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Pantothenate-p-nitroanilide as a Substrate for Pantetheinase Assay

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PANTOTHENATE-p-NITROANILIDE AS A SUBSTRATE
FOR PANTETHEINASE ASSAY

by

Robert T. Davidson

A thesis submitted in partial fulfillment of the requirements for the degree
of
MASTER OF SCIENCE
in
Nutrition and Food Sciences

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Logan, Utah

1994
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ABSTRACT

Pantothenate-p-nitroanilide has been synthesized for use as a substrate for pantetheinase assay monitoring absorbance at 410 nm. Pantothenate-p-nitroanilide is a crystalline compound with a molecular weight of 338.0 and a melting point of 146-149°C. Use of this substrate in the described assay is suitable for enzyme activity determination in high protein content media such as blood serum. Serum pantetheinase activity was determined for rats of varying pantothenate nutriture. Rats with mildly (but significantly, $p<0.05$) lower serum pantothenate levels had significantly higher
serum pantetheinase activities than the control group (6.24 nmol/min/ml ± 0.72 and 16.16 nmol/min/ml ± 4.14, respectively, p=0.0005).
INTRODUCTION

Pantetheinase is an amidase which converts one of the vitamin pantothenate's derivatives, namely pantetheine, back into pantothenate with cysteamine as a coproduct (Fig. 1).

There have been numerous methods utilizing various techniques devised to assay the activity of this enzyme, and they are mentioned briefly as follows: i) measurement of oxygen uptake by means of amine oxidase coupling (1), ii) chromatographic separation of $^{14}$C-labeled substrate and product (2,3), iii) electrophoretic separation of $^{14}$C-labeled substrate and product (4), iv) microbial assay of pantothenate (5), v) pH-stat titration of products (6), vi) amino acid analysis of the N-ethylmaleimide adduct of cysteamine (7), and vii) radioimmunoassay of pantothenate product (3). These methods are all quite time consuming and/or require specialized equipment.

With the exception of the assays using radiolabeled substrate, these previously mentioned assays are also prone to give high apparent activities if used to measure activity in a
Figure 1. Enzymatic hydrolysis of pantetheine by pantetheinase results in the products pantothenate and cysteamine.
medium, such as blood serum, containing no endogenous substrate but which may contain endogenous levels of enzymatic reaction product. This problem may be overcome by running an assay blank for each sample. This can be done by assaying endogenous product level before adding substrate and subtracting that from the value obtained from assay with substrate added. However, high blank values tax the limits of sensitivity on equipment, and small enzyme activities might not be seen.

Despite the fact that neither pantetheine nor its enzymatic hydrolysis products absorb light in the visible or ultraviolet light regions, there are three spectrophotometric continuous absorbance assays that are much easier and faster than the previously mentioned assays. They are i) cysteamine is measured by the absorbance of its fluoropyruvate adduct at 300 nm (3); ii) mercaptide ion is monitored at 240 nm as its concentration increases proportionately with enzymatic hydrolysis (3); and iii) S-pantetheine-3-pyruvate (pantetheine-S-pyruvate) forms a cyclic compound upon enzymatic hydrolysis, which is monitored as it absorbs strongly at 296 nm (8). Even though these three
spectrophotometric assays provide advantages such as decreased assay times, less specialized equipment, and no radiolabeled compounds, none are suitable for measurement of enzyme activity in high protein content media such as blood serum.

Because the spectrophotometric continuous absorbance assays are so simple and fast, and because protein in media such as blood serum has a high absorbance near 280 nm, it would be extremely convenient and advantageous to be able to use a simple spectrophotometric assay for pantetheinase that uses a substrate which, upon enzymatic hydrolysis, would absorb light in a region of the spectra in which protein has little or no absorbance.

It was the purpose of this study to synthesize and test a substrate which, upon enzymatic hydrolysis in a spectrophotometric assay, absorbs light at a wavelength such that there is minimal interference from protein-containing media (e.g. blood serum).
Pantothenic Acid

History

Pantothenic acid is a water-soluble vitamin that was first identified as a growth factor and isolated from yeast in 1933 by Williams (9). This growth factor for yeast was extracted from a variety of biological materials and consequently was called "pantothenic" acid, derived from the Greek word pantos, meaning "from everywhere." After both a chick "antidermatitis factor" (10,11) and a rat "liver filtrate factor" (12) were identified as pantothenic acid, inclusion of this compound as a vitamin was warranted because of its importance in animal nutrition. Studies soon led to its isolation and identification of its chemical structure as well as its synthesis (13).

Physiological Forms and Biochemical Functions

Pantothenic acid is the metabolic precursor of several coenzymes (see Fig. 2) and, as such, functions widely as a mediator in the metabolism of energy, carbohydrates, lipids, and amino acids and acts specifically as a carrier of acyl groups. Abiko (5) and
Figure 2. Biosynthetic and degradative pathways of coenzyme A.
Plesofsky-Vig and Brambl (14) have identified more than 100 intermediary metabolic reactions involving two pantothenate-derived coenzymes, namely coenzyme A and phosphopantetheine. As an example of an intermediary reaction in the Krebs cycle, coenzyme A (CoA) functions as an acyl acceptor for the pyruvate and α-ketoglutarate dehydrogenase complexes, forming acetyl-CoA and succinyl-CoA, respectively. Pantothenic acid coenzymes participate in fatty acid synthesis and degradation by carrying the acids as acyl groups through repetitive synthetic or degradative cycles.

**Coenzyme A.** In 1945, Lipmann (15) reported the acetylation of sulfanilamide was dependent upon an enzyme-coenzyme system present in liver homogenates. This coenzyme of acetylation, termed "coenzyme A," was found to be a derivative of pantothenate by Lipmann and co-workers in 1947 (16) and was also found to be widely distributed throughout the body (17). Coenzyme A structure was eventually enzymatically determined (18) and chemically synthesized (19).

**Pantetheine and pantethine.** Both pantetheine and pantethine
were originally identified as a single growth factor for *Lactobacillus bulgaricus* in 1949 (20) and shown to be a breakdown product of coenzyme A with a content of 65-75% pantothenate by weight (21). Structural determination and synthesis were accomplished the same year (22). The names "pantetheine" and "pantethine" were proposed for the growth factor's sulphhydryl and disulfide forms, respectively, indicating their relationship to pantothenic acid. Majerus et al. (23) later identified 4'-phosphopantetheine as the prosthetic group in the acyl carrier protein of fatty acid synthesis.

The structures of coenzyme A, pantothenate, and related compounds are shown in Fig. 3. Alternative names of selected compounds are given in Table 1.

**Biological Relevance**

Despite its importance and requirement to the human body, the vitamin pantothenate has no recommended dietary or daily allowance established by either the National Research Council's Food and Nutrition Board or the United States Government's Food
Figure 3. Structures and names of coenzyme A fragments.
Table 1. Synonyms for selected compounds in pantothenate metabolism

<table>
<thead>
<tr>
<th>Pantothenic Acid*</th>
<th>(R)-N-(2,4-dihydroxy-3,3-dimethyl-1-oxybutyl)-β-alanine**</th>
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<tr>
<td></td>
<td>D (+)-N-(2,4-dihydroxy-3,3-dimethylbutyryl)-β-alanine</td>
</tr>
<tr>
<td></td>
<td>chick antidermatitis factor</td>
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<td></td>
<td>vitamin B₃</td>
</tr>
<tr>
<td></td>
<td>Vitamin B₅</td>
</tr>
<tr>
<td>Pantetheine</td>
<td>N-(pantothenyl)-β-aminoethanethiol</td>
</tr>
<tr>
<td></td>
<td>2,4-dihydroxy-N-[3-[(2-mercaptoethyl)amino]-3-oxopropyl]-3,3-dimethylbutanamide*</td>
</tr>
<tr>
<td></td>
<td>2,4-dihydroxy-N-[2-[(2-mercaptoethyl)carbamoyl]ethyl]-3,3-dimethylbutyramide**</td>
</tr>
<tr>
<td></td>
<td>α,γ-dihydroxy-β,β-dimethylbutyryl-β-alanyl-β-aminoethanethiol</td>
</tr>
<tr>
<td>Pantethine (the disulfide of pantetheine)</td>
<td>bis(N-pantothenoyl-β-aminoethyl)disulfide</td>
</tr>
<tr>
<td></td>
<td>N,N'-[dithiobis[2,1-ethanediylimino(3-oxo-3,1-propanediyl)]bis-[2,4-dihydroxy-3,3-dimethylbutanamide]*</td>
</tr>
<tr>
<td></td>
<td>N,N'-[dithiobis(ethyleneimincarboxylethylene)]bis(2,4-dihydroxy-3,3-dimethylbutyramide)**</td>
</tr>
<tr>
<td>Lactobacillus bulgaricus factor (LBF)</td>
<td>Pantosin</td>
</tr>
<tr>
<td>Cysteamine</td>
<td>2-aminoethanethiol* ** mercaptamine</td>
</tr>
<tr>
<td></td>
<td>β-mercaptoethylamine</td>
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<tr>
<td></td>
<td>2-aminoethyl mercaptan</td>
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<td></td>
<td>thioethanolamine</td>
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<td></td>
<td>decarboxycysteine</td>
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<td>MEA</td>
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<td>Becaptan</td>
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<td>Cystamine (the disulfide of cysteamine)</td>
<td>2,2'-dithiobisethylamine* **</td>
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<td></td>
<td>2,2'-dithiobisanthamine</td>
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<td></td>
<td>β,β'-diaminodiethyl disulfide</td>
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<tr>
<td></td>
<td>bis[β-amoenoethyl]disulfide</td>
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<td></td>
<td>decarboxycystine</td>
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*Nomenclature used in Chemical Abstracts through the Eighth Collective Index, 1967-1971.

**Nomenclature used in Chemical Abstracts since the Eighth Collective Index, 1972-to date.
and Drug Administration (24). Consequently, these groups have alternatively published intake values deemed "safe and adequate" for normal, healthy individuals. The fact that there is no recommendation may not be of great concern as the diet seems to supply all the needed pantothenate, and deficiency symptoms in humans have only been noted in cases where an antagonist was introduced or in total parenteral nutrition (TPN) diets from which pantothenate has been mistakenly omitted.

For a recommended dietary allowance to be established for any nutrient, information pertaining to body pools as well as tissue status and nutrient requirement should be known. Except for blood serum, which contains only free pantothenate (25), pantothenic acid status in the body has been traditionally difficult to determine because of the several moieties into which pantothenate is converted (see Figures 2 & 3). In order to determine total pantothenate status, it is necessary to liberate pantothenate from these bound forms by enzymatic means (26). As can be seen in Fig. 2, there are three specific enzyme activities required to release pantothenate from coenzyme A and the other intermediaries:
pyrophosphatase, orthophosphatase, and an amidase, pantetheinase. The first two enzymes are available commercially in the form of calf intestinal alkaline phosphatase, but the third enzyme, the amidase, is not commercially available. It was the lack of a suitably pure amidase preparation that caused the American Association of Agricultural Chemists to abandon its four-year study to develop a standard assay for total pantothenate, stating:

Results using pigeon liver or hog kidney enzyme preparations were similar. However, a loss of activity of these enzyme preparations showed a need for establishing the activity of the enzymes before they could be relied upon for use in the assay procedure. (Toepfer, p. 29) (27)

The report concluded that "when sufficient information on the specific enzyme activity [pantetheinase] is available, it may be desirable to reactivate collaborative studies" (p. 29). Consequently, the amidase activities used in various preparations for total pantetheinase activity determination were not quantified and comparisons between different preparations and laboratories were practically impossible to make until a suitable amidase preparation procedure was developed.

It should be noted that the three-enzyme system of
Pantothenate liberation is designed to liberate pantothenate from coenzyme A, and use of this system in tissue determinations is an extrapolation of the assumption that bound pantothenate is present in the tissues as coenzyme A or other bound forms of the vitamin that are liberated by this three-enzyme system. This assumption is not entirely correct. Majerus et al. (23) reported that in the acyl carrier protein of fatty acid synthesis, phosphopantetheine is linked to serine via a phosphodiester bond that is not hydrolyzed by the dual (phosphatase and amidase) enzyme treatment. Likewise, Imamoto et al. (28) reported that pantothenate may be bound in other forms such as various natural glycosyl derivatives. However, while the three-enzyme treatment method may give lower total pantothenate values than other methods (29), much valuable information can be obtained concerning pantothenate body pools using this current three-enzyme approach.

In 1982, Wittwer et al. (3,37) published a procedure for sufficiently purifying the amidase from pig kidney for use in pantothenate determinations. The amidase was called pantetheinase because of its affinity for pantetheine as a
substrate. Whole blood pantothenate status has been determined using this three-enzyme process (25,30,31) coupled with radioimmunoassay. Dupre et al. (32) have also reported purifying the amidase to a lesser extent from horse kidney.

In the search for a suitably pure amidase preparation, there have been numerous assays developed to measure pantetheinase activity. These assays have been based on: oxygen uptake by means of amine oxidase coupling (1), chromatographic separation of $^{14}$C-labeled substrate and product (2), electrophoretic separation of $^{14}$C-labeled substrate and product (4), microbiological assay of pantothenate (5), pH-stat titration of products (6), amino acid analysis of the N-ethylmaleimide adduct of cysteamine (7), radioimmunoassay of pantothenate product (3), and chromatographic separation of $^{14}$C-labeled pantethine and product (3).

There are also the previously mentioned three simple spectrophotometric absorbance assays, two of which are continuous assays and are described in detail in the "Methods" section and all three described briefly here as follows: i) cysteamine is measured by the absorbance of its fluoropyruvate
adduct at 300 nm (3); ii) mercaptide ion is monitored at 240 nm as its concentration increases proportionately with enzymatic hydrolysis (3); and iii) the use of the substrate S-pantetheine-3-pyruvate (pantetheine-S-pyruvate), which forms a chromogenic, cyclic compound upon enzymatic hydrolysis, absorbing strongly at 296 nm (8).

While these three assays provide many advantages over other assays of pantetheinase activity, they utilize wavelengths in the ultraviolet range where absorbing interference from proteins is also encountered. In countering protein absorbance effects, sensitivity limitations of these assays are usually exceeded. Development of a new assay, or incorporation of a chromogenic substrate that absorbs light in a region different from that of protein, would be advantageous for enzyme activity assay of high-protein-content media.

Pantothenic acid and its bound forms administered orally are converted to free pantothenic acid in the intestinal lumen of the duodenum before entering the bloodstream (33). However, CoA and other bound forms of pantothenate administered intravenously led
to a rapid rise and then decline of CoA degradation products in the plasma until no CoA was detectable (17,34). Apparently, the degradative enzymes are present in blood plasma and their activities result in free pantothenate being the only form of degraded CoA in plasma under normal conditions (25,31).

Pantetheinase is usually described as an enzyme present in tissue. One explanation of its presence in blood plasma is for the conversion of pantetheine that has been transported out of a cell via an ATP dependent transport system.

While investigating total blood pantothenate content, Wittwer et al. (31) quantified human blood plasma pantetheinase activity. A mean plasma activity of $11.2 \pm 2.0 \mu\text{mol/min/ml}$ serum in 29 fasted subjects, as determined by radiolabeled pantothenate assay, was reported. It was determined that this plasma enzyme activity was "comparable to activity usually added from exogenous sources for liberation of pantothenate from whole blood" (p. 1072).

Rats fed a pantothenate-restricted or pantothenate-deficient diet have markedly lower serum pantothenate levels than rats fed a normal diet (35,36). Plasma pantothenate levels may affect plasma
pantetheinase activity in one or more of the following ways:

i) Pantothenate inhibits pantetheinase by a product feedback inhibition mechanism (5). A small decrease in plasma pantothenate levels might increase pantetheinase activity.

ii) An increase in enzyme substrate might trigger release (or transcription/production) of more enzyme into the bloodstream. When substrate concentration falls dramatically, the amount of new enzyme released into the bloodstream may be greatly decreased, causing an overall loss of plasma enzyme activity.

iii) Enzyme is activated by thiol compounds such as cysteine (5). Wittwer et al. (31) reported that maximal plasma activity rates were obtained after treatment with dithiothreitol; when dithiothreitol was omitted from the assay, hydrolysis occurred at 57% the maximal rate. The plasma flux pattern of enzyme activity may follow a similar plasma flux pattern of a thiol compound such as cysteine.
REVIEW OF LITERATURE

Pantetheinase Assays

Microbial Assay

Liberation of pantothenate was originally monitored by microbiological assay. This assay is most successful after ion exchange separation of pantothenate (5). This method of assay is very time consuming and gives inconsistent results (7). Abiko's use of this assay (5) did not specify whether the racemic mixture or biologically active (D) stereoisomer of pantethine was used in the assay.

Amine Oxidase Coupling Assay

Cavallini et al. (1) monitored the hydrolysis of pantetheine by coupling the production of cysteamine to its oxidation with amine oxidase and measuring the uptake of oxygen. Reducing compounds were also found to activate the reaction (2), making this assay unreliable.
pH-stat Titration Assay

Dupre et al. (6) reported an assay utilizing pantethine as a substrate and the quantization of hydrolysis products by a pH-stat procedure. This requires special equipment but is much faster than procedures using radiolabeled compounds.

Amino Acid Analysis

Orloff et al. (7) described an assay utilizing pantethine reduced to pantetheine as a substrate. Quantization of the n-ethylmaleimide adduct of cysteamine was by automated ion exchange chromatography using an amino acid analyzer.

$^{14}$C Labeled Assays

Chromatographic Separation Assay. Cavallini et al. (2) described an assay utilizing $^{14}$C DL-pantethine as substrate. Hydrolysis products are separated by paper chromatography and the radiolabeled products quantified. This assay requires the expensive radiolabeled substrate. The racemic mixture was used rather than the enzyme specific (D) form alone.
Electrophoretic Separation Assay. Dupre et al. (4) described an assay much like the $^{14}\text{C}$ chromatographic (paper) separation assay, but differing by an electrophoretic separation of hydrolysis products. This was the fastest procedure to date but still time consuming due to a 5-hour electrophoresis step. The racemic mixture substrate was used.

Radiolabeled Assay. Wittwer et al. (3) described a radiolabeled assay utilizing D-[$^{14}\text{C}$]-pantethine reduced to D-[$^{14}\text{C}$]-pantetheine as substrate and ascending paper chromatography used to separate resulting radiolabeled pantothenate from unhydrolyzed radiolabeled pantetheine. While this assay is similar to the previously mentioned paper chromatography radiolabeled assay, labeled D-pantethine, the biologically active form is used as enzyme substrate instead of the racemic DL-pantethine.

Pantothenate Assay. Wittwer et al. (3) described an assay utilizing D-[$^{14}\text{C}$]-pantethine reduced to D-[$^{14}\text{C}$]-pantetheine as the enzymatic substrate. Determination of the resulting radiolabeled pantothenate was by the radioimmunoassay of Wyse et al. (38). A scintillation counter and antibodies specific for pantothenate are
required for this assay.

Cysteamine Assay

Wittwer et al. (3) described a spectrophotometric assay utilizing 0-pantetheine as the substrate. The hydrolysis product, cysteamine, is measured by the absorbance of its fluoropyruvate adduct at 300 nm. The assay is specific for pantetheinase and fairly simple.

Mercaptide Assay

Wittwer et al. (3) described a continuous spectrophotometric assay utilizing D-pantetheine as the substrate and monitoring the absorbance of mercaptide ion at 240 nm. The mercaptide ion concentration increases proportionately with hydrolysis at a buffered pH because of a difference in pK (-SH) between pantetheine (9.9) and cysteamine (8.1) at 37°C. The assay is simple, does not require radiolabeled substances or specialized equipment other than a spectrophotometer, and due to the use of the enzyme's native substrate, it is very specific with an apparent Km of 20 µM.
Pantetheine-S-Pyruvate Assay

Dupre et al. (8) described a continuous spectrophotometric assay using 3-pantetheine-S-pyruvate (pantetheine-S-pyruvate) as a substrate for pantetheinase. The enzymatic hydrolysis of this substrate leads to the direct formation of S-cysteamine-3-pyruvate, which cyclizes in a non-rate-limiting step to give 2H-1,4-thiazin-5,6-dihydro-3-carboxylic acid (aminoethylcysteine ketimine), a compound exhibiting a strong absorbance at 296 nm. The assay is specific and much more simple and rapid than other assay procedures, but assay results give much lower activity values (ca. 40% lower) than activity values from the mercaptide assay which uses the enzyme's native substrate. The synthesis of pantetheine-S-pyruvate is quite simple but does not yield a crystalline compound or purify it (see synthesis scheme in methods section). Activity values might increase if the substrate were pure since any unreacted pantetheine in the substrate mixture would also compete for enzymatic hydrolysis. The apparent Km value of 28 µM was similar to the reported Km from the mercaptide assay (20 µM).
Pantetheinase

Pantetheinase (EC 3.5.1.15) is highly specific for pantetheine and does not hydrolyze any other carboamide bonds such as those in β-alanyl-cysteamine and pantothenic acid. Pantetheinase contains at least one thiol group at its activation site, is activated by thiol compounds, most effectively by mercaptoethanol and dithiothreitol, and undergoes product inhibition by pantothenate (5).

In specificity studies for pantetheinase, Wittwer et al. (3,39) synthesized and tested several analogs of pantetheine for enzyme specificity and concluded that the enzyme requires a coupled D-pantooyl-β-alanyl moiety for significant hydrolysis to occur. This research group further concluded that modification of the cysteamine portion had a lesser effect on enzyme specificity, when compared to pantetheine, after observing a 75 to 100% activity on pantetheine analogs altered at the cysteamine portion.

Wittwer concluded that pantetheinase specifically engages pantetheine-like substances on the chiral-carbon end of the substrate, thus allowing various analogs of pantetheine to be hydrolyzed at the amide bond specified in Fig. 3. This finding led
Dupre et al. (8) to develop a continuous absorbance spectrophotometric assay by utilizing pantetheine-S-pyruvate as a substrate.

For many peptidases and proteinases, enzyme specificity depends mostly on the substrate structure at the carboxylic side of the bond to be split. The substrate structure at the amino side is of much smaller influence (40). In artificial substrates for color indicator activity assays of proteases, chromogenic groups are usually located on the amino side of the hydrolyzed bond. Groups at the carboxylic side of this bond are selected to resemble natural substrates to maximize enzyme specificity. Many proteases accept esters and thioesters as substrates in addition to peptides, frequently resulting in lower Km values and higher V values as the reactions proceed faster.

The most commonly used chromogenic group for proteinases is p-nitroaniline and is typically used for the testing of trypsin and other proteases in peptide analysis (41,42). While the peptide-linked p-nitroaniline shows an absorption maximum at 315 nm, free p-nitroaniline has an absorption maximum at 380 nm and can
be conveniently measured at 410 nm with an extinction coefficient of 8,800 following enzymatic hydrolysis from peptide-like molecules (41).

The β-alanyl moiety of pantetheine (see Fig. 3) is bonded by amide (or peptide-like) bonds with pantetheinase hydrolyzing the outermost amide bond. Pantetheinase must therefore act by a mechanism similar to that of peptidases and proteinases and has in fact been described as a thiol protease (43). It would naturally follow that similar modifications might be made in pantetheinase substrates for use in color indicator activity assays. Indeed, the substrate of Dupre et al. (8), namely pantetheine-S-pyruvate, is a prime example.

With this in mind, it was hypothesized that synthesis of a molecule with the enzyme specific pantetheine moiety coupled to a p-nitroaniline chromogenic group, absorbing light in the visible light region upon enzymatic hydrolysis, would result in a substrate that could be utilized in a convenient, continuous spectrophotometric assay of pantetheinase activity. The enzyme should be specific for such a substrate and have a Km similar to
those of assays utilizing native substrates (ca. 20 \( \mu \text{M} \)). Furthermore, the assay procedure should be similar, if not identical, to the assay described by Dupre et al. utilizing pantetheine-S-pyruvate. The new assay should therefore be extremely simple and fast, yet should monitor a chromogenic compound in the visible light region, thus avoiding interference of protein in samples such as blood serum.
MATERIALS AND METHODS

Chemicals

For the synthesis of pantothenate-p-nitroanilide and assays of pantetheinase, chemicals and solvents used were reagent grade and obtained from the following companies: Aldrich Chemical Co., isopropanol, ethyl acetate, isooctane (2,2,4-trimethyl pentane), methylene chloride; Fisher Scientific Co., sodium hydroxide, potassium carbonate (anhydrous), sodium chloride, carbon tetrachloride; J.T. Baker, methanol, hydrochloric acid; Mallinckrodt, Inc., sodium sulfate (anhydrous), petroleum ether; Sigma Chemical Co., Inc., β-alanine, di-tert-butyl-pyrocarbonate, citric acid, p-nitroaniline, N,N'-dicyclohexylcarbodiimide, tri-fluoroacetic acid, D(-) pantoyllactone.

Synthesis

The synthesis of pantothenate-p-nitroanilide was accomplished through a three-part procedure that yielded two intermediary products and a final product, each of which was
crystalline. Figure 4 depicts the overall reaction scheme for the synthesis of pantothenate-p-nitroanilide.

Preparation of t-boc-β-alanine

The synthesis of t-boc-β-alanine as previously described by Kopelevich et al. (44) utilizes a reductive amination reaction common to peptide synthesis while adding t-boc protection, which is readily removed later by mild acid hydrolysis without cleavage of the labile amide bond. Only minor modifications were made to the published procedure and are outlined as follows.

First, 9.0 g of β-alanine and 14.0 g potassium carbonate dissolved in 100 ml water are stirred at room temperature for 30 min with 30.0 g di-tert-butyl-pyrocarbonate that has been previously dissolved in 100 ml isopropanol. Next, 400 ml of water is added to the reaction mixture after which the entire solution is washed with an equal amount of petroleum ether in a separatory funnel and the wash discarded. The wash removes any unreacted di-tert-butyl-pyrocarbonate. Then, 60 g of citric acid is slowly added to the solution (CO₂ is vigorously produced in this step). The citric
Figure 4. Reaction scheme for the synthesis of pantothenate-p-nitroanilide.
acid protonates any unreacted β-alanine, which reacts immediately with the potassium carbonate, forming a hydrophilic product that remains in the aqueous phase in the next step. Next, 150 g of sodium chloride is added to the solution, which is then extracted three times with ethyl acetate (300 ml each time) using a separatory funnel. The salt saturates the solution and "pushes" the t-boc-β-alanine into the organic phase during extraction. The combined extracts are washed with 600 ml saturated sodium chloride solution to remove traces of citric acid without loss of the desired t-boc-β-alanine. The extract is then dried over anhydrous sodium sulfate for 30 min, vacuum filtered, rotory-evaporated to an oily liquid, and allowed to recrystallize overnight in a 1:1 mixture of isooctane:carbon tetra-chloride (350-400 ml). The fine, white crystals are then filtered, freeze-dried overnight, and used in the second part of the synthesis.

Preparation of β-alanyl-p-nitroanilide

The second part of the synthesis is a N,N'-dicyclohexylcarbodiimide condensation reaction in which 3.0 g t-
boc-β-alanine, 3.91 g N,N'-dicyclohexylcarbodiimide, and 1.1 g p-nitroaniline are incubated in 15 ml methylene chloride at 40°C in a closed (screw-capped) test tube for 24 hrs with shaking. The solution is filtered and brought to a volume of 75 ml with methylene chloride and washed twice with 30 ml of 3M HCl and the wash discarded. This HCl wash removes any unreacted p-nitroaniline. A 20-ml mixture of 1:1::TFA:CH₂Cl₂ (trifluoroacetic acid:methylene chloride) is prepared and added to the solution with stirring for 1 hr to remove the t-boc group from the product (see Fig. 4). Then, 40 ml of distilled water is added to the solution and stirred. Saturated sodium carbonate (Na₂CO₃) is added (~18 ml) with mixing until the aqueous phase has a pH of 5.0-6.0 and the aqueous layer is separated off. Sodium carbonate neutralizes the unreacted TFA and adjusts the pH so that the product is optimally partitioned into the aqueous phase (see Table 2). The aqueous layer is then brought to a pH of 12.0 with sodium hydroxide solution to obtain free base product (15% NaOH is used to reduce volume required). Since the free base product has no charge associated with it, further purification from ionically charged molecules
(salts, etc.) is easily facilitated by extracting the solution three times with methylene chloride. The methylene chloride extractions are combined and rotary-evaporated to complete dryness. A minimal amount of warm water (~70°C) is added to dissolve the β-alanyl-p-NA and the solution placed at 3°C for at least 3 hrs to crystallize. Crystals are collected by vacuum filtration, freeze-dried overnight, and melting point and yield are determined. A 100% yield for this reaction would be 3.3 g while the expected yield is 0.8 grams (24% yield). The fiberglass-like, yellow crystals have a melting point of 146-148°C.

The yield of 24% is slightly improved (~6%) if, at the water extraction point, after Na₂CO₃ is used to raise the pH to 5-6, the precipitate that forms between the methylene chloride and water layers is retained by filtration, redissolved in methylene chloride, filtered, rotary-evaporated to complete dryness, dissolved in dH₂O, and further treated the same as (or combined with) the normal water extraction, that is, raise pH to 12.0 with NaOH, extract three times with methylene chloride, and continue as previously described.
Preparation of Pantothenate-p-Nitroanilide

The third part of the synthesis consists of a condensation reaction similar to reactions performed by Barnett and Robinson (45) in which the calcium and sodium salts of pantothenic acid and several analogues are prepared by refluxing β-alanine, or other amino acid, with the appropriate lactone for 1 hr in methanol. The part II product, β-alanyl-p-nitroanilide, and an equimolar amount of d(-)pantoyllactone, which has been freshly resublimed to exclude moisture, is refluxed for 1 hr in a minimal amount of absolute methanol (dried over anhydrous sodium sulfate and freshly redistilled before use) to dissolve the reactants at room temperature. The methanol is evaporated under vacuum and the pantothenate-p-nitroanilide is dissolved in a minimal amount of warm water (~60°C) and allowed to cool and crystallize at 5°C overnight.

Crystalline Product Analysis

Each of the crystalline products obtained from the synthesis of pantothenate-p-nitroanilide was characterized in the following ways: i) a melting point was obtained on a Unimelt capillary
melting point apparatus (Arthur H Thomas Co., Philadelphia, PA); ii) a proton nuclear magnetic resonance (H-NMR) was obtained on a JEOL 270 Fourier transform NMR spectrometer; and iii) elemental analysis was obtained from Atlantic Microlab Inc. (Norcross, GA) as reported.

Enzyme Purification

The pantetheinase enzyme is purified by the procedure described by Wittwer et al. (37), with some deviations to the published procedure, as outlined by Wittwer (39).

In this procedure, fresh pig kidneys (Thorn-Apple Valley, Hyrum, UT) are cooled on ice and slices of cortex frozen until use. Then, 550 g of semi-solid, thawed cortex are carried through the purification, though equipment limits the scale ofSteps 1 through 3 to ca. 275 g of cortex. Therefore, these steps are performed twice per purification to provide a pooled extract from 550 g of cortex at the end of Step 3 that is then used in the remaining purification. All steps are performed at 0-4°C unless otherwise indicated.
Step 1: Homogenate

Four volumes of 0.02 M Tris-HCl, pH 8.2, and 275 g of cortex are homogenized in a Waring blender for 4 min at high speed. The suspension is centrifuged for 10 min at 10,000 g and the supernatant is collected off the pelleted large particulate matter.

Step 2: Microsomal Agglutination

Microsomes in the supernatant are agglutinated at pH 4.2 by the addition of 1 M formic acid and centrifuged for 10 min at 20,000 g. The supernatant is decanted and the pellet resuspended in ca. 400 ml of deionized water by homogenization in a Waring blender at high speed for 10-20 sec. The suspension is brought to pH 5.75 with 0.5 M Tris-HCl, pH 9.0.

Step 3: Butanol Solubilization

One and a half volumes of n-butanol are added to the microsomal suspension and the mixture is homogenized in a Waring blender at low speed for 2 sec. The material is then centrifuged for 10 min at 10,000 g. The aqueous phase containing solubilized enzyme is aspirated and immediately passed through a column (5.5 x 65 cm) of Sephadex G-25 previously equilibrated with 0.02 M
phosphate, pH 7.0, to remove dissolved butanol.

Step 4: Heat Treatment

The void volume eluate from Step 3 is brought to pH 5.0 with 1 M formic acid and heated in a 75°C water bath up to 68°C and held at 68°C for 2 min. The suspension is rapidly cooled, centrifuged for 10 min at 20,000 g, and the supernatant is collected off from coagulated proteins.

Step 5: (NH₄)₂SO₄ Fractionation

The supernatant from Step 4 is saturated to 55% by adding 32.6 g solid (NH₄)₂SO₄ per 100 ml followed by centrifuging for 15 min at 20,000 g. The resulting supernatant is saturated to 70% by adding 9.3 g (NH₄)₂SO₄ per 100 ml and centrifuged for 30 min at 20,000 g. The precipitate is dissolved in ca. 20 ml of hydrophobic chromatography "application buffer" (114 g of (NH₄)₂SO₄ added to 1 liter of 0.02 M phosphate, pH 7.0).

Step 6: Hydrophobic Chromatography

The protein from Step 5 is applied to a 2.5 x 23-cm column of
Octyl-Sepharose CL-4B (Pharmacia) that had previously been equilibrated with application buffer. The protein is washed with application buffer at a flow rate of ca. 150 ml/hr until the $A_{280}$ of the eluate is less than 0.05. Enzyme is eluted with a solution of 20% ethylene glycol, 80% application buffer (v/v) at the same flow rate until the $A_{280}$ is less than 0.03. The column is regenerated by sequentially applying one column volume each of 50 mM NaOH, 95% ethanol, deionized water, and application buffer.

Step 7: Hydroxyapatite Chromatography

The enzyme eluate from Step 6 is concentrated to ca. 20 ml by ultrafiltration and diafiltered into 0.001 M phosphate, pH 7.0, with an Amicon PM ultrafiltration membrane (Lexington, MA). The solution is applied at ca. 25 ml/hr to a 1.5 x 5-cm Bio Gel HT hydroxyapatite column (Bio Rad, Richmond, CA) previously equilibrated with 0.001 M phosphate, pH 7.0. Enzyme is eluted at the same rate with 0.02 M phosphate, pH 7.0.

For specific activity determination, protein content of the enzyme solution is determined by using an extinction coefficient
as follows: $E^{\%}_{1\text{cm}}(280\text{ nm}) = 11.3 \ (37)$.

Assays

Pantetheinase activity was determined by both the mercaptide assay described by Wittwer et al. (3) and the pantetheine-S-pyruvate assay described by Dupre et al. (8), both of which follow absorbance at appropriate wavelengths as hydrolysis product accumulates. Synthesized pantothenate-p-nitroanilide was also used as a substrate in a continuous absorbance spectrophotometric assay monitoring accumulation of hydrolysis product. All spectrophotometric assays were performed on a Gilford model 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH).

Mercaptide Assay

In a 3-ml spectrophotometer cuvette, the enzyme is pre-reduced by incubating 240 $\mu$l enzyme/dH$_2$O solution with 30 $\mu$l 0.5 M Tris, pH 8.5, and 30 $\mu$l of 100 mM dithioerythritol for 20 min at 37°C. The amount of enzyme depends on an estimation of activity;
suspicion of high enzyme activity warrants inclusion of lesser amounts of enzyme in assay. After incubation, the 300 µl of prereduced enzyme solution is diluted with 2.69 ml of 0.05 M Tris-HCl, pH 8.1, which had been deoxygenated by bubbling nitrogen gas through the solution and prewarmed to 37°C. The A$_{240}$ is followed for 10 min or until a constant decrease with time can be determined. Pantetheine is added (15 µl of 100 mM) to initiate the reaction and the A$_{240}$ followed. The maximal ΔA$_{240}$ per minute (after linearization and before product inhibition) is proportional to the amount of enzyme present when the rate before substrate addition is taken as baseline.

D-Pantetheine is obtained by the reduction of D-Pantethine by the method of Butler et al. (46). Pantetheine solutions are stored under nitrogen and standardized by sulfhydryl analysis (47).

A reaction run to substrate exhaustion established a ΔA$_{240}$ corresponding to 100% hydrolysis. This ΔA$_{240}$ found with a known amount of substrate is used in a Beer's law calculation (A=εbc) to calculate the extinction coefficient for the assay.
PE-s-pyr Assay

In a 3-ml spectrophotometer cuvette, the enzyme is pre-reduced by incubating 300 µl enzyme/dH2O solution with 100 µl of 0.9 M mercaptoethanol and 100 µl of 0.5 M phosphate buffer, pH 8.0, for at least 10 min at 37°C. The amount of enzyme depends on an estimation of activity; suspicion of high enzyme activity warrants inclusion of lesser amounts of enzyme in assay. After incubation, the 500 µl of prereduced enzyme solution is diluted with 2.4 ml of 0.1M phosphate buffer, pH 8.0, which had been prewarmed to 37°C. Pantetheine-S-pyruvate is added (100 µl of 15mM) to initiate the reaction and the A296 followed. The maximal ΔA296 per minute (after linearization) is proportional to the amount of enzyme present (ε = 5950).

Pantetheine-S-pyruvate is synthesized as according to Dupre et al. (8) by reacting 10 mM pantetheine with 20 mM bromopyruvate in 10 ml of 0.1M phosphate buffer, pH 8.0, at room temperature. After 10 min, 20 mM mercaptoethanol was added. Since the product is not crystalline, the solution was quantified for pantetheine-S-pyruvate by acid hydrolysis (4 N HCl, 100°C, 20 min) and by
monitoring the change in absorbance at 296 nm after neutralization to pH=7.6.

Pantothenate-p-nitroanilide Assay

In a 3-ml spectrophotometer cuvette, the enzyme is prereduced by incubating 300 µl enzyme/dH₂O solution with 100 µl of 0.9 M mercaptoethanol and 100 µl of 0.5 M phosphate buffer, pH 8.0, for at least 10 min at 37°C. The amount of enzyme depends on estimation of activity; suspicion of high enzyme activity warrants inclusion of lesser amounts of enzyme in assay. After incubation, the 500 µl of prereduced enzyme solution is diluted with 2.47 ml of 0.1 M phosphate buffer, pH 8.0, which had been prewarmed to 37°C. Pantothenate-p-nitroanilide is added (30 µl of 12.4 mM) to initiate the reaction and the A₄₁₀ followed. The maximal ΔA₄₁₀ per minute (after linearization) is proportional to the amount of enzyme present (ε = 8,800) (41).
Biological Application

Assay of Pantetheinase Activity in Rat Serum

Frozen serum samples from Sprague-Dawley rats with varying degrees of pantothenate nutriture were obtained from Dr. Won Song (Michigan State University) and assayed for pantetheinase activity using the pantothenate-p-nitroaniline assay. The serum samples were from a study in which 3-week-old Sprague-Dawley rats were assigned to one of five groups and fed deficient and control diets ad. lib. or pair fed for 4 and 12 weeks. Groups were assigned experimental semi-purified AIN-76 diets (48) as follows:

Week 4: PA+ ad lib. group: 12 mg pantothenic acid/kg diet, administered ad. lib.

PA- ad lib. group: <0.8 mg pantothenic acid/kg diet, administered ad. lib.

PA+ pair fed group: 12 mg pantothenic acid/kg diet, pair fed to deficient group.
Week 12: PA+ group: 12 mg pantothenic acid/kg diet, administered ad. lib.

PA- group: <0.8 mg pantothenic acid/kg diet, pair fed to deficient group.

Blood samples were collected after a 24-hr fast at week 4, and an overnight (15-16 hrs) fast at 12 weeks. Results were statistically compared to investigate relationship between pantothenate status and pantetheinase activity in rat blood serum by analysis of variance (StatView 512+, Brainpower Inc., Calabasas, CA).

The pantothenate status for each serum sample was determined by radioimmunoassay (38). The low number of samples from the deficient group was due to many of the rats on the pantothenate-deficient treatment dying during the course of the experiment.
RESULTS

Synthesis

Preparation of t-boc-β-alanine

The synthesis of t-boc-β-alanine (molecular weight 189.0) yielded fine, white crystals with a melting point of 73-74°C in a typical yield of 73% (ca. 14 g), which is the same as previously reported by Kopelevich et al. (44). The melting point of 73-74°C is also in good agreement with the published values of 75-76°C. Elemental analysis of the crystals found %: C 50.82, H 7.87, N 7.60, O 33.74. \(\text{C}_{18}\text{H}_{15}\text{NO}_4\); calculated %: C 50.78, H 7.99, N 7.99, O 33.83.

A proton nuclear magnetic resonance (H-NMR) spectrum of t-boc-β-alanine in deuterated chloroform is given in Fig. 5 with peak assignments.

Preparation of β-alanyl-p-nitroanilide

The synthesis of β-alanyl-p-nitroanilide (molecular weight 209.2) yielded fine, greenish-yellow crystals with a melting point
Figure 5. H-NMR spectrum of t-boc-β-alanine. Deuterated chloroform is the solvent.

Assignment (PPM)

- a 1.42
- b 2.58
- c 3.39
- d 5.08
- e 6.12
of 146-149°C in a typical yield of 24% (ca. 0.8 grams). Elemental analysis of the crystals found %: C 51.58, H 5.34, N 20.03, O 22.94. C$_9$H$_{11}$N$_3$O$_3$; calculated %: C 51.67, H 5.30, N 20.09, O 22.94. A proton nuclear magnetic resonance (H-NMR) spectrum of β-alanyl-p-nitroanilide in deuterated water is given in Fig. 6 with peak assignments. When looking at the H-NMR spectrum obtained from a sample in D$_2$O, it should be noted that hydrogen atoms bound to nitrogen and oxygen will exchange with the D$_2$O solvent and no resonance spectrum will be seen for these hydrogen atoms.

One interesting feature that aided in purification of this product was its ability to be selectively soluble in either organic or aqueous solvents, depending on degree of protonation (a factor of pH) of the terminal nitrogen. Table 2 shows ratios of partitioning for β-alanyl-p-nitroanilide in organic and aqueous solvents. An aqueous extraction at pH=5 extracted virtually all of the product from the organic layer. When this aqueous extraction was raised to a pH of 12, organic extraction performed three times
Figure 6. H-NMR spectrum of β-alanyl-p-nitroanilide. Deuterated water is the solvent.
TABLE 2. β-alanyl-p-nitroanilide purification by solvent partitioning with pH

<table>
<thead>
<tr>
<th>pH</th>
<th>β-alanyl-p-nitroanilide (mmoles)</th>
<th>p-nitroanilide (mmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>organic*</td>
<td>aqueous</td>
</tr>
<tr>
<td>1</td>
<td>4.03</td>
<td>x</td>
</tr>
<tr>
<td>5</td>
<td>0.27</td>
<td>2.82</td>
</tr>
<tr>
<td>7</td>
<td>0.40</td>
<td>2.98</td>
</tr>
<tr>
<td>9</td>
<td>0.98</td>
<td>1.97</td>
</tr>
<tr>
<td>10</td>
<td>1.30</td>
<td>1.74</td>
</tr>
<tr>
<td>11</td>
<td>1.67</td>
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</tr>
<tr>
<td>12</td>
<td>1.57</td>
<td>1.31</td>
</tr>
</tbody>
</table>

*Organic phase is methylene chloride, aqueous phase is water.
extracted most (~85%) of the product out of the aqueous layer.

Preparation of Pantothenate-p-Nitroanilide

The synthesis of pantothenate-p-nitroanilide (molecular weight 338.0) yielded a precipitate-like crystalline product with a melting point of 74-76°C with an initial yield of 14% (ca. 0.2 grams). The yield is improved by concentrating the crystallization liquor. A proton nuclear magnetic resonance (H-NMR) spectrum of pantothenate-p-nitroanilide in deuterated water is given in Fig. 7 with peak assignments.

Stick H-NMR spectra of synthesis products as well as other similar compounds are given in Fig. 8 to show chemical shifts of corresponding protons.

Enzyme Purification

Enzyme purification resulted in a hydroxyapatite column chromatography eluate (Step 7) enzyme preparation with a protein content of 0.21 mg/ml, an activity of 3600 nmol/min/ml
Figure 7. H-NMR spectrum of pantothenate-p-nitroanilide. Deuterated water is the solvent.
Figure 8. Stick H-NMR regional spectra of pantothenate-p-nitroanilide and related compounds. D$_2$O was used as solvent. Chemical shifts of corresponding protons correlate with structural differences between compounds.
(pantothenate-p-nitroanilide assay), and a specific activity of 16,600 nmol/min/mg protein (see Table 3). This enzyme preparation was used exclusively in all assays (except as noted in the comparative assays where enzyme aliquots of varying purity from various purification steps were used).

Assays

Km Determination

A Lineweaver-Burk plot of initial rates with varying amounts of enzyme (Fig. 9) reveals an apparent Km of 12.6 µM for pantothenate-p-nitroanilide by the purified pantetheinase enzyme. The Lineweaver-Burke plot is quite linear with an R² value of 0.986.

Assay Comparisons

Comparative assays for pantetheinase activity were run on sample aliquots collected during the pantetheinase purification process using the mercaptide, pantetheine-S-pyruvate, and
Figure 9. Lineweaver-Burke plot for Km determination of pantetheinase with pantothenate-p-nitroanilide as substrate. Purified enzyme (enzyme purification Step 7 eluate) was used in the pantothenate-p-nitroanilide assay with 12.4 mM pantothenate-p-nitroanilide as substrate.

$K_m = 12.6 \mu M$
pantothenate-p-nitroanilide assays. Assay results are presented in Table 3. The pantothenate-p-nitroanilide assay has results similar to the mercaptide assay while the pantetheine-S-pyruvate assay tended to give activity results about 66% lower than either of the other assays.

Coefficients of Variation

Coefficients of variation were calculated for both the mercaptide assay and the pantothenate-p-nitroanilide assay by running each assay several times using purified enzyme from Step 7 of the enzyme purification procedure and calculating the coefficient of variation (activity average/standard deviation). Data and results are given in Table 4. While the coefficient of variation is much lower for the pantothenate-p-nitroanilide assay than the mercaptide assay (3.3 vs 6.1), it is worthwhile to note that Wittwer et al. published a coefficient of variance value of 3.2 for the mercaptide assay (3).
Table 3. Assay comparisons on pantetheinase of varying purity

<table>
<thead>
<tr>
<th>Stage of purification</th>
<th>Activities*</th>
<th></th>
<th></th>
<th></th>
<th>Protein† (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PA-p-NA assay</td>
<td>PE-s-Pyr assay</td>
<td>Mercaptide assay</td>
<td></td>
<td></td>
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<tr>
<td>((\text{NH}_4\text{H}_2\text{SO}_4)) fractionation</td>
<td>3600 (230)</td>
<td>1400 (92)</td>
<td>X</td>
<td></td>
<td>15.5</td>
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<tr>
<td>Hydrophobic chromatography</td>
<td>730 (1200)</td>
<td>320 (520)</td>
<td>X</td>
<td></td>
<td>0.60</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>2200 (2200)</td>
<td>1100 (1100)</td>
<td>X</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>Hydroxyapatite chromatography</td>
<td>3600 (16,600)</td>
<td>1200 (5600)</td>
<td>3600 (16,500)</td>
<td></td>
<td>0.22</td>
</tr>
</tbody>
</table>

* Activity given in terms of nmol/min/ml
Specific activity (in parenthesis) in terms of nmol/min/mg protein.
† Protein (mg/ml)=\(\frac{A_{280} \times \text{dilution factor}}{1.13}\) (References 37 & 39).

Note: Enzyme samples are from the pantetheinase purification with the \((\text{NH}_4\text{H}_2\text{SO}_4)\) fractionation being from Step 5, the hydrophobic separation from Step 6, the ultrafiltration from the first part of Step 7, and the hydroxyapatite chromatography sample being the final purified enzyme.
<table>
<thead>
<tr>
<th>Run #</th>
<th>Mercaptide assay</th>
<th>Activity (nmol/min/ml)</th>
<th>Pantothenate-p-nitroanilide assay</th>
</tr>
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<tr>
<td>1</td>
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</tr>
<tr>
<td>10</td>
<td>3600</td>
<td>3600</td>
<td></td>
</tr>
</tbody>
</table>

average: 3650                3665
st. deviation: 224.1        121.5
coeff. of variation: 6.1%   3.3%
Biological Application

Rat serum samples were assayed for pantetheinase activity using the pantothenate-p-nitroanilide assay. Data and results are given in Table 5. Week 4 serum pantetheinase activities were significantly different between the PA+ ad lib (average ± st. dev. = 8.8 ± 1.5) and the PA- ad lib group (ave. ± st. dev. = 16 ± 4.2) as well as the PA- ad lib group and the PA+ pair fed group (ave. ± st. dev. = 6.3 ± 0.7) (p=0.0022 and p=0.0005, respectively) by one-way analysis of variance. The PA- ad lib group had higher enzyme activities than the control groups. Serum pantothenate levels were also significantly different (p<0.02 and p<0.001, respectively) with the deficient group having lower values (ave. ± st. dev. PA- ad lib = 0.14 ± 0.04 vs. PA+ ad lib = 0.52 ± 0.22 and PA+ pair fed = 0.54 ± 0.15). The week 12 pantothenate-deficient group did not have a significantly different pantetheinase activity than the pantothenate-enriched group (ave. ± st. dev = 7.9 ± 2.0 and 10.5 ± 4.7, respectively; p=0.12) by one-way analysis of variance. Week 12 PA- group serum pantothenate values were significantly lower than the week 12 PA+ control group (ave. ± st. dev. = 0.10 ± 0.05 and
0.51 ± 0.20, respectively; p=0.001). Figure 10 shows a plot of serum pantothenate values versus serum pantetheinase activity.
Table 5. Pantetheinase activity for serum samples of rats with varying pantothenic acid nutriture

*Group & Rat # | Activity (nmol/min/ml) | Serum pantothenate (nmol/ml)
--- | --- | ---
4 wk. PA+ ad lib. 1 | 6.8 | 0.68
" " 2 | 9.4 | 0.40
" " 3 | 7.8 | 0.36
" " 4 | 9.6 | 0.26
" " 5 | 10.9 | 0.40
" " 6 | 7.4 | p=.0022
" " 7 | 9.4 | 0.64
ave. ± st. dev. = 8.8 ± 1.5

4 wk. PA- ad lib. 1 | 20.8 | 0.13
" " 2 | 12.8 | 0.18
" " 3 | 14.9 | 0.11
ave. = 16.2 ± 4.2

4 wk. PA+ pair fed 1 | 6.0 | 0.36
" " 2 | 6.7 | p=.0005
" " 3 | 6.9 | 0.76
" " 4 | 5.9 | 0.47
" " 5 | 6.9 | 0.63
" " 6 | 5.1 | 0.43
ave. = 6.3 ± 0.7

12 wk. PA+ 1 | 7.2 | 0.21
" 2 | 6.4 | 0.38
" 3 | 4.8 | 0.49
" 4 | 9.4 | 0.81
" 5 | 7.7 | 0.46
" 6 | 5.4 | 0.48
" 7 | 9.9 | 0.58
" 8 | 6.2 | 0.72
" 9 | 10.9 | 0.40
" 10 | 7.2 | 0.36
" 11 | 9.5 | p=.1174
" 12 | 9.6 | 0.36
ave. = 7.9 ± 2.0

12 wk. PA- 1 | 14.1 | 0.13
" 2 | 14.4 | 0.15
" 3 | 4.6 | 0.06
" 4 | 9.0 | 0.05
ave. = 10.5 ± 4.7

* All samples were collected after a 24-hr fast. Wk 4 PA+ ad lib. group was fed a control diet with 12 mg pantothenate/kg diet ad lib. Wk 4 PA- group was fed a deficient diet of <0.8 mg pantothenate/kg diet. Wk 4 PA+ pair fed group was fed the control diet, pair fed to corresponding deficient rats. 12-wk group samples were collected at end of the 12th week of the experiment, PA+ group having been fed the control diet and PA- group fed the deficient diet. p-values compare controls to its deficient group (ANOVA).
Figure 10. Plot of serum pantothenate status vs pantetheinase activity.
DISCUSSION

Synthesis

The main objective for the pantothenate-p-nitroanilide synthesis project was to obtain a pure crystalline product, specific for the enzyme pantetheinase, which would absorb light in the visible light region upon enzymatic hydrolysis. The result obtained was synthesis of a pure substrate compound with a sharp melting point, containing the most widely used chromogenic group in peptide chemistry, namely p-nitroaniline.

Synthesis of the pantothenate-p-nitroanilide was fairly simple and straightforward as presented here. The synthesis results in sufficient substrate for approximately 1500 assays (200 mg pantothenate-p-nitroanilide, MW=338.0, yields 47.7 ml of 12.4 mM substrate solution).

The proton nuclear magnetic resonance spectrum for the final product pantothenate-p-nitroanilide is shown in Fig. 7. The chiral stereocenter of the molecule is responsible for the a-b splitting patterns seen at 0.78 ppm and at 3.21 ppm. The unusual nine-peak multiplet splitting pattern at 3.43 ppm is harder to explain but
may be due to a cis-trans amide bond interaction. Comparative H-NMR spectra in Fig. 8 are from Wittwer (39), and demonstrate in particular how addition of a group on the amino end of the pantothenate moiety leads to resolution of superimposed peaks.

Pantothenate-p-nitroanilide is a crystalline product. As such, the compound should have a longer shelf-storage life than other substrates of spectrophotometric pantetheinase assays which are not crystalline compounds, namely, pantetheine and pantetheine-S-pyruvate. Furthermore, pure substrate solutions can be prepared from the pure pantothenate-p-nitroanilide crystals, a trait that has not been reported for the other spectrophotometric assays using impure, noncrystalline substrate compounds.

Enzyme Purification

The enzyme obtained from the purification process of Wittwer et al. (37,39) had an activity of 3600 nmol of pantothenate liberated/min/ml as determined by both the mercaptide assay and the pantothenate-p-nitroanilide assay, a specific activity of 16,600 nmol/min/ml by the pantothenate-p-
nitroanilide assay (sp. act. = 16,500 nmol/min/ml by the mercaptide assay), and a protein content of 0.22 mg protein/ml. This preparation of pantetheinase would appear to be of a higher purity than has been previously reported. Wittwer et al. reported a pantetheinase preparation having a protein content of 4.0 mg/ml and a specific activity of 14,000 nmol/min/mg protein (37). The enzyme preparation reported in this study has an 18% higher specific activity than that previously reported.

Assays

The pantothenate-p-nitroanilide proved to be a very good substrate for the enzyme pantetheinase, resulting in apparent activity values comparable to those obtained from the mercaptide assay, which uses the enzyme's physiological substrate, pantetheine.

The pantothenate-p-nitroanilide assay has all the advantages of the pantetheine-S-pyruvate assay, namely, speed and simplicity, but with a pure substrate, resulting in activity values identical to the mercaptide assay. The pantetheine-S-pyruvate assay
consistently gave activity values that were only one-third to one-half those obtained from either the mercaptide assay or the pantothenate-p-nitroanilide assay.

The ability to measure activity in high-protein-content media such as blood serum is perhaps the greatest asset of this assay, a trait other continuous spectrophotometric assays have not been capable of for this enzyme. This assay provides a faster and simpler method of determining pantetheinase activity in blood serum than any method reported to date. The advantages the pantothenate-p-nitroanilide assay provides over other assays should be found useful in furthering study of pantothenate homeostasis.

Biological Application

While there was not a great number of serum pantothenate deficient samples available, the week 4 samples tested had a significantly higher pantetheinase activity than the rat serum samples which were not pantothenate deficient (see Table 5). Week 12 samples might have been similar but for the exception of two
deficient samples which were grossly pantothenate deficient; these two samples had pantetheinase activities similar to the normal serum pantothenate samples.

Pantetheinase appears susceptible to product feedback inhibition by pantothenate (5). This might explain the increased activity of the mildly (but still significantly) deficient samples. As substrate levels decline, the activity of the enzyme increases, presumably so as to convert any substrate encountered. The amount of enzyme in the blood plasma may be regulated in such a way that when substrate levels increase dramatically, more enzyme is introduced into circulation. Conversely, with a dramatic decrease in substrate concentration, much less or no new enzyme is introduced, leading to a natural reduction of enzyme as it is cleared from the system. The two samples from week 12 with grossly low serum pantothenate levels were approaching serum levels associated with immediate death. These values may have been so low that despite being more "active" due to lack of product inhibition, the amount of plasma enzyme may have already been greatly reduced. However, half-life studies of pantetheinase in
blood serum were not found in the literature. Obviously, more knowledge about pantetheinase and its regulation in blood serum is required before any definite conclusions might be made.

The two samples from week 12 with grossly low pantothenate levels had enzyme activities similar to the control group values, suggesting that other methods may be more suitable for severe pantothenate deficiency monitoring. However, the pantothenate-p-nitroanilide assay might be of use in detecting mild (but significant) pantothenate deficiencies as plasma pantetheinase activity seems to increase under mildly deficient conditions (see Fig. 10).

More studies should be conducted to further investigate how pantetheinase activity varies with pantothenate status and nutriture. How serum enzyme activity may vary under an ingested meal load, intramuscular pantetheine injection, or injection into a vein may be of particular interest. The pantothenate-p-nitroanilide assay for pantetheinase activity is particularly well suited for use in studies such as this.
Summary

Pantothenate-p-nitroanilide was synthesized for use as a substrate in a simple, continuous spectrophotometric assay of pantetheinase activity. The substrate compound is crystalline and economical as 200 mg of crystalline pantothenate-p-nitroaniline will yield approximately 1500 assays in a 12.4 mM substrate solution. The substrate is suitable for assaying pantetheinase in high-protein-content media such as blood serum as the enzymatic hydrolysis product, p-nitroaniline, absorbs light in the visible light region where there is little or no absorbing inhibition from serum proteins.

This pantothenate-p-nitroanilide assay provides a faster and simpler method of determining pantetheinase activity in blood serum than any method reported to date. Pantetheinase activity results obtained from the assay may also prove to be a more sensitive indicator of pantothenate deficiency than current methods. The advantages the pantothenate-p-nitroanilide assay provides over other assays will perhaps prove to be an incentive
for further study of the role pantetheinase may play in pantothenate homeostasis.
REFERENCES


42. Svendsen, L. (1972) Thrombosis Research 1, 267-278.


