty acids released from adipocytes, bound to PA also. Therefore, the authors reported an inability to calculate the exact kinetics of PA uptake.

Smith and Milner (1985) investigated, in vitro, the mechanism of PA transport across plasma membranes of rat liver parenchymal cells by determining initial velocity of [^{14}C]PA influx and efflux. The authors found that transport occurred by a carrier mediated system, from 0.3 - 36.5 μmol PA/L, and primarily by passive diffusion with 50 - 200 μmol PA/L in the media. The uptake of [^{14}C]PA was measured in the presence of amino acids, carboxylic acids (i.e. carnitine, pyruvate, 2-oxoisovalerate, 2-oxoglutarate, n-butyrate, DL-3-hydroxybutyrate and 2-oxovalerate), pantothenol and unlabeled PA. Of these only DL-3-hydroxybutyrate, 2-oxoisovalerate and pantothenol were weak inhibitors.

To determine dependence of PA uptake on sodium, the media concentration of sodium was varied by substituting,

iso-osmotically, choline chloride or lithium chloride for 116 mmol/L sodium chloride. The K_m of PA transport decreased with increasing sodium concentration in the media, and the K_{mpant} at zero sodium concentration was 250 μmol/L. The effect of intracellular sodium concentration on PA uptake was examined using cells incubated with ouabain, an inhibitor of Na+, K+ -ATPase, or gramicidin D, a Na+ ionophore. Both decreased the uptake of PA.

Respiratory effectors (i.e. cyanide, azide, 2,4-dinitrophenol) decreased the initial velocity of [^{14}C]PA influx and the strength of the inhibitory effect increased with incubation time. Velocity of [^{14}C]PA uptake was not significantly affected by preloading cells with PA. The reported K_m and V_{max} of PA transport were 11 μmol/L and 350 nmol/mg protein/min, respectively. The efflux of [^{14}C]PA was measured with a K_m of 85 ± 29 μmol/L and a V_{max} of 0.015 ± 0.003 nmol/mg protein/min. PA efflux was not sensitive to sodium, PA, ouabain, gramicidin D or 2,4-dinitrophenol added to the external media. They concluded that PA is cotransported by the rat liver parenchymal cell in a 1:1 ratio with sodium on a carrier highly specific for PA; sodium decreases the apparent K_m for PA, and a sodium carrier complex forms only on the intracellular side of the membrane.

Transport of [^{14}C]PA by the choroid plexus and brain slices of rabbit was studied, in vitro, by Spector and Boose (1984). The choroid plexus accumulated [^{14}C]PA against a

concentration gradient and the accumulation was inhibited by unlabeled PA, probenecid and caproic acid. N-ethylmaleimide and poly-L-lysine, which block sodium transport, inhibited uptake of [^{14}C]PA, and dinitrophenol and iodoacetic acid almost stopped accumulation totally. Accumulation of [^{14}C]PA, in the brain, declined over time and was slower than in the choroid plexus. PA, dinitrophenol, iodoacetic acid, probenecid, N-caproic acid and N-ethylmaleimide at 37°C and at 1°C inhibited uptake of [^{14}C]PA by the brain slices. The amount of phosphorylated [^{14}C]PA after accumulation was determined in both brain and choroid plexus by separation of [^{14}C]PA from phosphorylated forms of PA with paper chromatography. After developing the chromatogram, labeled phosphorylated PA forms were detected by counting for radioactivity. It was found that up to 43% of [^{14}C]PA taken up by the tissue incubated in PA concentrations of 0.5 - 3 μmol/L in the media was phosphorylated. Efflux studies of the choroid plexus and brain slices, after incubation with [^{14}C]PA, showed that [^{14}C]PA was readily released whether PA was present in the media or not, but when the temperature was decreased the efflux decreased also. They concluded that the choroid plexus contains an active transport system for PA which may depend on sodium and the exact mechanism for brain accumulation of PA is uncertain although facilitated diffusion with intracellular trapping by phosphorylation was highly speculated.

Spector et al. (1986) also studied transport of [^{3}H]PA, in vitro, across the blood-brain barrier of the rat. They reported that PA is transported by a saturable system with a K_m of 19 μmol/L and a V_{max} of 0.21 nmol/g brain/min.

PA transport was studied, in vitro, across the renal brush-border membrane vesicles of the rat (Karnitz et al. 1984) and the rabbit (Barbarat and Podevin 1986). Both vesicles accumulated PA above equilibrium values in the presence of a sodium gradient. The K_m and V_{max} were reported as 7.3 μmol/L and 23.8 pmol/mg protein/min for rat, and 16 μmol/L and 6.7 pmol/mg/10s (40.2 pmol/mg/min) for rabbit. 4'-PPE and 4'-PPA were taken up and consequently inhibited PA uptake in the rat vesicles. The conclusion for the vesicles from both rat and rabbit was that PA is transported via a sodium dependent, active transport process. In the rabbit cell, the PA anion is transported with two sodium ions.

Lopaschuk et al. (1987) measured, in vitro, PA transport in perfused rat heart and sheep cardiac sarcolemmal vesicles. In rat heart the reported K_m was 10.7 μmol/L and the V_{max} was 418 nmol/g dry/30 min (13.9 nmol/g dry/min). Sodium decreased incrementally in the perfusate resulted in decreased PA uptake. Addition to the perfusate of a mixture of amino acids, whose uptake is sodium dependent, also resulted in decreased uptake of PA. When an inward sodium gradient was applied to the sarcolemmal vesicles, PA uptake was rapid, but uptake was markedly

decreased when sodium was replaced by potassium or if external sodium was below 40 mmol/L. Also, PA uptake in the vesicles increased with increasing PA concentration in the presence of sodium. They concluded that PA is transported across the myocardial sarcolemmal membrane and heart by a sodium dependent carrier mediated process. Beinlich et al. (1989) measured [^{14}C]PA uptake in perfused rat heart. The rate of PA transport was saturable, with a K_m of 11 μmol/L and V_{max} of 630 nmol/g dry/h (10.5 nmol/g dry/min), which is comparable to the Lopaschuk study.

Turner and Hughes (1962) did not detect transport of PA against a concentration gradient in everted sacs of rat intestine and concluded that PA was absorbed by passive diffusion through the intestine. Although, these studies were performed without radiolabeled PA and the high concentration of PA used (100 mmol/L) wasn't optimal for demonstrating transport mechanisms. Shibata et al. (1983) reported that in the rat gut, PA is hydrolyzed from CoA by the following series of reactions: CoA to 4'-PPE to PE to PA, and then passively diffuses at PA concentrations of 7.5 μmol/L - 0.15 mol/L into the rat small intestine. Fernstermacher and Rose (1986) reevaluated, in vitro, rat and chick intestinal absorption of PA, using 0.9 - 20.9 μmol [^{3}H]PA/L. They reported that PA absorption, in both tissues, demonstrates sodium dependence and saturation kinetics, with a K_m for PA of 17 ± 2 μmol/L and a V_{max} of 972 ± 80 pmol/cm^2/h (16.2 pmol/cm^2/min). They concluded, at

concentrations at or below 0.9 μmol/L, PA is absorbed by a sodium dependent active transport process. Stein and Diamond (1989) studied PA uptake in mouse intestine, in vitro, and reported a K_m of 21 μmol/L and V_{max} of 260 pmol/cm^2/min, for a sodium dependent saturable transport system.

Grassl (1992) investigated PA transport by epithelial cells of the maternal facing membrane of human placenta. He measured flux of [^{14}C]PA (2 μmol/L) across the membrane in the presence of the cations Na^+, Li^+, K^+ or choline (100 mmol/L) and found a marked stimulation of PA uptake with Na^+. After pretreatment of membranes with gramicidin, PA uptake in the presence of Na^+ was reduced, indicative of a mediated cotransport process coupling Na^+ and PA. Dependence of PA uptake on membrane voltage was demonstrated when PA accumulated markedly when conditions were favorable for an inside negative voltage and valinomycin was present. Kinetic measurements of sodium dependent PA uptake measured the K_m at 9 μmol/L and V_{max} of 3 nmol/mg protein/min. When biotin (20 μmol/L) was added to kinetic studies of sodium dependent PA transport, the K_m for PA was increased to 16.6 μmol/L while the V_{max} remained the same. Also biotin analogues had the same effect on sodium dependent PA transport as on sodium-dependent biotin transport, suggesting biotin and PA compete for the same transporter.

Table 1 summarizes all PA transport mechanism studies, most tissues or cells studied transported PA by a sodium

Table 1. Pantothenic acid transport mechanisms in various tissues*

Tissue/cell	Active Transport	Sodium Required	K_m μmol/L	V_{max}	Reference
adipocyte (rat)	yes**	ND	NR	NR	Sugarman and Munro (1980)
liver (rat)	yes	yes	11	350 nmol/mg prot/min	Smith and Milner (1985)
choroid plexus (rabbit)	yes	yes	NR	NR	Spector and Boose (1984)
brain (rabbit)	no***	NA	NR	NR	Spector and Boose (1984)
blood brain barrier (rat)	yes	ND	19	0.21 nmol/g brain/min	Spector et al. (1986)
kidney (rat)	yes	yes	7.3	23.8 pmol/mg prot/min	Karnitz et al. (1984)
kidney (rabbit)	yes	yes	16	40.2 pmol/mg/min	Barbarat and Podevin (1986)
heart (rat, sheep)	yes	yes	10.7	13.9 nmol/g dry/min	Lopaschuk et al. (1987)
heart (rat)	yes	ND	11	10.5 nmol/g dry/min	Beinlich et al. (1989)
intestine (rat)	no****	NA	NA	NA	Turner and Hughes (1962)
intestine (rat, chick)	yes	yes	17.2	16.2 pmol/cm^2/min	Ferstermacher and Rose (1986)
intestine (mouse)	yes	yes	21	260 pmol/cm^2/min	Stein and Diamond (1989)
placenta (human)	yes	yes	9	3 nmol/mg prot/min	Grassl (1992)

* Abbreviations used: ND - not determined, NA - not applicable, NR - not reported
** temperature dependence
*** facilitated diffusion
**** passive diffusion at PA concentrations near 100 mmol/L

dependent active transport system. The transport mechanism and metabolism of PA into the RBC have never been investigated and it can not be assumed that RBCs would transport PA by the same system as other tissues do, because the RBC is uniquely different from all other tissue cells in its organelle makeup and function.

METABOLISM OF WATER SOLUBLE VITAMINS IN THE RBC

Thiamin is transported into the RBC by facilitated diffusion (Hoyumpa 1982). Once inside the cell, thiamin is phosphorylated to the di- and tri-phosphate esters. The diphosphate ester, thiamin pyrophosphate, is the active cofactor for transketolase which functions in the hexose monophosphate pathway in the RBC.

Riboflavin enters the RBC in its free form, then it is phosphorylated to flavin mononucleotide. Flavin mononucleotide is converted to flavin adenine dinucleotide, which is the coenzyme form required for erythrocyte glutathione reductase (Beutler 1969).

Nicotinic acid or nicotinamide is transported into the RBC by facilitated diffusion. Nicotinic acid is converted to nicotinamide mononucleotide and finally to nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate, which are used in glycolysis and the hexose monophosphate pathway in the RBC.

Folate is found in the RBC in nine times the amount of serum (Grossowicz 1962). The transport system specific for

the forms 5-CH_3-tetrahydrofolate and 5-HCO-tetrahydrofolate is saturable and influenced by anions, although efflux is not energy dependent (Branda et al. 1978; Branda and Anthony 1979).

Pyridoxine and pyridoxal are transported into the RBC by passive diffusion. This transport is temperature dependent, due to phosphorylation of pyridoxine and protein binding of pyridoxal (Mehansho and Henderson 1980). Pyridoxine is phosphorylated to pyridoxine phosphate which is converted, via an oxidase to pyridoxal phosphate, the main coenzyme form (Anderson et al. 1971). Binding to hemoglobin by pyridoxal phosphate causes a change in oxygen affinity for hemoglobin.

Uptake of vitamin B_{12} bound to transcorrin, by the RBC is dependent upon reticulocytes, calcium and magnesium in the serum (Retief et al. 1966).

Ascorbic acid is oxidized on or near the surface of the RBC to dehydroascorbic acid. Dehydroascorbic acid then diffuses through the lipid portion of the cell wall and is reduced back to ascorbic acid, trapping it inside the cell (Wagner et al. 1987). Dehyroascorbic acid, therefore, acts as an antioxidant in the RBC.

Very little is known about PA metabolism in the RBC. Uptake and metabolism of PA by the RBC has never been investigated before although, it is known that the RBC does metabolize PA to an extent as attested to by the presence of bound forms of PA in the RBC.

SUMMARY

A study of the uptake and metabolism of PA by the RBC is necessary for the following reasons: 1) there is no information concerning the uptake and metabolism of PA in the RBC, 2) CoA has always been assumed to be the major form of PA in the RBC, but no evidence is available that this assumption is true and 3) the RBC has no known metabolic pathways requiring CoA; 4) the RBC is uniquely different from all other tissue cells in its organization and metabolism, and therefore can not be assumed to take up or metabolize PA by the mechanisms of other tissue cells that have been studied; and 5) we hypothesize that PA metabolism in the RBC mimics tissue PA metabolism and will thus serve as a reliable measure of PA nutriture in the animal, but this will have to be proven in subsequent studies.

The most effective and efficient way to determine the presence of PA derivatives, qualitatively and quantitatively, is with a combination of paper chromatography and RIA, so these methods were chosen for the metabolism studies.

MATERIALS AND METHODS

CHEMICALS

[1-^{14}C]Pantothenate (2.11 GBq/mmol) was purchased from New England Nuclear (Boston, MA). Soluene 360 and Picofluor 40 scintillation cocktail were purchased from Packard Instrument Co (Downers Grove, IL). A plasma hemoglobin kit, thiopropyl-Sepharose 6B, *Crotalus Atrox* venom, ninhydrin spray reagent, sodium nitroprusside, CoA, dpCoA, PA, pantethine and alkaline phosphatase were purchased from Sigma Chemical Co (St. Louis, MO). Pantetheinase was kindly provided by Drs. Wittwer and Wyse's lab at Utah State University.

ANIMALS AND DIET

Sprague-Dawley rats weighing 300 - 500 g were used in all studies. The rats were allowed free access to rat chow (Purina 5001 Laboratory Chow, Purina Mills Inc, St Louis, MO) and water, until killed by cardiac puncture under methoxyflurane anesthesia.

BLOOD COLLECTION

Blood was collected from the rat by a cardiac puncture in Vacutainer tubes containing heparin (Beckton Dickinson,

Rutherford, NJ) and centrifuged in a table top centrifuge at 1000 X g for 10 minutes. The plasma layer and buffy coat were removed. The RBCs were washed twice with 0.9% NaCl (physiological saline), in the same volume as the removed plasma layer, and centrifuged at 1000 X g for 10 minutes. The saline and residual buffy coat were discarded.

UPTAKE AND RELEASE OF PA BY THE RBC

Preparation of the RBCs

Packed RBCs were resuspended in phosphate buffered saline (PBS) containing 10 mmol glucose/L, in a 1:1 ratio (0.4 mL and 10 mL for uptake and release experiments, respectively). This RBC preparation will be referred to as RBC-PBS throughout the thesis. Each data point represents the average of 5 separate determinations.

Viability of the RBC membrane

The viability of the RBC membrane to withstand various incubation periods and centrifugation force was evaluated by measuring the hemoglobin content of the medium layer of RBC-PBS (1:1, 0.4 mL). The RBC-PBS samples, without added PA were incubated and centrifuged, along with the samples mixed with radiolabeled PA, during the uptake and release experiments. The viability of the RBC membrane was considered acceptable when < 1% of hemoglobin in the cell was released into the media. The measured hemoglobin concentration in the media layer was consistent between

experiments in the range from 22 - 186 μmol/L and 34 - 176 μmol/L in the uptake and release experiments, respectively.

Counting of RBCs

RBCs were counted in samples of 0.2 mL of RBCs using a hemocytometer, in all experiments. The RBC count was used to standardize all uptake and release data to 10^7 RBCs, in order to compare experiments. The RBC count, in 0.2 mL of RBCs, was equated with nmols PA taken up or released by 0.2 mL of RBCs, which was calculated from the measured radioactivity and the known specific activity of each sample. Then nmols PA was standardized to 10^7 RBCs.

Quantification of PA in the RBC

PA was quantitated in RBCs, in all experiments as a measure of homogeneity between experiments. Total PA includes the bound PA forms (4'-PPA, 4'-PPAcys, 4'-PPE, dpCoA, CoA and PE) as well as free PA. Initial free and bound PA concentrations were measured in the RBC after the cells were hemolyzed by three freeze-thaw cycles. For determination of the bound forms of PA, 0.5 mL of packed RBCs were incubated at 37°C for 8 hours, with 0.1 mol/L Tris buffer (400 μL, pH 8.1), alkaline phosphatase (50 units), pantetheinase (100 units) and distilled water to a final volume of 1.5 mL. The enzymatic hydrolysis of RBC samples was terminated by adding four times the RBC volume of equimolar amounts of saturated $Ba(OH)_2$ and 10% $ZnSO_4$, in

that order. The mixture was vortexed, centrifuged at 4000 X g for 10 minutes, and the supernatant was saved for analysis of PA by RIA (Wyse et al. 1979), as follows: A mixture of 100 μL of supernatant, 12.5 μL of rabbit antisera against PA, 10 μL of [^{14}C]PA (117 Bq) and 227.5 μL of 1.5% rabbit serum albumin in PBS was vortexed, and then shaken at room temperature for 15 minutes. Saturated $(NH_4)_2SO_4$ (350 μL) was added to the mixture, vortexed and centrifuged at 8500 X g at 10°C for 15 minutes. The supernatant, containing PA (both ^{14}C labeled and unlabeled) unbound to the antibody, was aspirated off. The pellet, containing antibody bound PA (both ^{14}C labeled and unlabeled), was resuspended in 500 μL of 50% $(NH_4)_2SO_4$, vortexed and centrifuged again at 8500 X g for 15 minutes. The supernatant was discarded. Tissue solubilizer, Soluene 360 (300μL), was added to the pellet, vortexed and incubated at 60°C for 30 minutes. The mixture was vortexed after incubation, and to it was added 3 mL of scintillation cocktail; radioactivity of the sample was counted in a Tricarb 4000 series scintillation counter (Packard Instrument CO, Downers Grove, IL). PA standards (0.5 pmol - 10 nmol) were processed in the same manner as the samples to obtain a standard curve which was used to determine the PA concentration of the samples.

Uptake of PA by the RBC

Effect of incubation time and PA concentration. In studying the uptake of PA by the RBC, 0.4 mL of RBC-PBS was incubated at 37°C with five concentrations of PA: 0.34, 1.7, 3.4, 17.0, 34.0 μmol/L RBC-PBS for varying durations. The PA concentrations were chosen to surround the physiological PA concentration of rat whole blood (approximately 3 μmol/L; Hatano et al. 1967; Israel and Smith 1986). All media contained 0.34 μmol [^{14}C]PA/L (2.11 GBq/mmol); the final PA concentration was adjusted with unlabeled PA.

After incubation for 3, 7, 10, 20, 30, 40, 50, 60, 120, 240 and 360 minutes, the samples were briefly placed on ice to impede further PA transport and then centrifuged at 5000 X g for 3 minutes. The supernatant was aspirated off and saved. The RBCs were then washed twice with 0.2 mL of ice cold physiological saline and centrifuged at 5000 X g for 3 minutes. The supernatant and subsequent two washes were pooled, mixed with 10 mL of Safety Solve scintillation cocktail and counted for radioactivity by a Tricarb 4000 series scintillation counter to account for all the radioactivity originally added.

Data collection. The radioactivity in the resulting RBCs was counted after the following preparation of the cells: Each sample of the resulting packed RBCs (0.2 mL) was mixed with 1 mL of isopropanol-Soluene 360 (1:1), vortexed and incubated at 40°C for 30 minutes. The RBC mixtures were then mixed with 1.5 mL of 30% H_2O_2, to bleach, and left at

room temperature for 1 hour. The samples were then incubated at 40°C for 15 - 30 minutes to expel any remaining H_2O_2. When cooled, the bleached RBC samples were combined with 15 mL of Picofluor 40 scintillation cocktail, vortexed, and counted for radioactivity by a Tricarb 4000 series scintillation counter.

Estimation of radioactive contamination. Radioactive contamination left on the outside membrane surface of the washed RBCs was estimated as follows. RBC-PBS (1:1, 0.4 mL) samples were combined with the various concentrations of PA, which contained labeled and unlabeled PA, immediately centrifuged at 5000 X g for 3 minutes, washed twice in 0.2 mL of ice cold physiological saline and centrifuged at 5000 X g for 3 minutes. The RBCs were prepared and counted for radioactivity as described above. The radioactivity that remained in the RBCs prepared in this manner was assumed to be from [^{14}C]PA attached to the outer surface of the RBC.

Effect of sodium. PA uptake by the RBC was determined in the presence of varied concentrations of sodium (0, 38, 76, 152 mmol/L) and at the four concentrations of PA: 0.34, 3.4, 17.0, 34.0 μmol/L RBC-PBS. As described earlier, the [^{14}C]PA in each sample was kept constant at 0.34 μmol/L, and the desired final concentration of PA was achieved by adding unlabeled PA. The physiological osmolarity of medium with varying sodium concentrations was maintained by replacing NaCl and Na_2HPO_4 with equimolar LiCl. After incubation in the media with varied sodium concentrations at 37°C for 7

minutes, the RBC-PBS (0.4 mL, 1:1) samples were briefly placed on ice, centrifuged at 5000 X g for 3 minutes, washed twice in PBS containing the respective sodium concentrations and centrifuged at 5000 X g for 3 minutes. The washed RBCs and PBS washes were prepared and counted for radioactivity as described above.

Effect of glucose. The effect of glucose on the uptake of PA by the RBC was determined. Samples of 0.4 mL RBC-PBS (1:1) were incubated with 0.34 μmol [^{14}C]PA/L and with or without 10 mmol glucose/L at 37°C for 3, 7, 10, 20 and 30 minutes. After incubation, the RBC-PBS samples were put briefly on ice, centrifuged at 5000 X g for 3 minutes, washed twice in 0.2 mL of ice cold physiological saline and centrifuged at 5000 X g for 3 minutes. The washed RBCs and PBS washes were prepared and counted for radioactivity as described above.

Effect of pH. The effect of pH on the uptake of PA by the RBC was investigated. Samples of 0.4 mL RBC-PBS (1:1) at various pH (7.2, 7.3, 7.4, 7.5 and 7.6) were incubated with 0.34 μmol/L of [^{14}C]PA at 37°C for 3, 7, 10 and 20 minutes. After incubation the RBC-PBS samples were put briefly on ice, centrifuged at 5000 X g for 3 minutes, washed twice in 0.2 mL of ice cold physiological saline and centrifuged at 5000 X g for 3 minutes. The washed RBCs and PBS washes were prepared and counted for radioactivity as described above.

Release of PA by the RBC

Effect of incubation time and PA concentration. In determining the release of PA from the RBC, four RBC-PBS samples (10 mL, 1:1) were mixed with 0, 0.34, 3.4 and 34.0 μmol/L of PA. Again the latter three samples contained 0.34 μmol/L of [^{14}C]PA and the final PA concentration was obtained by adding unlabeled PA. After incubation at 37°C for 2 hours, the RBCs were separated from the media by centrifugation followed by washing as described above. The average radioactivity in 0.2 mL of RBCs at this point (zero time) was 37 Bq. The sample with no added PA was used for RBC counting and hemoglobin analysis.

Aliquots (0.2 mL) from each of the RBC preparations containing different concentrations of PA were then mixed with 0.2 mL of PBS and incubated at 37°C for 10, 20, 30, 40, 50, 60 and 240 minutes. Upon termination of incubation, each sample was briefly set on ice, then centrifuged at 5000 X g for 3 minutes to separate the cells from the media. The cells were washed twice in ice cold physiological saline (0.2 mL) and centrifuged at 5000 X g for 3 minutes.

Data collection. The mixture of cell supernatant and two washes, which contain [^{14}C]PA released from the cell, were pooled, mixed with 10 mL of Safety Solve scintillation cocktail and counted for radioactivity.

METABOLISM OF PA BY THE RBC

Quantification of PA Derivatives

Identification and quantification of PA derivatives in the RBC was investigated with the RIA for PA. Lysed fresh RBCs were subjected to enzymatic treatment, with pantetheinase alone, alkaline phosphatase alone, and both enzymes together. These preparations along with lysed RBCs which had not been subjected to enzymatic degradation, were analyzed by RIA for PA, as described above. Pantetheinase degrades PE to PA providing information on PE content of the RBC, and alkaline phosphatase removes phosphate groups therefore 4'-PPA will be degraded by alkaline phosphatase to PA. Both enzymes applied simultaneously break down all PA derivatives (4'-PPA, 4'-PPAcys, 4'-PPE, PE, dpCoA, CoA) to PA.

Paper Chromatography

Qualitative identification of PA derivatives in the RBC after uptake of PA by the RBC was accomplished with paper chromatography.

Preparation of standards. PA, [^{14}C]PA, dpCoA and CoA standards were purchased. Standards for three PA derivatives that are not available commercially were prepared as below.

4'-PPA standard was synthesized by the modified method of Halvorsen and Skrede (1980): Fresh rat liver (2 g) was homogenized in 1.0 mL of 20 mmol/L potassium phosphate

buffer (pH 7.2). The homogenate was centrifuged (10,000 X g) at 0°C for 20 minutes. One mL of the supernatant, which contains pantothenate kinase, was incubated at 37°C for 40 minutes with 1.5 mL of a solution of 0.2 mmol PA/L, 10 mmol ATP/L, 5 mmol $MgCl_2$/L, 2 mmol dithiothreitol/L, and 50 mmol tris buffer/L (pH 7.4). After the incubation, protein was precipitated by addition of five drops of methanol and centrifugation at 10,000 X g at 0°C for 20 minutes. The supernatant was subjected to chromatography on a thiopropyl-Sepharose 6B column and allowed to react for 30 minutes. The thiol groups of the matrix hang onto any thiol containing PA derivatives, allowing 4'-PPA to pass through. The column was prepared as follows: 1 g of gel material was equilibrated with 10 mL of potassium phosphate buffer (20 mmol/L, pH 8.0). This gave about 3 mL of matrix and the column used was 1 cm in diameter. 4'-PPA in the column was eluted with 6 mL of 20 mmol potassium phosphate buffer/L (pH 8.0).

4'-PPE standard was synthesized by the method of Halvorsen and Skrede (1980): A 1 mL solution of 2 mmol CoA/L, 8 units *Crotalus Atrox* venom, 2 mmol $MgCl_2$/L, 0.1 mmol dithiothreitol/L and 50 mmol tris buffer/L (pH 7.0), was incubated at 37°C for 30 minutes. The mixture was cooled on ice and diluted to 2 mL with 20 mmol potassium phosphate buffer/L (pH 8.0). The pH was adjusted to 8.0 with 0.1 mol NaOH/L. The solution was loaded onto a thiopropyl-Sepharose 6B column, prepared as described above,

and allowed to react for 30 minutes. The column was then washed with 6 mL of 20 mmol potassium phosphate buffer/L (pH 7.0) and final elution of 4'-PPE was accomplished with 8 mL of 0.1 mmol dithiothreitol/L in 20 mmol potassium phosphate buffer/L (pH 8.0).

PE was synthesized by reduction of disulfide bonds of pantethine with dithiothreitol, at ten times the concentration of pantethine.

Paper chromatography of standards. Standards were spotted on DEAE cellulose paper and developed in 0.5 mol formic acid/L (pH 3.1, adjusted with NH_4OH) for 4 - 4.5 hours. This gave a solvent front at 25 cm.

Chemical visualization of standards. PA and 4'-PPA were visualized by heating the dried paper chromatogram at 160°C for 30 minutes, spraying with ninhydrin, and heating again briefly. Heating causes ß-alanine to break from pantoic acid, and the former reacts with ninhydrin to produce violet color. Sulfhydryl goups of CoA, dpCoA and 4'-PPE were visualized using a sodium nitroprusside spray, (5 mL of 30% sodium nitroprusside dissolved in 1 mol H_2SO_4/L, mixed with 95 mL of methanol and 10 mL of 28% ammonia, and filtered).

After subjecting the standards to the chromatography and chemical visualization, it was determined that the amount of RBCs needed for visualization of its PA and PA derivatives, was much greater than amounts practical for the present study. Detection of PA derivatives in the RBC was

subsequently accomplished by using the RBC incubated with radiolabeled PA.

RBC preparation. 20 mL of RBC-PBS (1:1) were incubated at 37°C for 6 hours with 3.4 μmol [^{14}C]PA/L. After incubation the RBCs were separated by centrifugation at 1000 X g for 10 minutes. The media layer was removed, and the RBCs were washed twice with 10 mL of physiological saline, centrifuged at 1000 X g for 10 minutes and the saline wash was discarded.

Each sample (1 mL of packed RBCs) was combined with 2 mL of 6% $HClO_4$ containing 30 mmol dithiothreitol/L, to lyse the cells and precipitate proteins. The mixture was centrifuged at 10,000 X g for 5 minutes and the supernatant was neutralized to pH 6 - 7 with 3 mol KOH/L and centrifuged again. The supernatant was saved and dried in a vacuum oven. The dried sample was dissolved in 50 μL of 0.2 mol dithiothreitol/L (pH 3.1, adjusted with formic acid). The 50 μL samples were subjected to paper chromatography as the standards desribed above.

Data collection. The paper chromatogram of the prepared RBCs, after having dried, was cut into sample columns and each column was cut into 1 cm pieces, from base to solvent front. The 1 cm pieces were added to 5 mL of scintillation cocktail and 100 μL of distilled water, to increase solubility of radioactivity from paper (application note ABA-006, Packard Instrument CO, Meriden, CT). The mixture was counted for radioactivity in a scintillation

counter and the distribution of radioactivity was then compared to the visualized standards for identification.

Enzymatic degradation. Enzymatic degradation was also applied to RBCs after incubation with [^{14}C]PA for 6 hours as follows: Each RBC sample (1.0 mL) was mixed with 6% $HClO_4$ to lyse cells and precipitate proteins, as described above. The supernatant of each sample was incubated at 37°C for 8 hours with 0.1 mol/L tris buffer (400 μL, pH 8.1), either 200 units of pantetheinase, 100 units of alkaline phosphatase or both and distilled water to a final volume of 2.0 mL. At termination of enzymatic hydrolysis, all samples were boiled for 5 minutes and centrifuged at 10,000 X g for 5 minutes. The supernantant was dried in a vacuum oven, resuspended in 50 μL of 0.2 mol dithiothreitol/L and subjected to paper chromatography, as described above.

The subsequent data were suggestive of either pantetheinase breaking PA down to pantoic acid and β-alanine, or contamination in the pantetheinase preparation shifting the location of paper chromatography peaks. Pursuant to the answer, chromatograms were run on the following 8 samples: 1) 0.68 nmol of [^{14}C]PA was incubated with 20 units of low purity (182 units/mL) pantetheinase and 0.1 mol/L tris buffer (400 μL, pH 8.1) at 37°C for 5 hours. The solution was then boiled 5 minutes and centrifuged at 10,000 X g for 5 minutes. 2) 20 units of low purity (182 units/mL) pantetheinase was incubated with 0.1 mol/L tris buffer (400 μL, pH = 8.1) as for sample 1. After

incubation, to the solution was added 0.68 nmol of [^{14}C]PA, immediately boiled for 5 minutes and centrifuged at 10,000 X g for 5 minutes. 3) 0.68 nmol of [^{14}C]PA was incubated with 20 units of high purity (807 units/mL) pantetheinase and 0.1 mol/L tris buffer (485 μL, pH 8.1) at 37°C for 5 hours. The solution was then boiled 5 minutes and centrifuged at 10,000 X g for 5 minutes. 4) 20 units of high purity (807 units/mL) pantetheinase was incubated with 0.1 mol/L tris buffer (485 μL, pH 8.1) as for sample 3. After incubation, to the solution was added 0.68 nmol of [^{14}C]PA, immediately boiled for 5 minutes and centrifuged at 10,000 X g for 5 minutes. 5) 0.68 nmol of [^{14}C]PA was incubated with 10 units of alkaline phosphatase (40,820 units/mL) and 400 μL of 0.1 mol/L tris buffer (pH 8.1) for 5 hours at 37°C, boiled and centrifuged for 5 minutes at 10,000 X g for 5 minutes. 6) 0.68 nmol of [^{14}C]PA was incubated with 10 units of alkaline phosphatase (40,820 units/mL), 20 units of low purity pantetheinase (182 units/mL) and 400 μL of 0.1 mol/L tris buffer (pH 8.1) at 37°C for 5 hours, boiled and centrifuged for 5 minutes at 10,000 X g. 7) 0.68 nmol of [^{14}C]PA was added to 300 μL of 6% $HClO_4$, then neutralized to about pH 7 with 3 mol KOH/L. 8) 500 μL of 6% $HClO_4$, neutralized with 3 mol KOH/L, was mixed with 0.68 nmol of [^{14}C]PA. Supernatant from all 8 samples were subjected to paper chromatography, as described above.

STATISTICS

All data points in uptake and release experiments represent 5 replicates computed as mean ± SD. Regression analysis was performed on all uptake and release data with a significance level of $p < 0.001$. Data analysis for this study was done using the following statistics software packages: Statgraphics 3.0 (STSC Inc and Statistical Graphics Corp, Rockville, MD) and Crunch 4.0 (Crunch Software Corp, Oakland, CA). RIA data are reported as the mean ± SD of 4 - 6 replicates.

RESULTS AND DISCUSSION

PA UPTAKE AND RELEASE BY THE RBC

All data have been standardized to 10^7 RBCs which is from approximately 2 mL of whole blood, assuming a 50% hematocrit. Uptake of total PA followed the classical hyperbolic pattern of increasing rapidly for the first 2 hours then slowing down or leveling off at all PA concentrations (**Figure 2**). Uptake of total PA by the RBCs continuously increased with the amount of PA added (**Figure 3 & 4**); statistical analysis indicated significant least squares regressions at all time points (r^2 = 0.77 - 0.99, $p < 0.001$), which indicated a nonsaturable influx process. In each of these experiments, 0.34 μmol [^{14}C]PA/L was used with varying amount of unlabeled PA added to obtain the desired concentration. The same data were analyzed for the uptake of radioactive PA per 10^7 RBCs, rather than by the uptake of total PA (both labeled and unlabeled; **Figure 5 & 6**). A significant linear decrease was seen with labeled PA uptake with increasing initial PA concentration, after incubation for 20, 30, 50, 60 and 120 minutes ($p < 0.05$, with r^2 = 0.21 - 0.64). Since this effect was not seen at all time points and r^2 was low, it was concluded that total PA in the media had no affect on uptake of [^{14}C]PA, which is

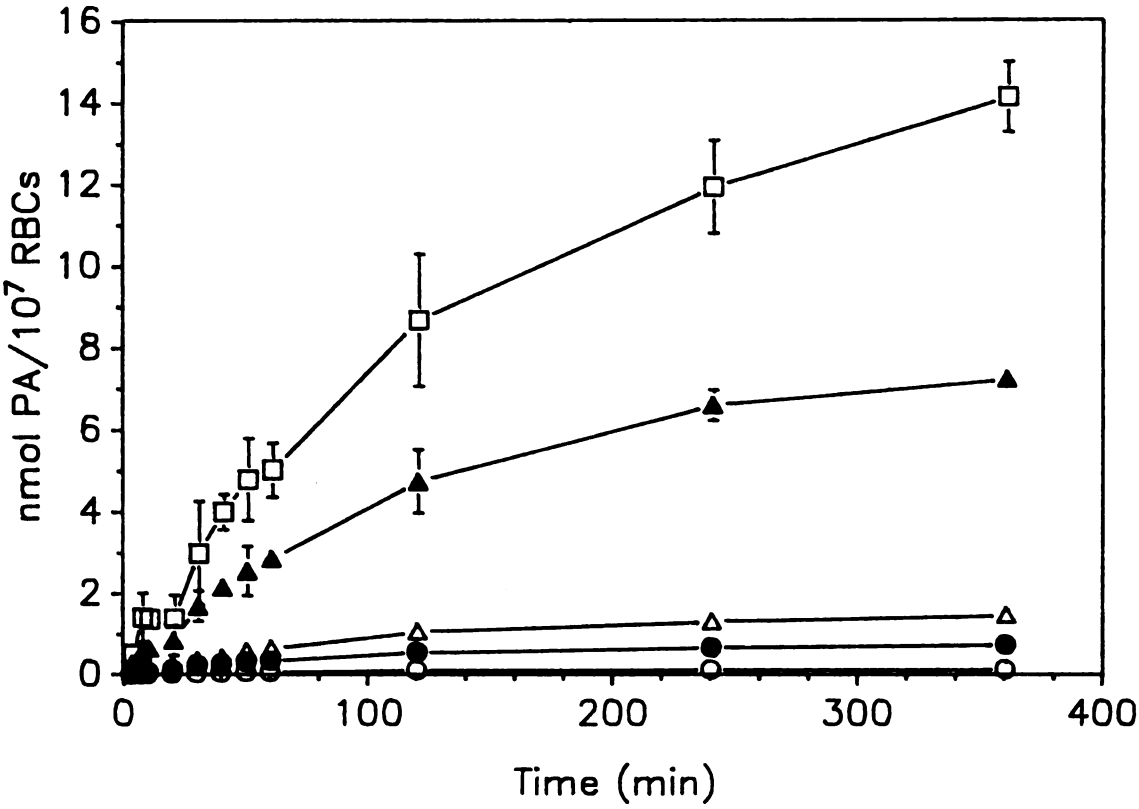


Figure 2. RBC uptake of PA vs incubation time.
The RBCs were incubated with PA concentrations of 0.34 (○), 1.7 (●), 3.4 (△), 17.0 (▲) and 34.0 (□) μmol/L at 37°C for 3, 7, 10, 20, 30, 40, 50, 60, 120, 240, and 360 minutes. All media contained a constant [^{14}C]PA concentration of 0.34 μmol/L, with varying specific activity achieved with unlabeled PA. PA concentrations were chosen to surround the average blood PA concentration in rats of 3 μmol/L. Each point represents the mean and SD of 5 replicates.

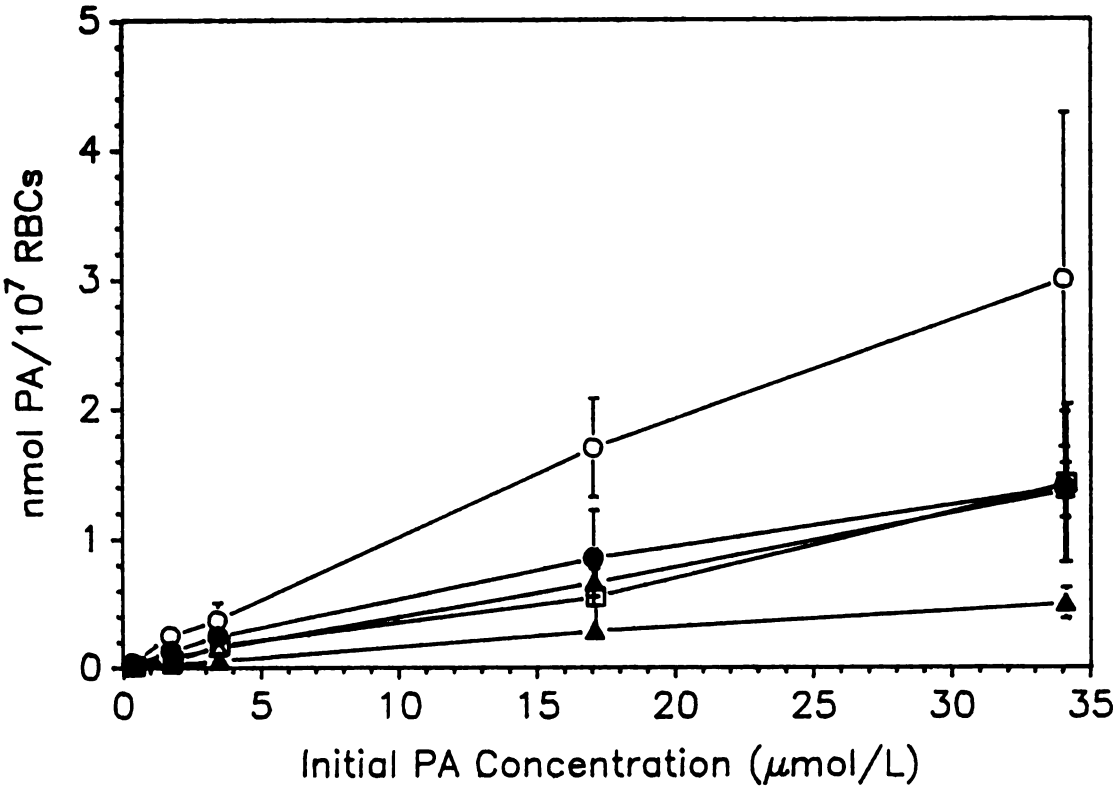


Figure 3. Total PA uptake by RBCs (3-30 min) vs initial PA concentration in media. The RBCs were incubated with PA concentrations of 0.34, 1.7, 3.4, 17.0 and 34.0 μmol/L at 37°C for 3 (▲), 7 (□), 10 (△), 20 (●) and 30 (○) minutes. All initial PA concentrations included 0.34 μmol [^{14}C]PA/L. All time points increased linearly (r^2 = 0.77 - 0.96, $p < 0.001$) which indicated a nonsaturable influx process, and each point represents the mean and SD of 5 replicates.

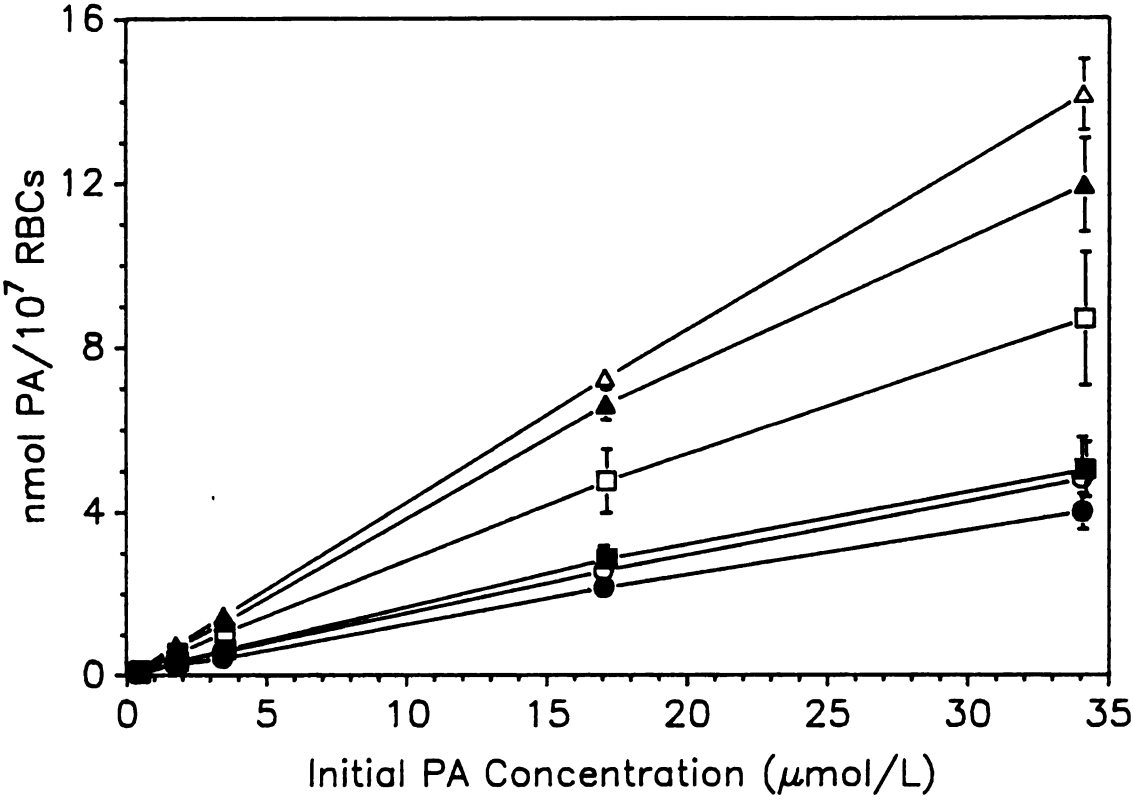


Figure 4. Total PA uptake by RBCs (40-360 min) vs initial PA concentration in media. The RBCs were incubated with PA concentrations of 0.34, 1.7, 3.4, 17.0 and 34.0 μmol/L at 37°C for 40 (●), 50 (○), 60 (■) 120 (□), 240 (▲) and 360 (△) minutes. All initial PA concentrations included 0.34 μmol [^{14}C]PA/L. All time points increased linearly (r^2 = 0.93 - 0.99, $p < 0.001$) which indicated a nonsaturable influx process, and each point represents the mean and SD of 5 replicates.

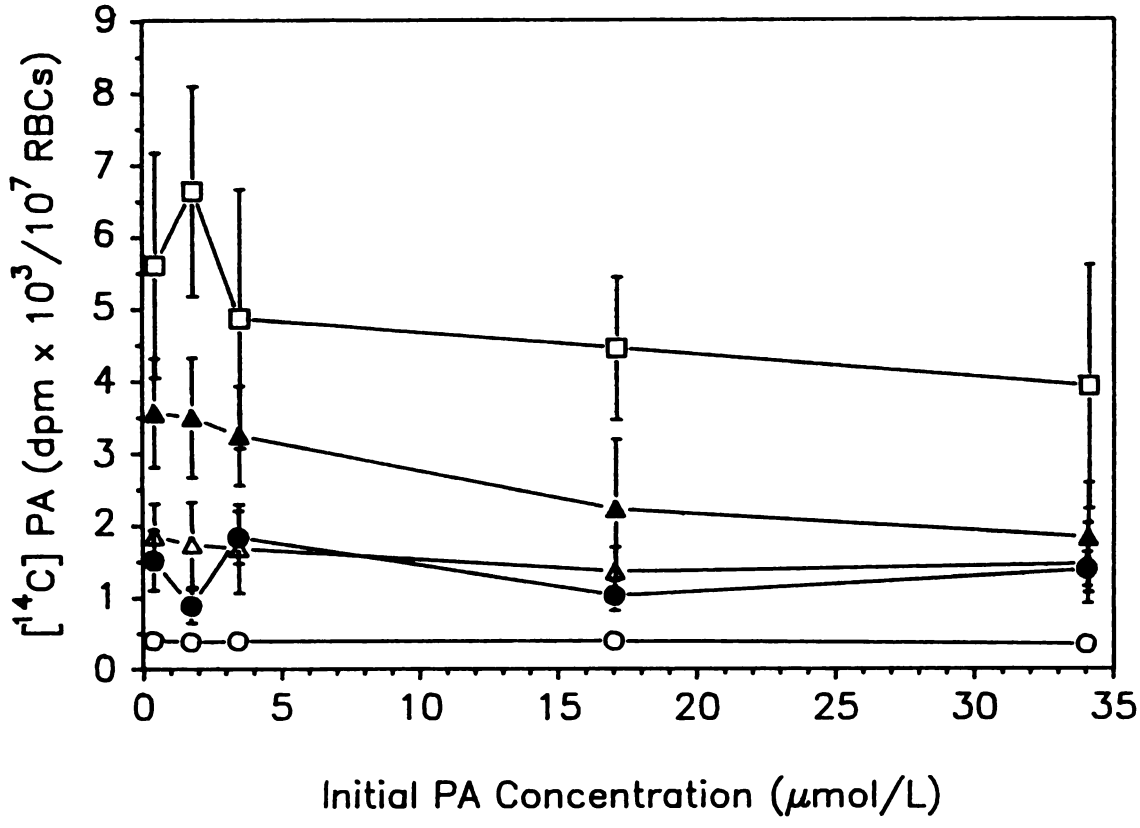


Figure 5. [^{14}C]PA uptake by RBCs (3-30 min) vs initial PA concentration in media. The RBCs were incubated with several PA concentrations (0.34, 1.7, 3.4, 17.0 and 34.0 μmol/l) all of which contained 0.34 μmol [^{14}C]PA/L, at 37°C for 3 (○), 7 (●), 10 (△), 20 (▲) and 30 (□) minutes. Regression analysis of [^{14}C]PA uptake showed a significant linear decrease at 20 and 30 min ($p < 0.05$, $r^2 = 0.21 - 0.46$). Since the effect was not seen at all time points and r^2 was very low, it was concluded that total PA in the media has no affect on labeled PA uptake. Each point represents the mean and SD of 5 replicates.

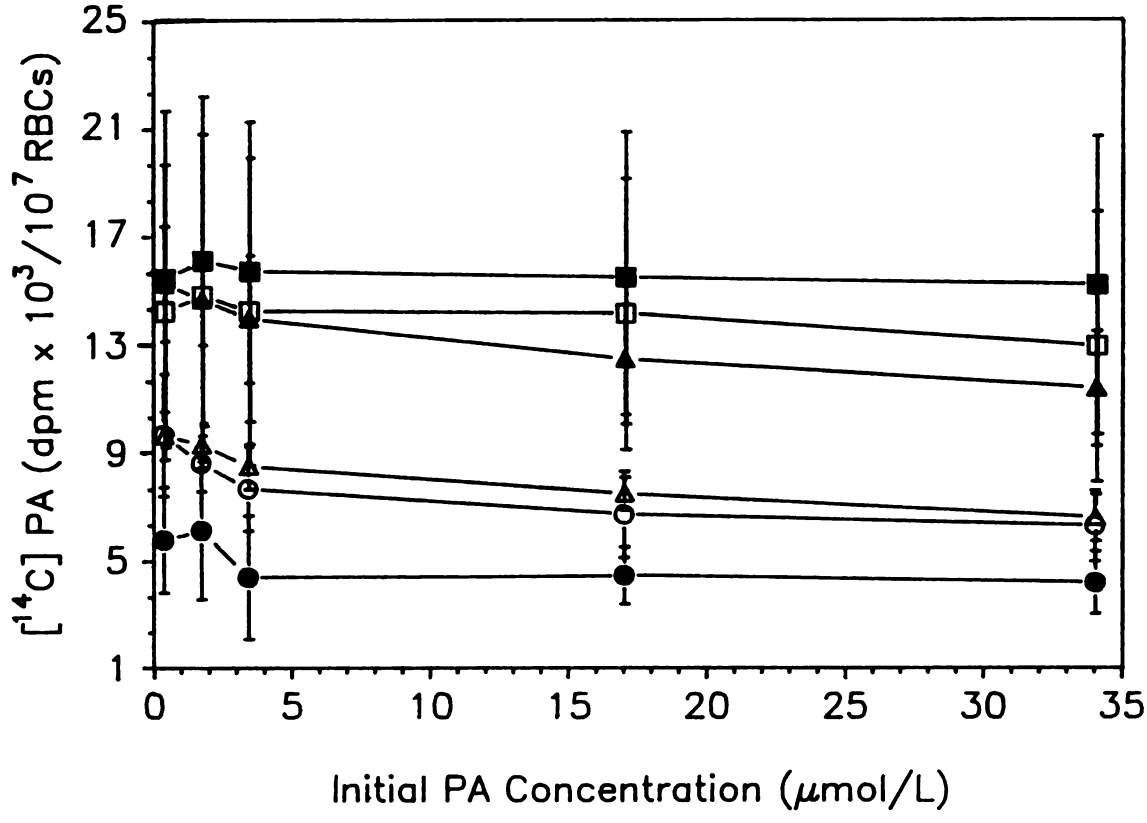


Figure 6. [^{14}C]PA uptake by RBCs (40-360 min) vs initial PA concentration in media. The RBCs were incubated with several PA concentrations (0.34, 1.7, 3.4, 17.0 and 34.0 μmol/L) all of which contained 0.34 μmol [^{14}C]PA/L, at 37°C for 40 (●), 50 (○), 60 (△), 120 (▲), 240 (□) and 360 (■) minutes. Regression analysis of [^{14}C]PA uptake showed a significant linear decrease at 50, 60 and 120 min ($p < 0.05$, $r^2 = 0.31 - 0.64$). Because the effect was not seen at all time points and r^2 was low, it was concluded that total PA in the media had no affect on labeled PA uptake. Each point represents the mean and SD of 5 replicates.

expected in a nonsaturable system.

Previous PA uptake studies in the adipocytes (Sugarman and Munro 1980), liver (Smith and Milner 1985), kidney (Karnitz et al. 1984), heart (Lopaschuk et al. 1987) and intestine (Fernstermacher and Rose 1986), reported that PA was transported via a sodium dependent active transport system. Brain, however, was reported to transport PA via facilitated diffusion (Spector and Boose 1984). The PA uptake experiment in **Figure 7**, showed that at each sodium concentration of 0, 38, 76 and 152 mmol/L RBC-PBS (7 minute incubation time), the total PA uptake increased linearly (r^2 = 0.70 - 0.76, $p < 0.001$) with no differences at different sodium levels. The data indicated that sodium is not necessary for the uptake of PA by the RBC.

We compared the uptake of total PA by the RBC with and without 10 mmol glucose/L RBC-PBS, to insure that energy is not required for the uptake of PA by the RBC. Regression analysis showed that presence of glucose in the media did not affect uptake kinetics (**Figure 8**). We also investigated the uptake of PA by the RBC at various pH of the media (7.2, 7.3, 7.4, 7.5 and 7.6). No differences were seen in total PA uptake among the pH levels studied (**Figure 9**).

Figure 10 shows that the total PA release from the RBC was linear for about 50 minutes at initial PA concentrations of 0.34 - 34.0 μmol/L RBC-PBS. At zero time the PA concentrations of the RBCs which were pre-incubated in 0.34, 3.4 and 34.0 μmol PA/L RBC-PBS, all of which contained

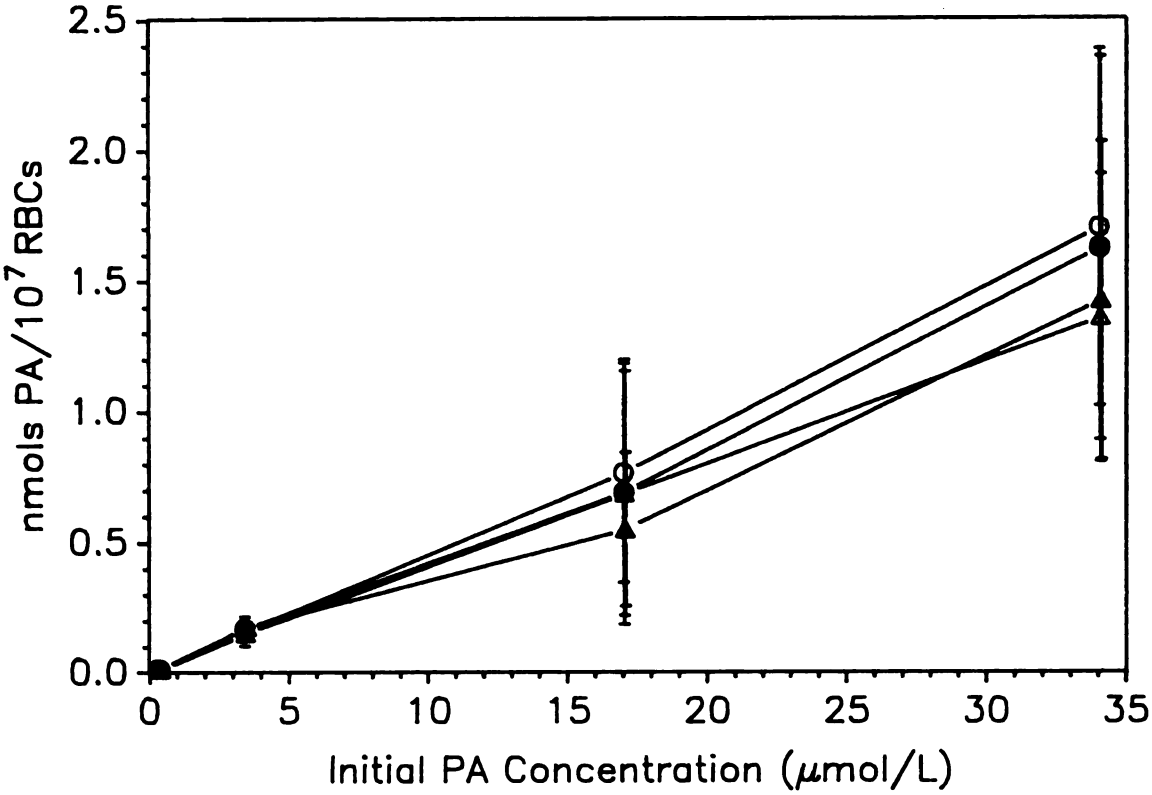


Figure 7. Effect of sodium on RBC uptake of PA. The RBCs were incubated with PA concentrations of 0.34, 3.4, 17.0 and 34.0 μmol/L at 37°C in media containing 4 sodium concentrations (0 (○), 38 (●), 76 (△) and 152 (▲) mmol/L) for 7 minutes. All initial PA concentrations included 0.34 μmol [^{14}C]PA/L. The osmolarity of medium with varying sodium concentration was kept constant by replacing NaCl and Na_2HPO_4 with equimolar LiCl. Each point represents the mean and SD of 5 replicates. At each sodium concentration the uptake was linear (r^2 = 0.70 - 0.76, $p < 0.001$) and there were no differences between sodium concentrations.

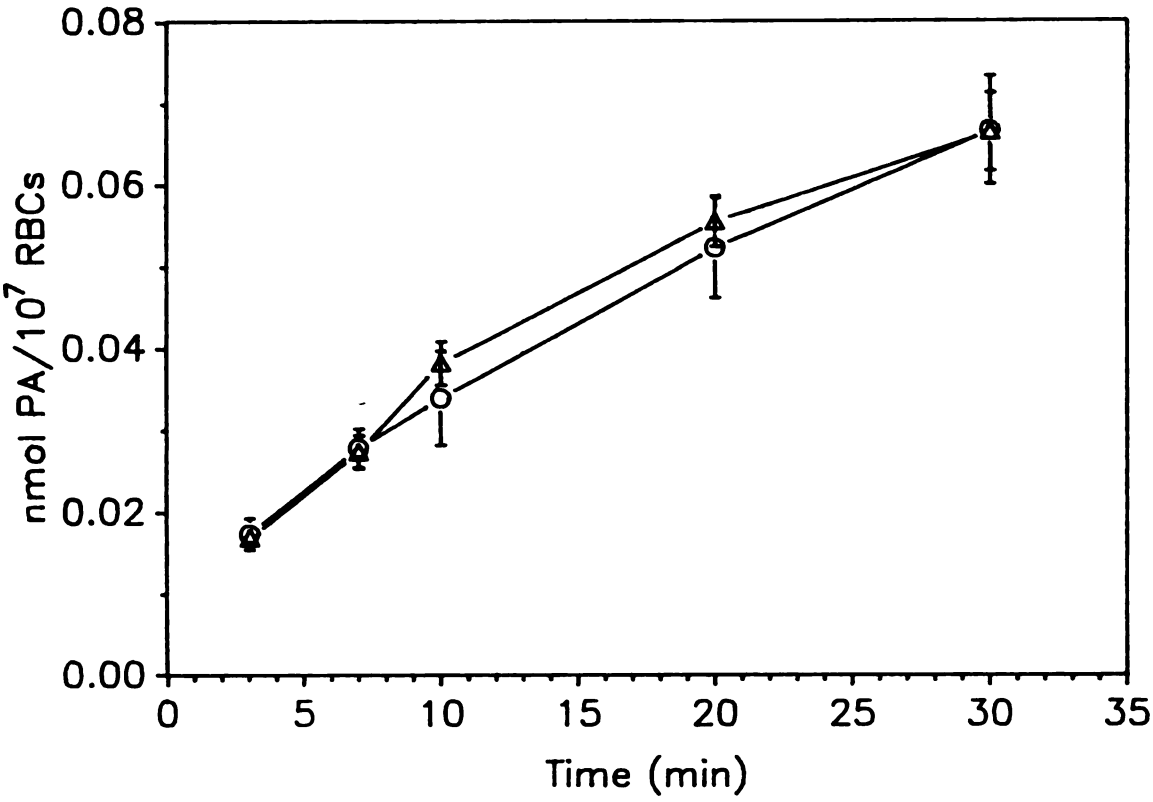


Figure 8. Effect of glucose on uptake of PA by the RBC. The RBCs were incubated with 0.34 μmol [^{14}C]PA/L and either 0 (○) or 10 (△) mmol glucose/L at 37°C for 3, 7, 10, 20 and 30 minutes. Each point represents the mean and SD of 5 replicates. Regression analysis showed no differences in PA uptake whether glucose was present or not.

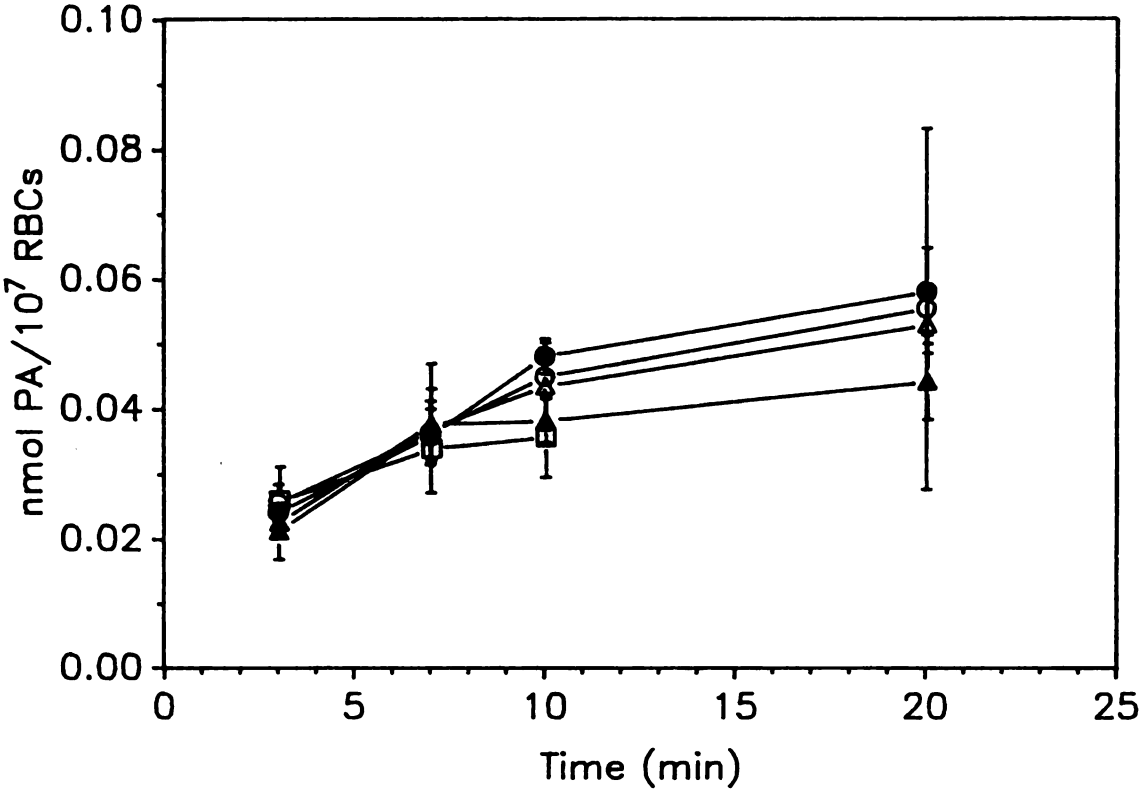


Figure 9. Effect of pH on uptake of PA by the RBC. The RBCs were incubated with 0.34 μmol [^{14}C]PA/L at pH levels of 7.2 (○), 7.3 (●), 7.4 (△), 7.5 (▲) and 7.6 (□) at 37°C for 3, 7, 10 and 20 minutes. Each point represents the mean and SD of 5 replicates. Regression analysis showed no differences in PA uptake between the various pH conditions examined.

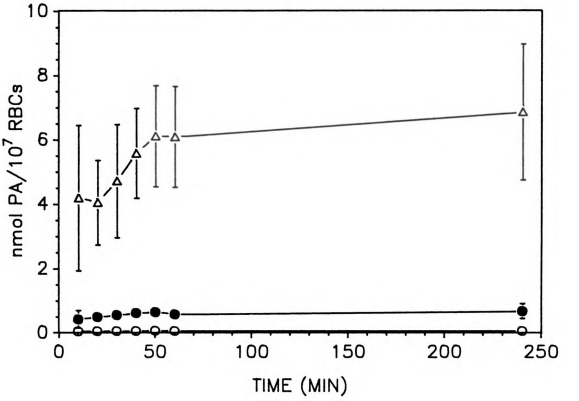


Figure 10. Release of total PA by RBCs vs incubation time. The RBCs were incubated with PA concentrations of 0.34 (○), 3.4 (●) and 34.0 (△) μmol/L at 37°C for 2 hours. All initial PA concentrations included 0.34 μmol [^{14}C]PA/L. The radiolabeled RBCs (37 Bq), which contained 0.104, 1.04 and 10.4 nmols PA/10^7 RBCs, were then washed and reincubated in fresh media for 10, 20, 30, 40, 50, 60 and 240 minutes. Each point represents the mean and SD of 5 replicates.

0.34 μmol [^{14}C]PA/L RBC-PBS, were 0.104, 1.04 and 10.4 nmols PA/10^7 RBCs, respectively. With increasing initial PA concentrations in the RBC, the release of total PA from the cell increased linearly (**Figure 11**, r^2 = 0.71 - 0.93, $p < 0.001$), which indicated that efflux of PA is also nonsaturable. The data were analyzed by regression analysis in terms of total activity (**Figure 12**), as in the uptake experiment. With increasing initial PA concentrations in the RBC, release of radioactive PA was not changed which indicated total PA in the RBC had no affect on release of labeled PA, expected in a nonsaturable system.

In summary, PA uptake and release by the RBC were found to be occuring by passive diffusion based on the following evidence: 1) uptake and release of PA from the RBC continuously increase with PA concentrations from 0.34 - 34 μmol/L, 2) total PA had no affect on uptake or release of labeled PA, 3) energy, pH and sodium did not affect PA uptake under our experimental conditions.

METABOLISM OF PA BY THE RBC

Figure 13 shows the structure of CoA and its bonds that are hydrolyzed by pantetheinase, alkaline phosphatase and pyrophosphatase. The alkaline phosphatase preparation purchased from Sigma contains both alkaline phosphatase and pyrophosphatase.

PA derivatives present in the RBC were determined qualitatively and quantitatively by subjecting the RBCs to a

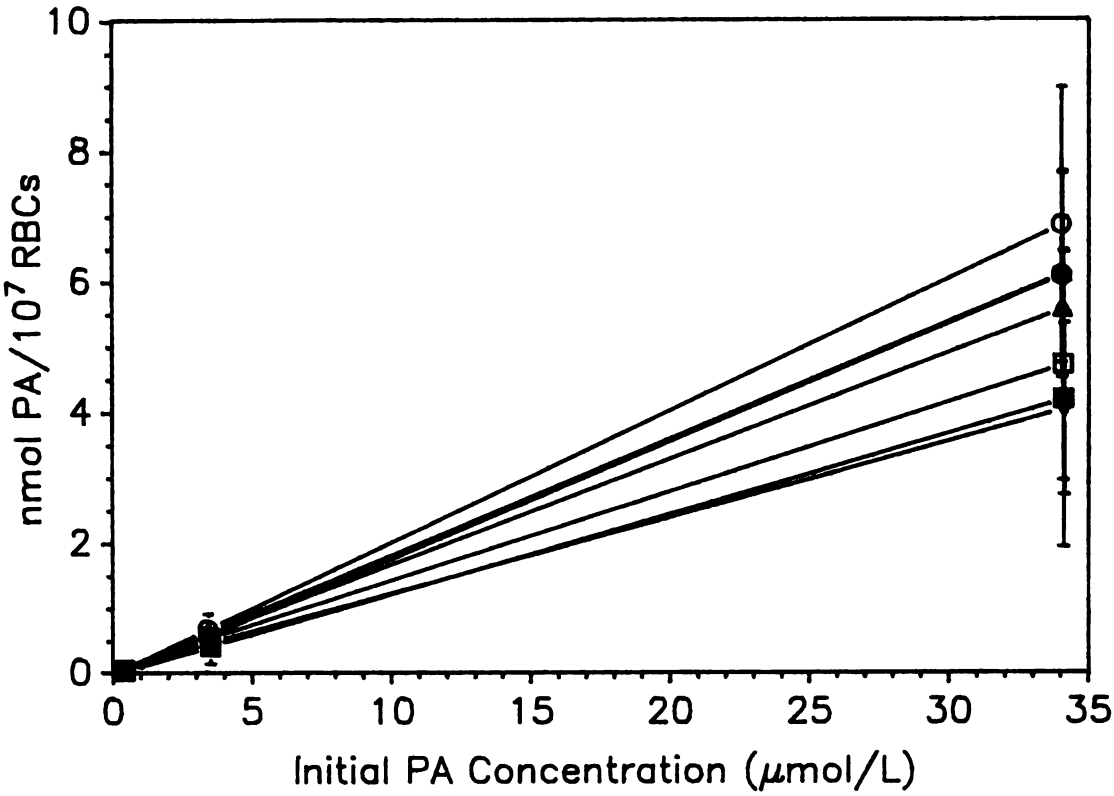


Figure 11. Release of total PA by RBCs vs initial PA concentration in media. The RBCs were incubated with PA concentrations of 0.34, 3.4 and 34.0 μmol/L at 37°C for 2 hours. All initial PA concentrations included 0.34 μmol [^{14}C]PA/L. The radiolabeled RBCs (37 Bq), which contained 0.104, 1.04 and 10.4 nmols PA/10^7 RBCs, were then washed and reincubated in clean media for 10 (■), 20 (▽), 30 (□), 40 (▲), 50 (△), 60 (●) and 240 (○) minutes. Each point represents the mean and SD of 5 replicates, and all time points increased linearly (r^2 = 0.71 - 0.93, $p < 0.001$) which indicated a nonsaturable efflux process.

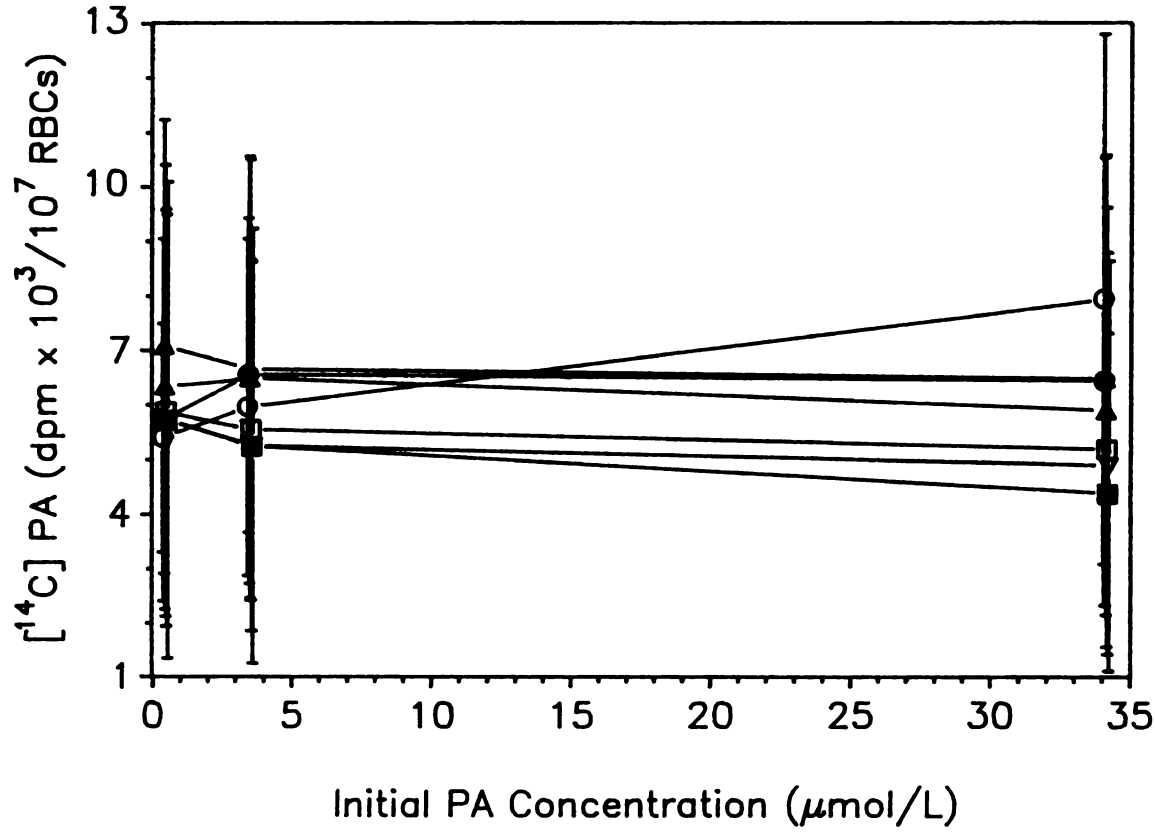
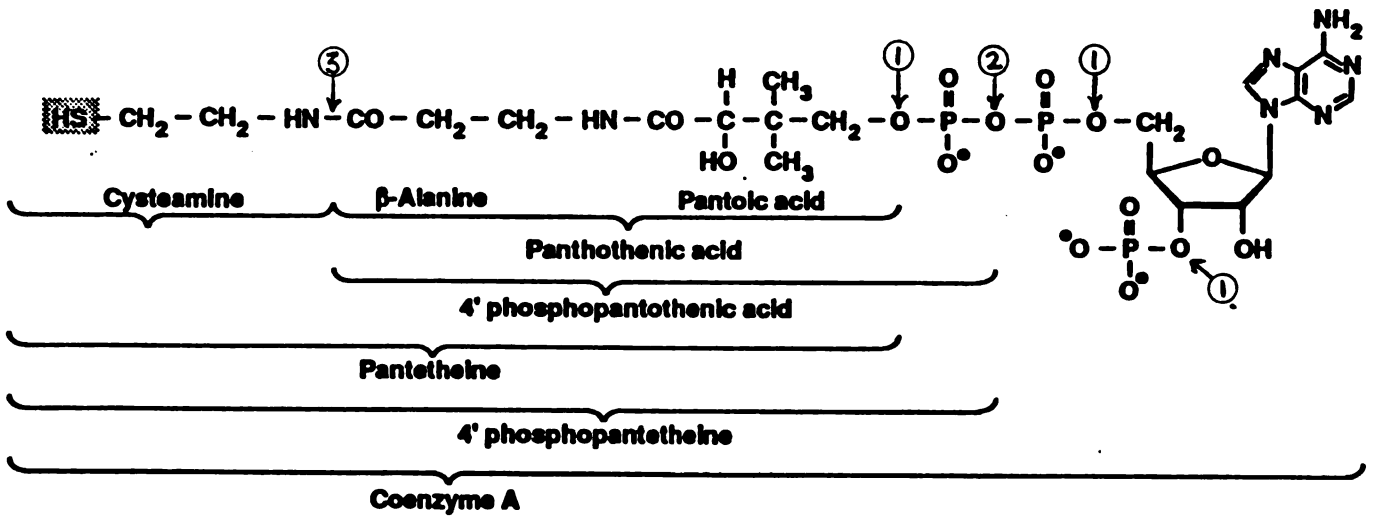


Figure 12. Release of [^{14}C]PA by RBCs vs initial PA concentration in media. The RBCs were incubated with varied PA concentrations (0.34, 3.4 and 34.0 μmol/L) at 37°C for 2 hours. All initial PA concentrations included 0.34 μmol [^{14}C]PA/L. The radiolabeled RBCs (37 Bq) were then washed and reincubated in clean media for 10 (■), 20 (▽), 30 (□), 40 (▲), 50 (△), 60 (●) and 240 (○) minutes. Regression analysis of [^{14}C]PA release showed no change with initial PA concentration which indicated total PA had no affect on release of labeled PA. Each point represents the mean and SD of 5 replicates.



1 - alkaline phosphatase
2 - pyrophosphatase
3 - pantetheinase

Figure 13. Bonds broken in CoA after enzymatic hydrolysis.

differential enzymatic hydrolysis, then by quantitating the total PA generated by RIA (**Table 2**). All enzymes applied simultaneously, break down all PA derivatives (4'-PPA, 4'-PPE, 4'-PPAcys, dpCoA, CoA and PE) to PA, representing total PA in the cell. Alkaline phosphatase applied to PA derivatives breaks all phosphate group bonds (see Figure 13), so 4'-PPA is the only PA derivative which will be degraded to PA and can then be quantitated by RIA. Pantetheinase applied to PA derivatives catabolizes the amide bond, breaking off cysteamine, so PE is the only PA derivative degraded to PA. Free PA made up only 17% (0.78 ± 0.37 nmol PA/10^7 RBCs) of the total PA (4.58 ± 2.00 nmol PA/10^7 RBCs) in the RBC. Seventy eight percent (3.56 ± 2.51 nmol PA/10^7 RBCs) of the total PA, which was measured by RIA after hydrolysis by the alkaline phosphatase preparation, include PA and 4'-PPA only. Therefore, it was concluded that 61% of the total PA in the RBC is in the form of 4'-PPA. Forty eight percent (2.21 ± 0.80 nmol PA/10^7 RBCs) of total PA in the RBC, which was quantitated by RIA after hydrolysis by pantetheinase, include PA and PE only. We thus conclude that 31% of total PA in the RBC is in the form of PE. The sum of free PA, 4'-PPA and PE added to 109%.

Next, the identity of the PA derivatives present in the RBC was determined qualitatively with paper chromatography and using authentic standards. The R_f values of standards were determined by visualization procedures after paper chromatography or by using radiolabeled PA and paper

Table 2. Pantothenic acid and it's derivatives in RBCs quantified after different enzyme treatments[1]

Enzyme	PA derivatives*	PA[2]	% of total
		nmol/10^7 RBCs	
none	PA	0.78 ± 0.37	17
alkaline phosphatase & pantetheinase	PA, 4'-PPA, 4'-PPAcys 4'-PPE, dpCoA, CoA, PE	4.58 ± 2.00	100
alkaline phosphatase	PA, 4'-PPA	3.56 ± 2.51	78
pantetheinase	PA, PE	2.21 ± 0.80	48

[1] Total PA (bound and free) in RBCs was subjected to specific enzyme treatments and measured subsequently for PA by RIA.

[2] Values are mean ± SD of 4 - 6 replicates.

* Abbreviations used: (PA), pantothenic acid; (4'-PPA), 4'-phosphopantothenic acid; (4'-PPAcys), 4'-phosphopantothenoylcysteine; (4'-PPE), 4'-phosphopantetheine; (dpCoA), dephosphoCoA; (CoA), Coenzyme A; (PE), pantetheine.

chromatography. The R_f values obtained were 0.8, 0.64, 0.35, 0.4, 0.15 and 0.76, for PA, 4'-PPA, 4'-PPE, dpCoA, CoA and PE, respectively.

The RBCs incubated in the PBS media with [^{14}C]PA (3.4 μmol/L) for 6 hours were processed (see Materials and Methods), and then analyzed for PA derivative with paper chromatography. **Figure 14** shows the distribution of radioactivity on the paper chromatogram of the native RBCs extracted without prior enzyme treatment. No peaks appeared below 11 cm (R_f = .44) from the base which indicated that 4'-PPE, dpCoA and CoA were not present in the RBCs. Present in the RBCs were PA at 20 cm (R_f = 0.8), 4'-PPA at 16 cm (R_f = 0.64) and, possibly, PE at the same peak as PA; PE and PA could not be clearly separated from each other by the paper chromatography method.

A small possibility also exists that the PE in the RBC was in an unlabeled form that was not synthesized from the PA taken up, but had entered the cells from an unlabeled pool. Both PA and PE can cross the intestinal cell membrane (Shibata et al. 1983), which would make it probable that PE can cross other cell membranes including the RBC membrane. PE is a degraded product of 4'-PPE in the CoA degradative pathway (see Figure 1), and no known pathways exist to allow PA to be synthesized directly to PE in any cells studied, but PE was detected in the RBC by the RIA, without the intermediate 4'-PPE present. Although, it is unlikely because pantetheinase activities exist in serum (Wittwer et

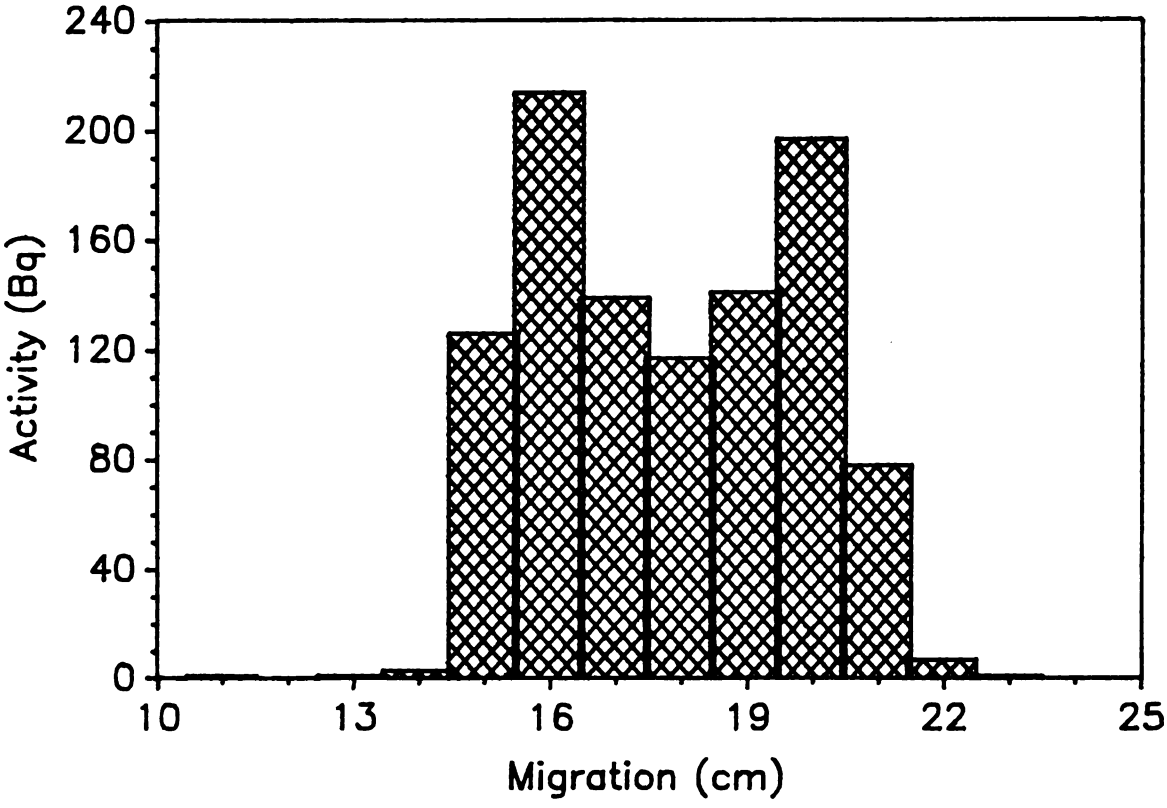


Figure 14. Paper chromatogram of PA derivatives in the native RBC. The RBCs were incubated with 3.4 μmol [^{14}C]PA/L at 37°C for 6 hours. The RBCs were washed, prepared and subjected to paper chromatography on DEAE cellulose paper, using as solvent 0.5 mol/L formic acid (pH = 3.1, adjusted with NH_4OH), for 4 - 4.5 hours which produced a solvent front at 25 cm. After drying, the paper chromatogram was cut into 1 cm pieces and counted for radioactivity. Standards of PA, 4'-PPA, 4'-PPE, dpCoA, CoA and PE produce peaks at 20, 16, 9, 10, 4 and 19 cm, respectively, based on a 25 cm solvent front. No peaks appeared below 10 cm, therefore 4'PPE, dpCoA and CoA were not present. PA, 4'-PPA and possibly PE were identified as the PA derivatives in the native RBCs.

al. 1989) and only free PA has been found in serum (Song et al. 1985).

The absence of CoA in the RBCs, as indicated by the paper chromatogram (Figures 14) as well as by the RIA (Table 2), is also in agreement with CoA measurements in whole blood made in our lab (unpublished data) using a radiochemical assay (Knights and Drew 1988) and by Smith (1987) using the ∝-ketoglutarate dehydrogenase method (Garland et al. 1965). Both labs found no CoA present in whole blood.

The presence of 4'-PPA in the RBC was further confirmed, directly and indirectly, by the combined analytical procedures, differential enzymatic hydrolysis and paper chromatography, and PA kinase activities in the RBC. The RBCs were incubated with [^{14}C]PA for 6 hours, then subjected to enzymatic degradation with alkaline phosphatase before being prepared and applied to paper chromatography (**Figure 15**). The alkaline phosphatase treated RBCs had a single peak at 20.5 cm (R_f = 0.82), which indicated that the peak at 16 cm (R_f = 0.64) which appeared in the native RBCs sample (Figure 14) was completely converted to PA.

Figure 16 is the chromatogram of prepared RBCs which had been incubated with [^{14}C]PA for 6 hours and subjected to pantetheinase alone. The data were unexpected. Pantetheinase breaks down PE to PA, but these two peaks could not be clearly distinguished from each other by paper chromatography, so there should have been no significant

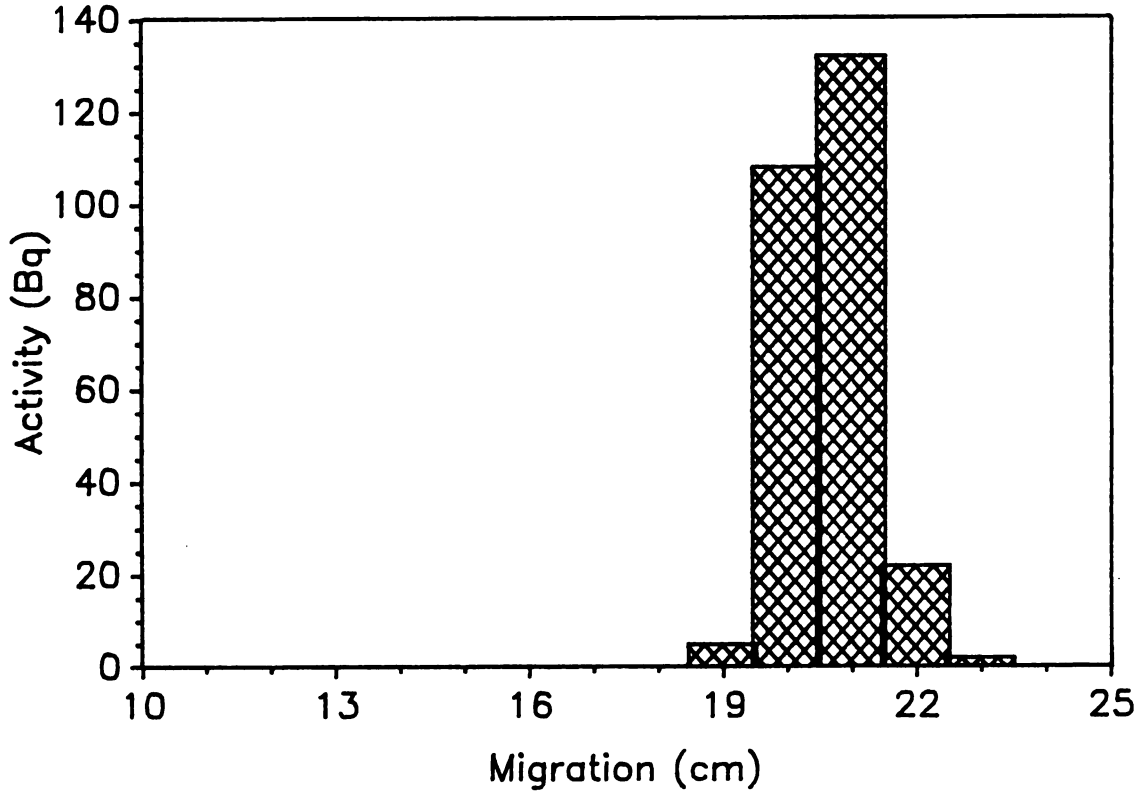


Figure 15. Paper chromatogram of PA derivatives in RBCs after incubation with alkaline phosphatase. The RBCs were incubated with 3.4 μmol [^{14}C]PA/L at 37°C for 6 hours. The RBCs were lysed, subjected to enzymatic degradation with alkaline phosphatase, which causes 4′-PPA to convert to PA, prepared and chromatographed. Standards of PA, 4′-PPA and PE produce peaks at 20, 16 and 19 cm, respectively, based on 25 cm solvent front. No peaks appeared below 10 cm, but the single peak at 20.5 cm (R_f = 0.82) indicated that everything was converted to PA, confirming that 4′-PPA was present.

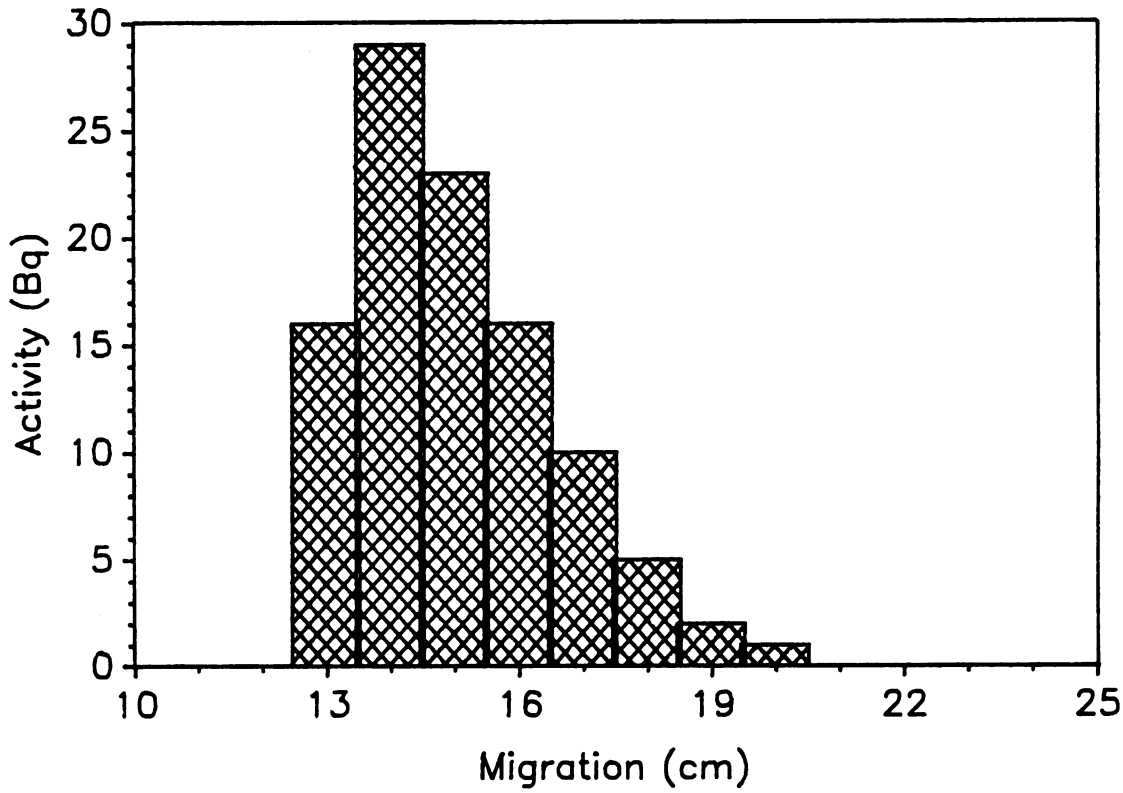


Figure 16. Paper chromatogram of PA derivatives after incubation of RBCs with pantetheinase. The RBCs were incubated with 3.4 μmol [^{14}C]PA/L at 37°C for 6 hours. The RBCs were lysed, subjected to enzymatic degradation with pantetheinase, which causes PE conversion to PA, prepared and chromatographed. Standards of PA, 4'-PPA and PE appeared at 20, 16 and 19 cm, respectively, based on a 25 cm solvent front.

shift of peaks from the chromatogram of the native RBCs. The peak at 14 cm (R_f = 0.56) might represent a combination of free PA and derivatives because there was no PA peak at 20 cm (R_f = 0.8), though 4'-PPA does fall within this peak at 16 cm (R_f = 0.64). **Figure 17** is the chromatogram of prepared RBCs which were incubated with [^{14}C]PA for 6 hours and subjected to enzymatic degradation with a combination of two enzymes: alkaline phosphatase and pantetheinase. Application of both enzymes to the RBC was expected to convert all PA derivatives to PA, generating a single peak. Although a PA peak is definitely seen, another peak appeared at 12.5 cm (R_f = 0.50) which was similar to the peak at 14 cm (R_f = 0.56) in Figure 16. Since both chromatograms (Figure 16 and Figure 17) were from the samples treated with pantetheinase, we suspected that the pantetheinase caused the unexpected chromatograms. We then questioned if pantetheinase hydrolyzes the amide bond in PA to give pantoic acid and β-alanine. Since the ^{14}C is in the β-alanine molecule, the new peak at 12.5 - 14 cm (R_f = 0.53) could be explained by presence of β-alanine. An alternative possibility was that the pantetheinase preparation contained substances which caused a slower migration of PA and its derivatives during paper chromatography. To answer these questions, several control chromatograms were run, by procedures described in Materials and Methods; the results are shown in **Table 3**. Since no peaks appeared below 10 cm, the radioactivity for each chromatogram is listed from 10 cm

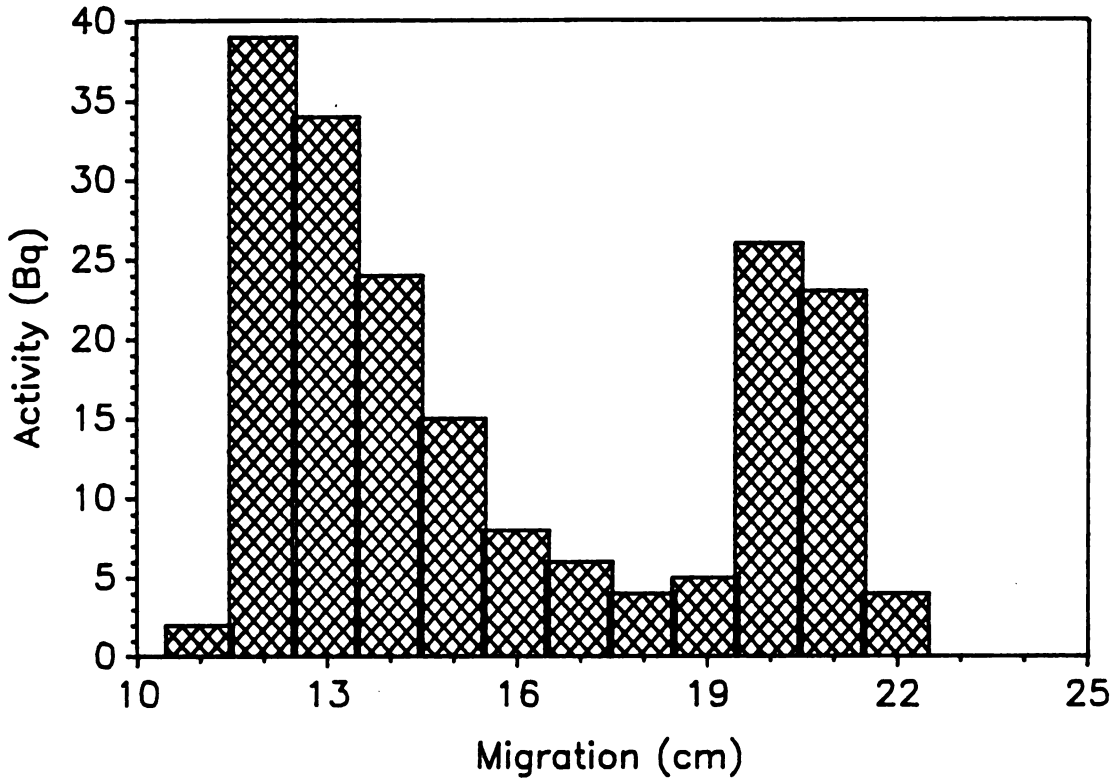


Figure 17. Paper chromatogram of PA derivatives after incubation of RBCs with alkaline phosphatase, pyrophosphatase and pantetheinase. The RBCs were incubated with 3.4 μmol [^{14}C]PA/L at 37°C for 6 hours. The RBCs were lysed, subjected to enzymatic degradation with pantetheinase and alkaline phosphatase, which causes degradation of all PA derivatives to PA, prepared and chromatographed. Standards of PA, 4'-PPA and PE appeared at 20, 16 and 19 cm, respectively, based on a 25 cm solvent front.

Table 3. Distribution of radioactivity in paper chromatograms after [^{14}C]PA was treated with various enzymes or perchloric acid[1].

Migration	Samples[2]								
	1	2	3	4	5	6	7	8	9
cm	Becquerels								
10	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	1	0	0
13	0	0	0	0	0	0	8	0	0
14	6	0	0	0	0	2	10	0	0
15	20	5	0	0	0	15	9	0	0
16	21	20	0	0	0	24	7	0	0
17	17	26	0	0	0	19	5	0	0
18	11	18	0	0	0	14	5	1	0
19	7	11	0	8	1	8	4	12	0
20	4	5	6	27	10	4	3	20	1
21	1	2	59	40	57	1	3	36	48
22	0	0	28	7	25	0	2	28	141
23	0	0	1	0	1	0	2	1	3
24	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0
Total	88	88	94	84	95	87	60	98	194

[1] Radioactivity eluted between 10 - 26 cm of paper chromatogram, for 8 samples plus standard [^{14}C]PA. Migration of the radioactivity was measured in cm; no peaks appeared below 10 cm.

[2] Samples were prepared as follows: 1) 0.68 nmols of [^{14}C]PA and 20 units of low purity pantetheinase (182 units/mL) were incubated together (37°C, 5 hours) and the reaction was terminated by boiling. 2) 20 units of low purity pantetheinase (182 units/mL) was incubated (37°C, 5 hours) and mixed with 0.68 nmols of [^{14}C]PA immediately before termination of reaction by boiling. 3) 0.68 nmols of [^{14}C]PA and 20 units of high purity pantetheinase (807 units/mL) were incubated together (37°C, 5 hours) and boiled for termination of reaction. 4) 20 units of high purity pantetheinase (807 units/mL) was incubated (37°C, 5 hours) and mixed with 0.68 nmols of [^{14}C]PA immediately before termination of reaction by boiling. 5) 0.68 nmols of [^{14}C]PA and alkaline phosphatase were incubated together (37°C, 5 hours) and boiled. 6) 0.68 nmols of [^{14}C]PA, 20 units of low purity pantetheinase (182 units/mL) and alkaline phosphatase were incubated together (37°C, 5 hours) and boiled. 7) 6% $HClO_4$ was mixed with 0.68 nmols of [^{14}C]PA, then neutralized with 3 mol/L KOH. 8) 6% $HClO_4$ was neutralized to pH 7 with 3 mol/L KOH, then mixed with 0.68 nmols of [^{14}C]PA. 9) Standard [^{14}C]PA (0.27 nmol, 296 Bq).

to the solvent front. Sample 1 (0.68 nmol of [^{14}C]PA incubated at 37°C for 5 hours with 20 units of low purity pantetheinase, 182 units/mL) and sample 2 (20 units of low purity pantetheinase, 182 units/mL, to which 0.68 nmol of [^{14}C] PA was added immediately before termination) produced practically the same chromatograms with peaks appearing at 15 - 16 cm (R_f = 0.58) and 16 - 17 cm (R_f = 0.62) compared to the [^{14}C]PA standard peak at 21 - 22 cm (R_f = 0.81; Sample 9, Table 3). The two chromatograms (Sample 1 & 2) indicated that pantetheinase does not catabolize PA, but caused a slower migration of PA. The chromatogram for Sample 3 (0.68 nmol of [^{14}C]PA incubated at 37°C for 5 hours with 20 units of high purity pantetheinase preparation, 807 units/mL) was compared with that of sample 4 (20 units of high purity pantetheinase, 807 units/mL, mixed with labeled PA immediately before termination). The PA peak appearing at 21 cm (R_f = 0.81) in chromatograms for both samples 3 & 4, supported the conclusion drawn from samples 1 and 2, that pantetheinase did not catabolize the amide bond in the PA, and probably the amount of salts that were present in the low purity enzyme, but not the high purity enzyme, decreased the migration of [^{14}C]PA. Chromatograms for sample 5 (0.68 nmol of [^{14}C]PA incubated at 37°C for 5 hours with 10 units of alkaline phosphatase) and sample 6 (0.68 nmol of [^{14}C]PA incubated with 20 units of low purity pantetheinase, 182 units/ml, and alkaline phosphatase) confirmed the conclusion on the effect of pantetheinase on the paper chromatogram,

suggesting the importance of controlling salt concentrations in the sample extract to be chromatographed. A single PA peak appeared in sample 5 at 21 cm (R_f = 0.80) which, as expected, is very similar to the standard PA peak. Sample 6 produced a single peak at 16 cm (R_f = 0.62) which is similar as expected, to that in samples 1 and 2, because they also contain the low purity pantetheinase.

We further evaluated if the buffer system used in the [^{14}C]PA extraction procedure from the sample RBCs had any effect on paper chromatography for [^{14}C]PA. Sample 7 (0.68 nmol of [^{14}C]PA added with 6% $HClO_4$, then neutralized) and sample 8 (0.68 nmol of [^{14}C]PA added to neutralized 6% $HClO_4$) indicated that 6% $HClO_4$ broke PA down to pantoic acid and β-alanine when [^{14}C]PA alone was reacted with the acid. The decreased migration of the [^{14}C]PA was not seen in the samples (Figure 14 & Figure 15) which contained protein to react with the $HClO_4$ added and hence "protect" PA. The findings on the effect of the extraction procedure with $HClO_4$ on [^{14}C]PA points out that precautions must be taken against using high concentrations of $HClO_4$ or to prolong the length of time for the extraction procedures for PA and its derivatives from samples, as erroneous results are obtained.

Based on the data presented in Table 3, we can assume that excessive salt present in the low purity pantetheinase interfered with the migration of 4'-PPA and PA peaks (Figure 16). An additional peak at 12.5 cm (R_f = 0.5) seen in Figure 17 compared to the [^{14}C]PA standard (sample 6, Table

3), may be due to some of the PA in the RBC sample (Figure 17) was buffered against the effects of the salt by other compounds left in the supernatant after protein precipitation, therefore we assume Figure 17 represents PA only.

In our studies we found that PA passively diffuses into and out of the RBC and that free PA, 4'-PPA and PE are present in the cell.

The transport of other B-vitamins by the RBC seems to be based on the RBC's need for the vitamin: facilitated diffusion, if there is a metabolic need for the vitamin. Examples are nicotinic acid which is required for both glycolysis and the hexose monophosphate pathway, and thiamin which is required for transketolase in the hexose monophosphate pathway. Passive diffusion occurs when the vitamin effect is incidental, for example, when pyridoxal is bound to hemoglobin it causes a change in oxygen affinity for hemoglobin, although 2,3-diphosphoglycerate provides the same effect but is found in the RBC at > 1000 times the concentration of pyridoxal. We observed that PA diffuses passively into the RBCs and that it is not metabolized completely to CoA. The finding was not unexpected because the mature RBCs do not have metabolic pathways requiring CoA. Perhaps the role of PA is also incidental, possibly as PE, whose active sulfhydryl site could conceivably be used in protective roles in the RBC similar to those of glutathione. The chemically active site of glutathione is

the sulfhydryl of a cysteine residue, as in PE. Glutathione preserves red cell deformability, protects -SH groups of proteins (mainly hemoglobin), serves as a free radical scavenger and detoxifies foreign compounds. The concentration of glutathione in the RBCs (2.2 mmol/L), however, is 1000 times higher than that of PA, thus PA would have a minor role for this function in the cell. The functions, of PA, 4'-PPA and PE in the RBC need further study.

PA uptake by the RBCs is very different from PA uptake in other tissues studied. The adipose tissue, liver, kidney, heart and intestine all transport PA via a sodium dependent active transport system and the brain transports PA by facilitated diffusion. Also, the metabolism of PA in the RBCs is not to the same extent it is in these other tissues, which metabolize PA completely to CoA. These differences are due to the uniqueness of the RBC in its organelle makeup, metabolism and function. Since the RBC is taking up and metabolizing PA to 4'-PPA, the first and rate limiting step of CoA synthesis, the RBC is likely to mimic PA metabolism in the tissues. RBC metabolism of PA must be further investigated and compared to PA metabolism in other tissues in an effort to identify reliable and sensitive measures of PA nutriture. The RBC, if found to be a sensitive indicator of PA status, is easily accessible and will serve as a useful biological assay system in clinical studies.

CONCLUSIONS

PA is taken up and released by the RBCs by passive diffusion based on the following evidence: 1) uptake and release of PA from the RBCs continuously increases at PA concentrations from 0.34 - 34.0 μmol/L; 2) unlabeled PA added to the media or in the cell had no effect on the rate of uptake or release of labeled PA, respectively; and 3) energy, sodium and pH have no effect on PA uptake.

RBCs metabolize PA upon entry into the RBC to 4'-PPA, the product of the first and most important rate-limiting step in the conversion of PA to CoA in all nucleated cells, as supported by the following data: 1) the RIA for PA, after enzymatic hydrolysis of PA derivatives, indicated the presence of free PA, 4'-PPA and PE in the RBCs, 2) the paper chromatograms of the RBCs with and without alkaline phosphatase hydrolysis showed that PA, 4'-PPA and, possibly, PE are present in the RBCs, and 3) both RIA for PA and paper chromatography of the PA derivatives in the RBCs indicated that 4'-PPE, dpCoA and CoA are not present in the RBCs, from which PE can be generated as a degradative product.

Further study of PA metabolism in the RBCs is required to determine how PE can be present in the cell without the presence of 4'-PPE, the metabolic precursor of PE, and if

4'-PPA is broken down to PA in the cell. Also, the functions and metabolic roles of PA, 4'-PPA and PE in the RBC needs to be further studied.

LIMITATIONS

1) Uptake and release experiments do not include time points less than 3 minutes. The radioactivity detected in the RBC was too low to be accurate after a 3 minute incubation with 0.34 μmol [^{14}C]PA/L with a specific activity of 2.11 GBq/mmol as purchased. Use of higher concentrations of radiolabeled PA, which can offset the technical difficulty addressed will not, however, be in the range of the physiological concentration of PA in rat whole blood.

2) The effect of sodium on uptake is addressed, but not on release of PA by the RBC. It was not considered important after seeing the results of the uptake and release experiments, namely that PA is taken up and released by passive diffusion.

3) In the present study, the wash and centrifuge procedure instead of the oil drop centrifugation technique (Wohlhueter et al. 1978) was used to separate RBCs from the media. We found that the oil drop separation technique yielded a larger within treatment variability, especially at short time points, than the wash and centrifuge procedure.

4) The limitations of the paper chromatography method used were the inability to detect nonradiolabeled PA derivatives in the RBC samples by chemical visualization and

to accurately separate PA (R_f = 0.80) from PE (R_f = 0.76). The amounts of PA derivatives that could be detected by the chemical visualization would require approximately 100 mL of blood per sample.

RECOMMENDATIONS FOR FUTURE STUDIES

The next step in answering the overall hypothesis, would be to use the HPLC for separation and quantification of PA, 4'-PPA and PE in the RBC. Separation of PA, 4'-PPA, 4'-PPE, dpCoA and CoA has been demonstrated with the HPLC in rat heart (Robishaw et al. 1982). The HPLC is more sensitive than paper chromatography which means 1) the amount of RBC per sample required for analysis would be smaller, 2) separation of PA derivatives would be sharper (paper chromatography could not separate PA and PE), and 3) quantification of PA derivatives by the HPLC, which was not possible with paper chromatography, would be highly accurate. Furthermore, the clinical applications of the procedure must be considered. With the HPLC, many samples can be run quickly, as compared to the paper chromatography followed by RIA for quantification.

The next step would be to determine the sensitivity and reliability of the PA derivatives in the RBC to a PA deficiency. To do this PA, 4'-PPA and PE should be quantitated by HPLC methods, in the rat RBC, at a regular interval during the course when the rat is fed a PA deficient diet. Tissue concentrations of individual and total PA derivatives should also be measured at the same

regular interval to compare with RBC concentrations to determine if they do indeed mimic tissue concentrations.

To further investigate metabolism of PA in the RBCs, the HPLC would be very useful. Still to be determined is if PE is synthesized in the RBC. To do this RBCs can be incubated with radiolabeled PA, as we did in our experiments. Then prepare the cells and inject them into the HPLC after separation of PA, 4'-PPA and PE standards is accomplished. Fractions of eluate would be collected at the retention times of the standards and counted for radioactivity to determine if radiolabeled PE had been synthesized in the RBCs.

The question also exists of how does the PA get back out of the cell after being trapped by phosphorylation? We can investigate if a phosphatase is present in the RBC which converts 4'-PPA or 4'PPE to PA or PE, respectively, by incubating RBCs containing radiolabeled PA with several concentrations of a phosphatase inhibitor and counting radioactivity released into the media. If a phosphatase is present the release of PA (or PE) will be inhibited and show a decrease with increasing concentrations of the phosphatase inhibitor.

Another metabolic question to be pursued: Is PA kinase made only in the reticulocyte and if so, how long does it last? To partially answer this we can collect reticulocytes, incubate them with several concentrations of radiolabeled PA, then prepare and inject them into the HPLC.

4'-PPA could then be quantitated by collection of eluate at the corresponding retention time and counting for radioactivity for each PA concentration, generating a rate of 4'-PPA synthesis. The same procedure could be done on mature RBCs, so the rates could be compared. If the rate is faster in the reticulocyte, we can assume that PA kinase is not refreshed in the mature RBCs. The life expectancy of PA kinase in the mature RBC would not be possible to determine unless mature RBCs could be separated based upon their age. Also, this experiment would require a large blood sample to collect the number of reticulocytes needed and may only be feasible using the HPLC, since small samples are required for the HPLC.

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