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Assay, Purification, and Characterization of a Pantetheine Hydrolyzing Enzyme from Pig Kidney

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ASSAY, PURIFICATION, AND CHARACTERIZATION OF A PANTETHEINE HYDROLYZING ENZYME FROM PIG KIDNEY

by

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of the requirements for the degree

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Carl T. Wittwer

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ABSTRACT

Assay, Purification, and Characterization of a
Pantetheine Hydrolyzing Enzyme
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by
Carl T. Wittwer, Doctor of Philosophy
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Department: Chemistry and Biochemistry

A microsomal glycoprotein hydrolyzing pantetheine to pantothenate and cysteamine has been solubilized and purified to homogeneity as determined by sodium dodecylsulfate electrophoresis. Four rapid, independent assays of pantetheine hydrolysis are described and compared along with a method for localizing enzymatic activity on polyacrylamide gels. The enzyme is solubilized on exposure to butanol and purified by heat treatment, $(NH_4)_2SO_4$ fractionation, hydrophobic chromatography, and hydroxyapatite chromatography. The glycoprotein, purified 5600-fold in 22% yield, has a specific activity of 14 μmoles pantothenate produced/min/mg of protein, 35 times that previously reported. The enzyme has a pH optimum of 9.0-9.5 and a K_m of 20 μM for D-pantetheine. Its molecular weight has been estimated by gel filtration (54,000) and sodium dodecylsulfate electrophoresis (60,000). Amino acid analysis indicates a high content of isoleucine (11.3 mole %) and phenylalanine (7.4 mole %) with an absorbancy index $E^{1\%}_{1cm}$ (280 nm) of 11.3. Galactose, mannose, fucose, and glucose are present in decreasing concentrations totaling 90 μg of neutral hexoses

per mg of protein. Galactosamine and sialic acid are also present. The enzymatic hydrolysis of various pantetheine analogs indicate the enzyme's specificity is high for the pantothenate moiety but low for the cysteamine portion. The enzyme should be useful in routine "total" pantothenate determinations in tissues.

(100 pages)

CHAPTER I

INTRODUCTION

D-Pantetheine is an intermediate compound between the vitamin pantothenic acid, and the coenzymes, phosphopantetheine, and coenzyme A. Pantothenate derivatives function widely in central metabolism and mediate many reactions in carbohydrate, lipid, and amino acid metabolism. Abiko (1975) has listed 72 enzymatic reactions that involve coenzyme A or phosphopantetheine as cofactors. The enzymatic hydrolysis of pantetheine to pantothenate and cysteamine is of interest as part of the catabolic pathway of these cofactors.

The purpose of this study is to reliably assay, purify to homogeneity, and characterize a pantetheine hydrolyzing enzyme from pig kidney.

Pantothenic Acid and Its Coenzymes

Discovery and Characterization

Pantothenic acid. Williams et al. (1933) identified an unknown substance in diverse biological sources that was a potent growth factor for yeast. Because of its widespread occurrence and acidic properties, it was called "pantothenic" acid, derived from the Greek, meaning "from everywhere". When both the chick anti-dermatitis factor (Woolley et al. 1939) and the liver filtrate factor of rats (Subbarow and Hitchings 1939) turned out to be pantothenic acid, its importance in animal nutrition warranted its inclusion as a vitamin. Studies on its isolation and chemical structure eventually led to its synthesis

(Williams and Major 1940). Although it was assumed that pantothenic acid functioned as part of a coenzyme like other vitamins, no pantothenate coenzyme was found until 14 years after its initial discovery.

Coenzyme A. Lipmann (1945) found the acetylation of sulfanilamide to depend on an enzyme-coenzyme system present in liver homogenates. The coenzyme of acetylation, or "coenzyme A" (CoA) was later shown to be a pantothenate derivative (Lipmann et al. 1947) that was also widely distributed (Kaplan and Lipmann 1948). The structure of CoA was determined enzymatically (Novelli et al. 1954), and it was eventually chemically synthesized (Moffatt and Khorana 1961).

Pantetheine and pantethine. Williams et al. (1949) described a previously unidentified growth factor for Lactobacillus bulgaricus. It was later found to contain 65-75% pantothenic acid by weight and to be a breakdown product of CoA (Brown et al. 1950). Its structural determination and synthesis were achieved (Snell et al. 1950). The names "pantetheine" and "pantethine" were suggested for the sulfhydral and disulfide forms, respectively, to indicate their relation to pantothenic acid. Majerus et al. (1965) later identified 4'-phosphopantetheine as the prosthetic group in the acyl carrier protein of fatty acid synthesis.

The structures of these compounds and their relation to each other are shown in Fig. 1. Alternative names of selected compounds are given in Table 1.

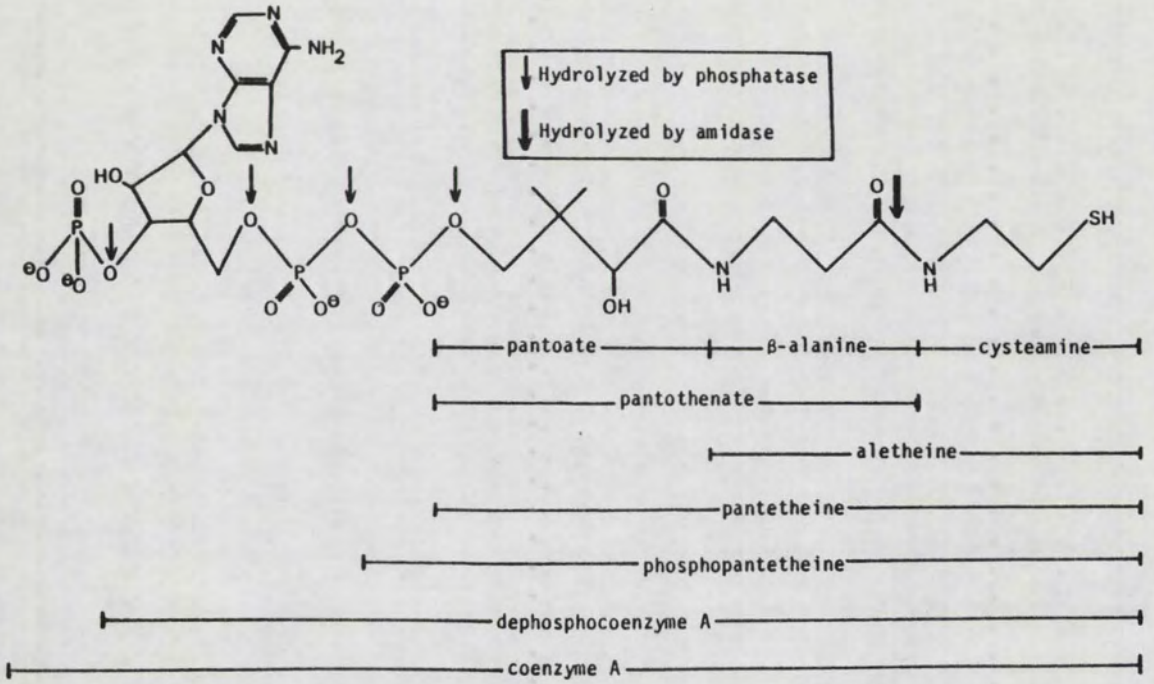


Figure 1. Structures and names of coenzyme A fragments.

Table 1. Synonyms for some Compounds in Pantothenate Metabolism.

Pantothenic Acid*

(R)-N-(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)-β-alanine**
D(+)-N-(2,4-dihydroxy-3,3-dimethylbutyryl)-β-alanine
chick antidermatitis factor
vitamin B_3
vitamin B_5

Pantetheine

N-(pantothenyl)-β-aminoethanethiol
2,4-dihydroxy-N-[3-[(2-mercaptoethyl)amino]-3-oxopropyl]-3,3-dimethylbutanamide*
2,4-dihydroxy-N-[2-[(2-mercaptoethyl)carbamoyl]ethyl]-3,3-dimethylbutyramide**
α,γ-dihydroxy-β,β-dimethylbutyryl-β-alanyl-β-aminoethanethiol

Pantethine (the disulfide of pantetheine)

bis(N-pantothenyl-β-aminoethyl)disulfide
N,N'-[dithiobis[2,1-ethanediylimino(3-oxo-3,1-propanediyl]]bis-[2,4-dihydroxy-3,3-dimethylbutanamide]*
N,N'-[dithiobis(ethyleneiminocarbonylethylene)]bis(2,4-dihydroxy-3,3-dimethylbutyramide)**
Lactobacillus bulgaricus factor (LBF)
Pantosin

Cysteamine

2-aminoethanethiol* **
mercaptamine
β-mercaptoethylamine
2-aminoethyl mercaptan
thioethanolamine
decarboxycysteine
MEA
Becaptan

Cystamine (the disulfide of cysteamine)

2,2'-dithiobisethylamine*
2,2'-dithiobisethanamine**
β,β'-diaminodiethyl disulfide
bis[β-aminoethyl]disulfide
decarboxycystine

*Nomenclature used in Chemicals Abstracts through the Eighth Collective Index, 1967-1971.

**Nomenclature used in Chemical Abstracts since the Eighth Collective Index, 1972-to date.

Biochemical Functions

Pantothenic acid is the vitamin moiety of CoA and phosphopantetheine, both of which carry acyl groups through thioester bonds. The S-acyl derivatives of these pantothenate coenzymes are formed on oxidation of aldehydes, on oxidative decarboxylation of α-keto acids, and from organic acids activated by ATP or GTP. The activated acyl groups may be transferred to other acceptors, or modified by oxidation/reduction, carboxylation/decarboxylation, hydration/dehydration, racemization, carbon skeleton rearrangement, or formation of new carbon to carbon bonds. The versatility of these acylated coenzymes arises in part from the extended configuration of pantetheine and in part from the reactivity conferred by the thioester bond.

Generally, pantothenate derivatives mediate the metabolism of carbohydrates, lipids, and amino acids. Specifically, in the tricarboxylic acid cycle, CoA functions as an acyl acceptor for the pyruvate and α-ketoglutarate dehydrogenase complexes, forming acetyl CoA and succinyl CoA, respectively. These CoA thioesters are also formed on oxidative degradation of several amino acids. The acetyl group from acetyl CoA is the carbon source for the biosynthesis of fatty acids, prostaglandins, ketone bodies, cholesterol, steroid hormones, and other compounds. Succinyl CoA is an essential precursor for porphyrins and hence, hemoglobin and cytochromes. In fatty acid synthesis and degradation, pantothenate coenzymes carry the acids as acyl groups through repetitive synthetic or degradative cycles. A CoA thioester is a necessary intermediate in the degradation of

cholesterol to bile acids. Phosphopantetheine has been implicated in the synthesis of peptide antibiotics (Lee and Lipmann 1977) through an oligopeptide synthetic system that may be an evolutionary forerunner of modern ribosomal protein synthesis (Lipmann 1971). Coenzyme A thioesters are the acyl donors for many acceptors, including choline and cholesterol. Some important compounds synthesized from pantothenate derivatives are shown in Fig. 2.

Metabolic Regulation

Intermediary metabolism is inundated with pantothenate coenzymes. There are several mechanistically unexplained correlations in the literature between altered metabolic states and altered pantothenate metabolism. Rheumatoid arthritis results in low whole blood total pantothenate (Barton-Wright and Elliott 1963) and patients with systemic lupus erythematosus have lower bound/free ratios of whole blood pantothenate (Slepyan et al. 1957). In human chronic ulcerative and granulomatous colitis, diseased colonic mucosa has low CoA activity, even though blood pantothenate levels are normal (Ellestad-Sayed et al. 1976). Of particular current interest are correlations between pantothenate derivatives and lipid metabolism.

Interest in pantothenate derivatives has increased recently as associations between the derivatives and lipid metabolism have appeared. Altering pantothenate metabolism by oral administration of pantethine effects lipid levels. Pantethine has been shown to decrease cholesterol ester synthesis in rat arterial wall extracts (Shirai et al. 1979) and to decrease the serum cholesterol, β-lipoprotein, and triglyceride levels in rats fed a high cholesterol

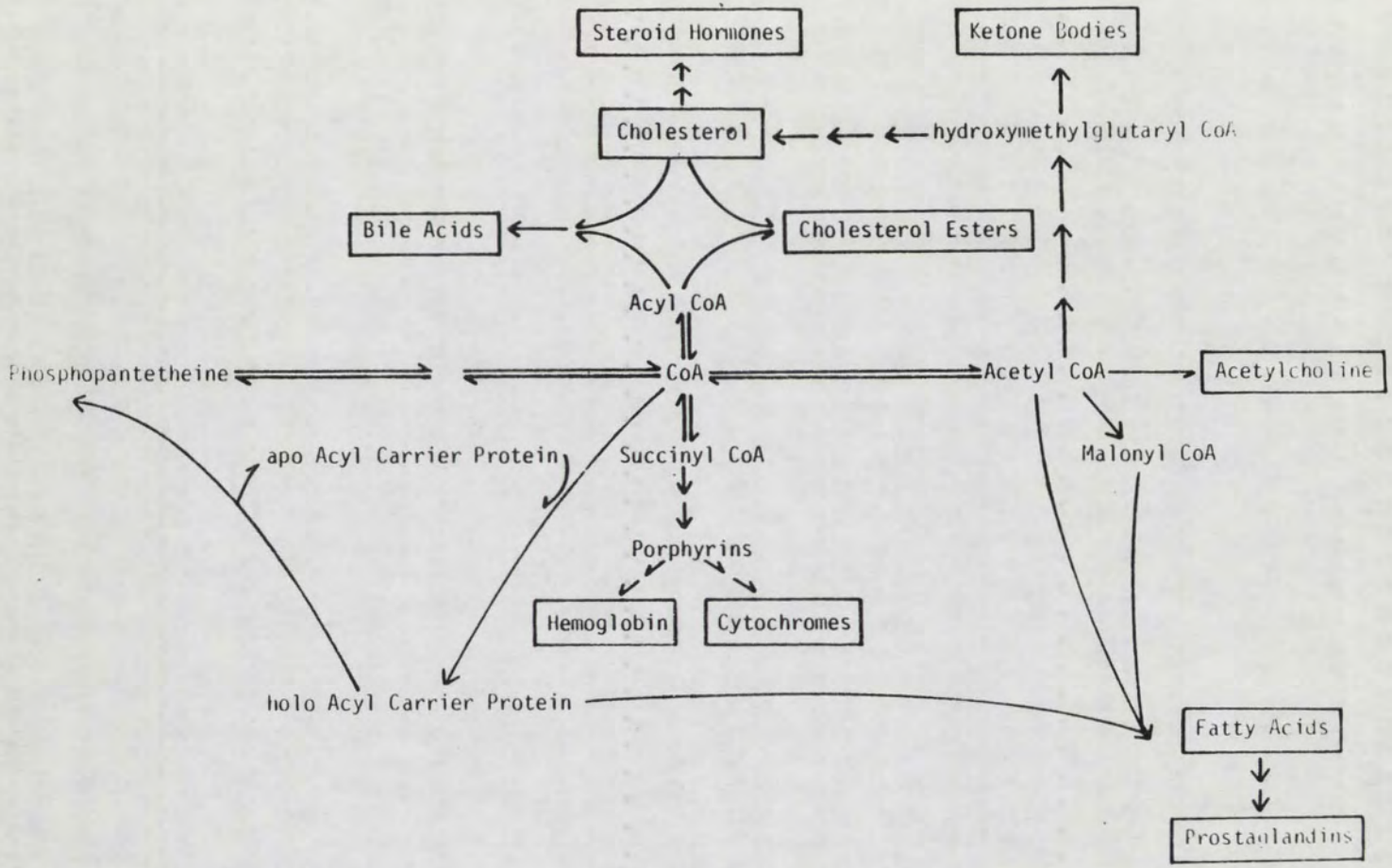


Figure 2. Some important compounds synthesized from pantothenate derivatives.

diet (Shinomiya et al. 1980; Kajiyama et al. 1981). Pantethine fed to streptozotocin diabetic rats reduced levels of serum triglycerides, free fatty acids, and β-hydroxybutyrate (Kameda and Abiko 1980). Rabbits fed a high cholesterol diet had low density lipoprotein cholesterol normalized upon treatment with pantethine, but high density lipoprotein was increased (Tomikawa et al. 1982). In diabetic patients with hyperlipidemia, pantethine decreased serum cholesterol, increased high density lipoprotein cholesterol, and normalized serum and platelet lipid compositions (Hiramatsu et al. 1981). In May of 1981, Dr. John Gilbert of Inveresk Research International, Edinburgh, Scotland was sent antisera for the radioimmunological determination of pantothenate in blood (Wyse et al. 1979) after administration of pantethine to human volunteers. In the past 5 years, Chemical Abstracts lists over 30 patents granted on the synthesis and uses of pantethine.

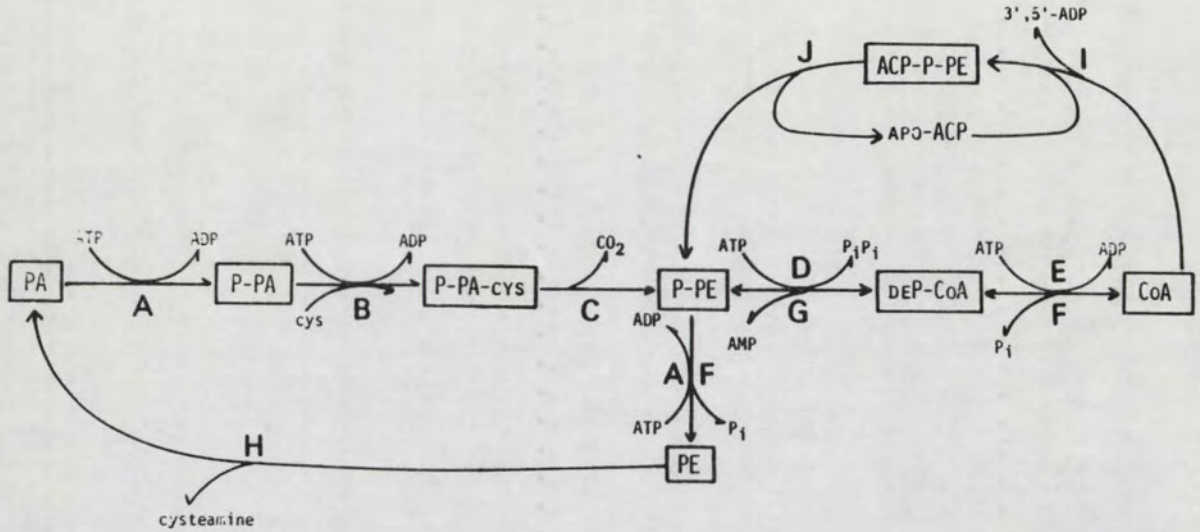
Not only does modified pantothenate metabolism effect lipid levels, but altered lipid metabolism effects levels of pantothenate derivatives. The altered lipid metabolism of fasting and diabetes (Smith et al. 1978; Reibel et al. 1981b), and clofibrate administration (Voltti et al. 1979), correlates with grossly elevated liver and heart CoA levels in rats. Changes in tissue CoA levels could conceivably arise from changes in pantothenate supply or transport. However, concentration and radioactive incorporation studies show that supply and transport are unimportant in most cases (Smith et al. 1978; Smith 1978; Smith and Savage 1980; Reibel et al. 1981a; Voltti et al. 1979; Ellestad-Sayed et al. 1976). Alterations

in the activity of biosynthetic or degradative enzymes of CoA are suspected.

Biosynthesis and Degradation of Coenzyme A

The biosynthesis and degradation of CoA have been reviewed by Abiko (1975). The relevant reactions are shown in Fig. 3. Intracellular control of CoA levels occurs within hours, following the fasting/feeding cycles of rats (Smith et al. 1978). The phosphopantetheine prosthetic group of acyl carrier protein also turns over in a few hours, whereas the half life of the protein is a matter of days (Tweto and Larabee 1972). A very dynamic control process is apparent that has only begun to be studied.

The enzymatic control point varying CoA levels remains unknown. It may be in either the biosynthesis, or the degradation of CoA, or both. In the case of clofibrate induced hyperlipidemea, Voltti et al. (1979) favored inhibition of the enzymes of CoA degradation, whereas Reibel et al. (1981a), studying diabetes and fasting, believed the regulation to be biosynthetic. Most workers hypothesize control points from indirect evidence of intermediate levels or kinetic incorporation studies. Only Skrede and Halvorsen (1979), working with clofibrate treated rats, have attempted to measure the specific activity of the enzymes involved, and they measured only pantothenate kinase, acid phosphatase, and nucleotide pyrophosphatase activities (Fig. 3). An unambiguous identification of the enzymatic control point would allow more detailed regulation studies on lipid metabolism



ENZYMES (IUB, 1979)

A	EC 2.7.1.33	Pantothenate kinase
B	EC 6.3.2.5	Phosphopantothenoylcysteine synthetase
C	EC 4.1.1.36	Phosphopantothenoylcysteine decarboxylase
D	EC 2.7.7.3	Pantetheinephosphate adenyltransferase
E	EC 2.7.1.24	Dephospho-CoA kinase
F	EC 3.1.3.2	Acid Phosphatase
G	EC 3.6.1.15	Nucleotide pyrophosphatase
H	--	Pantetheinase
I	EC 2.7.8.7	Holo-[acyl-carrier-protein] synthetase
J	EC 3.1.4.14	[Acyl-carrier-protein] phosphodiesterase

SUBSTRATES

PA	pantothenate
P-PA	4'-phosphopantothenate
cys	L-cysteine
P-PA-cys	4'-phosphopantothenoyl-L-cysteine
P-PE	4'-phosphopantetheine
deP-CoA	dephospho-CoA
CoA	coenzyme A
PE	pantetheine
ACP-P-PE	holo-acyl-carrier-protein
ACP	apo-acyl-carrier-protein

Figure 3. Biosynthetic and degradative pathways of coenzyme A.

in diabetes and hyperlipidemia with eventual academic, if not clinical, implications.

Metabolic Significance of Pantetheine Hydrolysis

The enzymatic hydrolysis of pantetheine to pantothenate and cysteamine may be part of the control process regulating CoA levels. It is the only specific step in the degradation of CoA (Abiko 1975; Skrede 1973). It is also the only catalytic step in Figure 3 whose enzyme has not been studied sufficiently to assign an Enzyme Commission code number (IUB 1979).

Cysteamine, the other product besides pantothenate in the enzymatic hydrolysis of pantetheine, is also of interest. It has long been known as a radioprotective agent (Bacq et al. 1953) and shows promise in treating cystinosis (Yudkoff et al. 1981). It is an antidote for acetaminophen (Tylenol, Paracetamol) poisoning (Prescott et al. 1976) and is a potent depletor of pituitary prolactin (Millard et al. 1982). Cysteamine is also a precursor of taurine (2-aminoethanesulfonic acid), and the production of taurine via pantetheine and cysteamine may be important physiologically (Federici et al. 1980).

Liberation of Pantothenate from Biological Samples

Assay of Pantothenate

Bioassay, or more often microbiological assay, of pantothenic acid are the usual methods of assay, although a radioimmunoassay has recently been reported (Wyse et al. 1979). Early comparisons between

bioassay and microbiological assay were disparate (Jukes 1947). Pantothenate available on bioassay was greater than that determined microbiologically. Various chemical and enzymatic treatments of biologic samples were used to increase the pantothenate available to microorganisms. Success was limited. Acid or base hydrolysis destroyed the vitamin, and enzymatic procedures were empirical without knowledge of how pantothenate was bound in tissues. A rational enzymatic approach would require at least one enzyme for each bound form of pantothenate. With the discovery of CoA as a major source of bound pantothenate, a rational approach became possible.

Enzymatic Liberation of Pantothenate from Coenzyme A

Coenzyme A inactivation by either intestinal phosphatase or an extract of pigeon liver led to the use of these two preparations in the enzymatic liberation of pantothenate from CoA (Novelli et al. 1949). Inactivation by intestinal phosphatase was accompanied by a complete liberation of organically bound phosphate. However, inactivation by the pigeon liver extract did not release phosphate and was not inhibited by fluoride (an inhibitor of phosphatase). The pigeon liver extract was later found to function by releasing cysteamine at the other end of the molecule (Fig. 1). Both preparations were necessary to release pantothenate from CoA because pantothenate was bound by two distinct linkages. Microbiological assay of the pantothenate content of CoA correlated well with the bioassay after this "dual enzyme" treatment.

The Amidase in Pantothenate Liberation

All known forms of pantothenate coenzymes function through the thiol group of cysteamine. Cysteamine is attached by amide bonding to pantothenate. Liberation of bound pantothenate prior to assay therefore requires an amidase to split this bond. As pantothenate also contains an internal amide bond, the problem is not trivial, and enzymatic methods are usually employed. Optimal characteristics for this amidase are listed below and are covered in the following paragraphs:

1) Readily obtainable source
2) Low pantothenate content
3) High stability
4) Quantifiable activity
5) Determined specificity

Only preparations commonly used to liberate pantothenate from biological samples will be covered in this section. These amidase sources were developed from 1945-1955 and are still used today in "total" pantothenate determinations. Current research on the enzymatic hydrolysis of pantetheine to pantothenate and cysteamine, which is concerned with the metabolic implications of the reaction rather than its practical significance in pantothenate assay, will be covered in Chapter II.

Source. The original source of the amidase was an extract of an acetone powder of pigeon liver (Kaplan and Lipmann 1948). Neilands and Strong (1948) described a preparation at least as effective from

the more readily available chicken liver. Rabbit, hog, and beef liver were not good sources (Novelli et al. 1949), but hog, lamb, and rat kidney were (Schweigert and Guthneck 1953; Novelli 1953). In general, avian liver or mammalian kidney appeared to be good sources.

Pantothenate content. If the amidase is to be used to liberate and determine pantothenate, it should not contain much pantothenate itself. This "enzyme blank" limits the sensitivity of the determination. Neilands and Strong (1948) suggested purifying the enzyme to remove high pantothenate levels, and Novelli et al. (1949) unsuccessfully tried ammonium sulfate fractionation.

The problem was at least partially solved by Novelli and Schmetz (1951) who used an anion exchange resin to bind pantothenate containing anions in the extract. Many investigators have since used resin treated liver extracts with apparent success for "total" pantothenate determination (Zook et al. 1956; Slepyan et al. 1957; Ishiguro et al. 1961; Barton-Wright and Elliott 1963; Nakamura et al. 1967; Cohenour and Calloway 1972). However, others do not find the pantothenate blanks of the resin treated extracts as satisfactory. Clegg (1958) reports troublesome growth stimulation from the extract and Hatano (1962) uses only half as much enzyme to reduce blank values. Markkanen (1973) cites the unavailability of the enzyme, "in a sufficiently high grade of purity" (p. 313), as a possible explanation for the wide variation in reported pantothenate levels in blood (Pearson 1980). Baker and Frank (1968) find use of the amidase unreliable and use "Clarase", an amylase with low pantothenate content, for liberation.

Stability. Kaplan and Lipmann (1948) first noticed the lability of the CoA inactivating enzyme from pigeon liver. Although claimed stable when frozen, standing for 4 hours at room temperature, or dialyzing 24 hours at 5°C destroyed most, if not all, of the activity (Novelli et al. 1949). Jannes (1950) could not prepare the amidase from pigeon or chicken liver, stating, "the work proved to present many technical difficulties. The enzyme produced ... proved to be very labile, and the possibilities of storing it were quite poor" (p. 60).

Activity measurement. The American Association of Agricultural Chemists abandoned its four year study to develop a standard assay for total pantothenate because of difficulty with the amidase preparation, 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 1960, p. 29).

The report is concluded by suggesting that, "when sufficient information on the specific enzyme activity is available, it may be desirable to reactivate collaborative studies" (p. 29). Markkanen (1973), also concerned about the activity of the amidase, states that, "the various enzyme preparations may have differed decisively in activity" (p. 313).

The amidase activity in preparations used for total pantothenate assay has never been quantified. "Enough" enzyme is added to give maximal liberation. Novelli (1955) suggests "titration" of the pigeon

liver enzyme to produce maximal liberation and then using double the amount for routine assay. Exact quantitation of the amount of activity required in terms of a specific substrate would be preferable and allow comparison between different preparations and laboratories.

Specificity. The specificity of the various amidase preparations used in total pantothenate assay is unknown. Novelli et al. (1954) do mention indications that avian liver enzymes prefer CoA as substrate, while hog kidney preparations act primarily on pantetheine. However, no experimental results are given. Known specificity of the amidase would determine whether the order of addition of phosphatase and amidase is important in total pantothenate determinations. It would also help define the meaning of "total" pantothenate measurements.

Total Pantothenate Assay

Most investigators studying pantothenate levels in tissues use the dual enzyme system of amidase and phosphatase to determine "total" pantothenate. This conventional dual enzyme system was designed to liberate pantothenate from CoA and its use in tissue determinations is an extrapolation. The assumption is that bound pantothenate is present in tissues as CoA or other bound forms of the vitamin that are liberated by the dual enzymes. This assumption is wrong. 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 enzymes (Majerus et al. 1965). There may be other bound forms of pantothenate that are not liberated, for example, various natural glycosyl derivatives (Imamoto et al. 1979). In a recent article

reminiscent of early studies, "substantially and consistently higher values of pantothenate" (p. 131), were found by chick assay than by microbiological assay with prior dual enzyme treatment (Latymer and Coates 1982). Apparently, significant amounts of bound pantothenate are not hydrolyzed by the dual enzymes.

Significance and Objectives of the Study

Assay

Available enzyme assays for the hydrolysis of pantetheine to pantothenate and cysteamine are either lengthy or require special equipment (see Chapter II). Simple assays would expedite purification of the enzyme as well as allow quantitation of amidase activity from different sources.

Purification

A stable purified source of the amidase with low pantothenate content would be useful in routine pantothenate assay. The enzyme has never been purified for use in pantothenate assay, and homogeneous preparations from horse kidney could not be prepared "routinely" (Dupre and Cavallini 1979, p. 266). A reliable homogeneous preparation would allow detailed characterization.

Characterization

Certain characteristics of the amidase, such as its pH profile and substrate specificity, are important for the liberation of pantothenate from biological sources. Other characteristics, including subcellular localization, apparent K_m, molecular weight,

amino acid analysis, and carbohydrate analysis may help to clarify this enzyme's place in the metabolism and regulation of CoA levels.

CHAPTER II

REVIEW OF LITERATURE

Review of the significance of pantetheine hydrolysis in coenzyme A metabolism and in the liberation of pantothenate for assay is covered in Chapter I. This chapter deals with the work of three contemporary research groups interested in the enzymatic hydrolysis of pantetheine. Dupre and Cavallini (1979), based in Italy, have been interested in pantetheine hydrolysis as a source of cysteamine for taurine biosynthesis. Abiko (1975) of Japan has studied the hydrolysis of pantetheine as part of the metabolism of coenzyme A. Finally, Orloff et al. (1981), of the United States have looked at altered pantetheine hydrolysis as a possible mechanism of cystine accumulation in cystinosis.

Assay

The liberation of pantothenate was originally monitored microbiologically, a method used recently by Abiko (1975) after ion exchange separation of pantothenate. The tedium of the procedure has led to the development of other assays. Cavallini et al. (1964) measured the hydrolysis of pantethine by coupling the production of cystamine to its oxidation with amine oxidase and measuring the uptake of oxygen. When reducing compounds were found to activate the reaction (Cavallini et al. 1968), other methods were developed. These included assays based on the chromatographic (Cavallini et al. 1968) and electrophoretic (Dupre et al. 1970a) separation of ^{14}C-labeled

substrate and product. Orloff et al. (1981) found the microbiological assay, "time consuming and erratic ... whereas the radiochemical assay required an expensive radiolabeled substrate that must be specially synthesized" (p. 1063). The fastest procedure available was still limited by a 5-hour electrophoresis step.

Recently, methods with a fast turnaround have been developed. A pH-stat procedure was reported by Dupre et al. (1976), and Orloff et al. (1981) have measured hydrolysis through amino acid analysis of the N-ethylmaleimide adduct of cysteamine. These procedures do however require special equipment.

Current assays all employ a thiol reducing compound as an activator, and an incubation temperature around 37°C (Table 2). However, the substrate concentration used varies widely and its exact form is often vague or unspecified. The racemic substrate is used by Dupre et al. (1970a), but Abiko (1975) and Orloff et al. (1981) do not specify whether they used the racemic compound or the biologically active (D) stereoisomer.

The reduction state of the substrate is often unclear. Dupre et al. (1970a) add 5 mM DL-pantethine and 15 mM cysteine simultaneously into the reaction mixture at the start of the incubation period. An equilibrium mixture of pantetheine and pantethine results as thiol-disulfide exchange occurs. Their product vs. time curve shows an initial lag period before linearity is established. Alternative explanations include insufficient enzyme activation by cysteine or preference of the enzyme for pantetheine over pantethine as a substrate. Dithiothreitol quantitatively reduces disulfides

Table 2. Literature Assay Conditions.

Reference	Buffer	Activators	Substrate	Miscellaneous
Dupre et al. 1970a	0.05 M phosphate pH 7.6	15 mM cysteine	5 mM DL-pantethine	60 min at 38°C
Abiko 1975	0.08 M tris-maleate pH 7.0	20 mM cysteine	0.1 mM pantetheine	30 min at 37°C
Dupre et al. 1976	(pH-stat)	134 mM DTT*	6.6 mM D-pantetheine	37°C
Dupre and Cavallini 1979	(pH-stat)	14 mM DTT*	6.6 mM pantetheine	38°C
Orloff et al. 1981	0.05 M phosphate pH 7.6	0.4 mM DTT* 1 mM EDTA	0.8 mM pantetheine	90-240 min at 37°C

*Calculated as the amount of reduced DTT (dithiothreitol) remaining after substrate reduction.

(Cleland 1964). Although it is not clear Dupre and coworkers allowed sufficient time in their pH-stat assay for complete pantethine reduction before substrate addition to the enzyme, Orloff et al. (1981) do specify a 10 minute preincubation at 37°C.

Purification

Initial attempts to purify the amidase from avian liver were mentioned in Chapter I. Partial success has recently been achieved with mammalian kidney as an enzyme source. Abiko (1975) solubilized the enzyme from rat kidney microsomes with 0.5% Triton X-100 in the presence of 0.1 mM cysteamine. Repeated gel filtration on Sephadex G-200 resulted in a 75-fold purification with respect to the homogenate. A 2-fold purification of the enzyme found in the 100,000 g supernatant of rat kidney homogenates was also achieved by a 25-50% $(NH_4)_2SO_4$ fractionation.

Dupre et al. (1970b) reported a 3300-fold partial purification of the enzyme from horse kidney in 11% yield. Purification steps included homogenization of the frozen-thawed tissue, heating at 60°C (pH 7.0) for 10 minutes, fractionation with $(NH_4)_2SO_4$ (45-65% saturation), CM-cellulose chromatography (pH 5.0), DEAE-cellulose chromatography (pH 7.6), and zone electrophoresis at pH 6.0 on a cellulose column. The highest specific activity obtained from selected fractions of the cellulose column was 70-100 nmoles of pantothenate produced per minute per mg of protein. Gel electrophoresis showed the presence of three main components.

Complete purification was claimed in an abstract from the Ninth

International Congress of Biochemistry (Dupre et al. 1973a). Homogeneity on ultracentrifugation and disc-electrophoresis, a molecular weight of 55,000, and an amino acid composition were apparently presented, although no specific data are recorded. Details of a modified purification have been reported by Dupre and Cavallini (1979). Although the final step could not be done "routinely" (p. 266), a 16,000-fold purification with 12.8 % yield gave a preparation claimed to be homogenous on ultracentrifugation and disc electrophoresis. No experimental evidence for homogeneity was presented. Purification steps included homogenization, heating at pH 5.5 in a boiling water bath up to 55°C, fractionation with $(NH_4)_2SO_4$ and ion exchange chromatography as in the original procedure, gel filtration on Sephadex G-200, and recycling on Sephadex G-75. The highest specific activity obtained was 400 nmoles of pantothenate produced per minute per mg of protein.

Reported specific activities are difficult to compare. Not only do the enzyme assays differ, but protein determinations vary between, and sometimes within, a purification. Abiko (1975) does not specify his method of protein assay. Dupre et al. (1970b) calculated the protein concentration of the starting homogenate by the dry weight of samples kept at 110°C to constant weight. Protein in the other fractions, as well as in the modified purification scheme (Dupre and Cavallini, 1979) was determined by the biuret reaction with bovine serum albumin as standard (Gornall et al. 1949).

Reported homogenate specific activities from various sources are presented in Table 3. Dupre et al. (1970a) present data for assay

Table 3. Homogenate Specific Activities.

Reference	Source	Protein Assay	Specific Activity*
Dupre et al. 1970a	horse kidney	Gornall et al. 1949	0.42
Dupre et al. 1970b	horse kidney	dry weight at 110°C	0.017
Dupre and Cavallini 1979	horse kidney	Gornall et al. 1949	0.025
Abiko 1975	rat kidney	unspecified	0.25
	rat liver		0.076
Orloff et al. 1981	human WBC	Lowry et al. 1951	0.87
	human fibroblasts		0.040

*Given as nmoles of pantothenate liberated per minute per mg of protein.

validation indicating a horse kidney homogenate specific activity of 0.42 nmol pantothenate/min/mg, yet in their purification schemes, they list 0.017 (Dupre et al. 1970b) and 0.025 nmol/min/mg (Dupre and Cavallini, 1979). A low homogenate specific activity would give a high apparent degree of purification.

Characterization

Sources

In addition to the enzyme sources given in Table 3, the enzymatic hydrolysis of pantethine has been demonstrated in homogenates of liver, kidney, heart, and muscle of horses, cattle, pigs, and rats (Cavallini et al. 1964). However, the absence of activating thiol

compounds in the amine oxidase coupled assay makes quantitative comparisons with more recent assays difficult. Horse and cattle kidneys contain about the same amount of enzyme, whereas pig kidney shows an activity about three times as high (Dupre et al. 1970b).

Intracellular Location

In rat liver, pantetheine splitting activity is exclusively located in the microsomal-lysosomal fraction, whereas it distributes to both the soluble and microsomal-lysosomal fractions in rat kidney (Abiko, 1975). The intracellular location of the enzyme purified from horse kidney (Dupre and Cavallini, 1979) has not been studied.

Michaelis Constant and pH Optimum

Available Michaelis constants and pH optima for the enzyme from various sources are given in Table 4. A neutral to acidic pH optimum is observed, depending on the source. Reported K_m values vary over a 100-fold range.

Substrate Specificity

All relevant studies show a high degree of substrate specificity for D-pantetheine. Dupre et al. (1970b) showed by amino acid analysis of β-alanine that aletheine (<1%) and pantothenate (ca. 2%) are poor substrates relative to pantetheine. Pantothenoyl-L-cysteine, 4'-phosphopantothenoyl-L-cysteine, pantetheine thiazoline, and CoA were hydrolyzed very slowly if at all (Dupre et al. 1973b; Abiko 1975). L-pantetheine was apparently hydrolyzed at a rate less than 2% of D-pantetheine (Bellussi et al. 1974). However, these results are

Table 4. Literature Michaelis Constants and pH Optima.

Reference	Source	pH Optimum	K_m (pantetheine)
Dupre et al. 1970b	horse kidney	4.0-5.5	10 mM DL
Dupre et al. 1976			3 mM D
Dupre and Cavallini 1979			5 mM
Abiko 1975	rat kidney supernatant	7.5	0.031 mM
	rat kidney microsomes	7.0	0.036 mM
	rat liver	6	0.095 mM
Orloff et al. 1981	human WBC	6.5-7.5	0.44 mM
	human fibroblasts	6-7	0.11 mM

questionable as L-pantetheine was obtained by exhastive enzyme hydrolysis of DL-pantetheine.

Dupre et al. (1973b) found 4'-phosphopantetheine to be hydrolyzed at 10% the rate of pantetheine. The presence of contaminating alkaline phosphatase activity and an incubation of 3 hours make prior hydrolysis of the phosphate a possibility. The enzyme from rat liver cannot hydrolyze 4'-phosphopantetheine at all if 1 mM sodium fluoride (an inhibitor of phosphatase) is included in the incubation mixture (Abiko, 1975).

Dupre et al. (1973b) concluded that the real substrate for the enzyme from horse kidney, previously called pantethinase, was pantetheine, the reduced form of pantethine. However from their data it is impossible to separate the preference of the enzyme for

the reduced substrate from the thiol activating properties of pantetheine. The enzyme is apparently a thiol protease (Dupre et al. 1973a) and disulfide exchange with the active site may be the sole reason for decreased activity with pantethine.

Activators and Inhibitors

The activation by thiol compounds has already been mentioned. This activation is not specific; mercaptoethanol and dithiothreitol give the highest effect (Dupre et al. 1970b). Iodoacetate and iodoacetamide are inhibitors at high concentrations when preincubated with the enzyme and the excess is removed by dialysis before activity determination (Dupre and Cavallini, 1979). Fifteen millimolar sodium azide inhibits 90%.

Inhibition by the products of the reaction, pantothenate and cysteamine, has been observed. Fifty millimolar pantothenate inhibits 60% (Dupre et al. 1970b). Inhibition by cysteamine has been reported as 10% with 50 mM cysteamine (Dupre et al. 1970b) and 44% with 5 mM cysteamine (Dupre and Cavallini, 1979).

Stability

The enzyme from horse kidney is stable indefinitely when frozen at neutral pH or lypholyzed in the absence of salts (Dupre et al. 1970b). Dilute solutions at 0°C lose activity at about 5% per week. The stability is good between pH 5.0 and pH 7.6, but rapidly decreases outside these limits (Dupre and Cavallini, 1979).

Other Properties

A molecular weight of 55,000 has been obtained by ultra-

centrifugation. The enzyme has an isoelectric point of 4.8 and an extinction coefficient determined by amino acid analysis of $E^{1\%}_{1cm}$ (280 nm)=15.2 (Dupre and Cavallini, 1979). Evidence for these properties was apparently presented at the Ninth International Congress of Biochemistry (Dupre et al. 1973a), but only an abstract survives.

Summary

The enzymatic hydrolysis of pantetheine to pantothenic acid and cysteamine has been previously studied. Quantitative assays have been developed, a complete purification from horse kidney has been reported, and many characteristics of the enzyme have been determined. However, the assays are time consuming or require special equipment, the purification is not performed "routinely" (Dupre and Cavallini, 1979, p. 266) and experimental evidence for homogeneity is lacking. Different authors report widely different K_m values and pH optima. Attempts in our laboratory to repeat the published purification from horse kidney have been unsuccessful (Chapter III). Its repetition has not been reported.

CHAPTER III

MATERIALS AND METHODS

The objectives of this study include development of rapid and reliable assays, routine purification to homogeneity, and characterization of a pantetheine hydrolyzing enzyme.

Preliminary Studies

Initial studies with horse kidney were performed in order to replicate the extensive work of Dupre and coworkers (Chapter II). The time and labor required for the radiolabeled assay (Dupre et al. 1970a) led to the development of a simpler radiolabeled assay reported later in this chapter. Use of this modified assay and estimation of protein by the method of Lowry et al. (1951) gave a specific activity for horse kidney homogenates of 0.8-1.4 nmols of pantothenate produced per min per mg of protein. This is close to the value of 0.42 nmoles/min/mg calculated from data given by Dupre et al. (1970a). Pig kidney homogenates gave higher specific activities (1.8-3.2 nmol/min/mg), similar to the 3-fold increase reported by Dupre et al. (1970b).

Purification according to Dupre et al. (1970b) was begun. The first step (heat treatment) is reported to give a 9-fold purification in 87.5% yield. However, in our laboratory no increase in specific activity and a loss of about two thirds of the total activity was repeatably found. Similarly, $(NH_4)_2SO_4$ fractionation on the heat treated enzyme (the second step) resulted in loss of activity without

significant gain in specific activity. Inability to repeat the published purification led to the eventual development of an independent scheme. As pig kidney was a richer source of the enzyme and more readily available than horse kidney, it was used as the enzyme source.

Assay

Four rapid, independent assays of enzymatic pantetheine hydrolysis were developed. Two assays detected specifically the hydrolysis products: cysteamine was measured by the absorbance of its fluoropyruvate adduct at 300 nm and pantothenate was measured by radioimmunoassay. Methods of [^{14}C] pantethine synthesis were reviewed and the labeled substrate employed in a third enzymatic assay. A fourth assay continuously monitored the absorbance of mercaptide ions at 240 nm. The mercaptide ion concentration increased proportionally with hydrolysis at a buffered pH because of a difference in pK(-SH) between pantetheine (9.9) and cysteamine (8.1) at 37°C.

Materials

The sodium salt of fluoropyruvic acid, dithiothreitol, dithioerythritol, cysteamine diHCl, 1-hydroxybenzotriazole, and Tris (Trisma Base) were obtained from Sigma (St. Louis, Mo.). D-Pantethine and D-calcium pantothenate were from U.S. Biochemical (Cleveland, Ohio) and DL-calcium pantothenate from ICN Pharmaceuticals (Cleveland, Ohio). Dicyclohexylcarbodiimide and cysteamine HCl were from Aldrich (Milwaukee, Wisc.). The sodium salt of D-[1-^{14}C] pantothenic acid,

ca. 50 mCi/mmol was supplied by New England Nuclear (Boston, Mass.). Other chemicals were analytical or scintillation grade. Sephadex G-25, 50-150 μm, was from Pharmacia (Piscataway, N.J.) and type BA85 nitrocellulose filters (25 mm) were from Schleicher and Schuell (Keene, N.H.). A glass calomel combination electrode (Sigma) was used for pH measurements. Absorbance was determined on a Gilford Model 240 spectrophotometer with silica cuvettes of 1-cm light path.

Substrate Preparation

D-[1-^{14}C] pantethine was synthesized by direct condensation of the salts of D-[1-^{14}C] pantothenic acid and cystamine by the 1-hydroxybenzotriazole dicyclohexylcarbodiimide method of Konig and Geiger (1970). DL-pantethine was similarly prepared. The application of this method to pantethine synthesis and the purification of pantethine have been the subject of numerous recent patents which can be consulted for details.[1] The concentration of pantethine in solution was standardized by disulfide analysis (Zahler and Cleland, 1968).

D-Pantethine was reduced to D-pantetheine by the method of Butler et al. (1976) for the reduction of disulfide-containing amines. The partition coefficients (H_2O/ethyl acetate) of pantetheine and dithiothreitol were ca. 9 and 0.5, respectively. Pantetheine

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[1]The first of several Japanese patents to appear was Japan. Kokai 76, 113,821 October 7, 1976, 8 pp. (Chemical Abstracts 86:171840d). A version in English exists: Brit. 1,561,047 February 13, 1980, 4 pp. (Chemical Abstracts 93:47198f).

solutions were stored under nitrogen and standardized by sulfhydryl analysis (Ellman, 1959).

Enzyme Preparation

Enzyme taken after $(NH_4)_2SO_4$ fractionation (see this chapter under, "Purification") and desalted through Sephadex G-25 into 0.02 M phosphate, pH 7.0 was used in validating the enzyme assays. This preparation had an activity of 0.50 µmoles of pantothenate produced/min/ml by the radiolabeled assay presented below and will be referred to hereafter as the "$(NH_4)_2SO_4$ enzyme solution."

Radiolabeled Assay

Both substrate and enzyme were reduced before assay. D-[^{14}C] pantethine (60 µCi/mmol) was reduced to D-[^{14}C] pantetheine by preincubating 12.5 mM pantethine with 25 mM dithioerythritol for 20 min or more at 37°C in 0.05 M Tris-HCl, pH 8.1. The enzyme was preincubated at 37°C for 20 min with 10 mM dithioerythritol in 0.05 M Tris-HCl, pH 8.1. The assay was initiated by adding 20 µl of prereduced substrate solution to 80 µl of prereduced enzyme solution.

The reaction was stopped after 15 min at 37°C by the addition of 200 µl of acetone. After centrifugation, the supernatant was spotted near one end of a 20 cm strip of Whatman No. 4 filter paper (2.5 cm in width). Spotting was done quickly over a 2- to 3-cm area with a hot-air dryer. The paper was chromatographed (ascending) with acetone/H_2O (90/10) in a small chromatography jar (6 X 10 X 15 cm) open to the atmosphere. The solvent traveled quickly up the paper, then slowed and stopped from evaporation. Under these conditions,

pantetheine moved with the solvent front and pantothenate remained where spotted. After 15 min the filter paper was removed and dried.

The distribution of radioactivity was determined qualitatively by gas-flow radiochromatoscanning (Actigraph III, Nuclear Chicago, 6 mm slit width), or quantitatively by liquid scintillation counting. For quantitation, the dried strip was cut halfway between the origin and front. Each half was placed in a separate scintillation vial, toluene/PPO/POPOP[2] (1 liter/4 g/50 mg) added to cover the strip, and each vial counted to 10,000 total counts in a Packard 3255 liquid scintillation spectrometer.

Blanks were run without enzyme and with excess enzyme to determine the separation limits achieved under conditions of 0 and 100% hydrolysis. Under 1%, and over 90%, respectively, of the counts were found in the origin half of the strip. Linearity was demonstrated by using various proportions of labeled pantothenate and labeled pantethine without active enzyme present.

Pantothenate Assay

Substrate and enzyme preincubation were the same as in the radiolabeled assay except that 5 mM D-pantethine was reduced with 10 mM dithioerythritol for 20 min or more at 37°C in 0.05 M Tris-HCl, pH 8.1. Ten microliters of prereduced substrate solution was added to 90 µl of prereduced enzyme solution to start the assay.

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[2]Abbreviations used: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyloxazol-2-yl)benzene.

The reaction was stopped after 15 min at 37°C by the addition of 100 µl of 0.3 N $Ba(OH)_2$, followed by an equivalent amount (Somogyi, 1945) of $ZnSO_4$, and H_2O to 1.0 ml. After centrifugation, the supernatant was analyzed for pantothenate by the radioimmunoassay of Wyse et al. (1979), modified to use nitrocellulose filters in the separation of antibody-bound and free hapten as follows. Unknown or standard was added, in a volume of 500 µl, to 500 µl of the antisera dilution (made without added rabbit albumin) containing 10,000 dpm of D-[1-^{14}C] pantothenate instead of D-[3-^{3}H] pantothenate. After 1 min or more at room temperature, the sample was cooled on ice and evenly vacuum filtered through a prewetted nitrocellulose filter into a scintillation vial. A Gelman No. 4204 filter funnel (Ann Arbor, Mich.) mounted on a cork, with vacuum introduced through a hypodermic needle was found convenient. The filter was immediately washed with 500 µl of ice-cold 10 mM phosphate, pH 7.0, containing 140 mM NaCl. The vacuum was released by lifting the filter (forcing the wash in the stem into the vial) and the filter transferred to another scintillation vial. Appropriate scintillation cocktails were added (Gershman et al., 1972) and both fractions radioassayed to determine the percentage binding.

Cysteamine Assay

Substrate and enzyme preincubation were the same as in the pantothenate assay. Reaction was initiated by diluting 150 µl of prereduced enzyme solution with 300 µl of 0.05 M Tris-HCl, pH 8.1, and adding 50 µl of prereduced substrate solution.

The reaction was stopped after 15 min at 37°C by adding 50 µl of 50% trichloroacetic acid. After centrifugation, the supernatant was analyzed for cysteamine by a modification of the method of Herrington et al. (1967) for aminoethanethiols in biologic media. Five hundred microliters of the supernatant was added to 1.5 ml of 0.8 M Tris-HCl, pH 9.0, and warmed to 37°C. One milliliter of 0.16 M fluoropyruvate in 0.05 M EDTA (pH 5.5) at 37°C was added. After 10 min at 37°C, the absorbance was read at 300 nm.

Blanks without enzyme, but with pantethine or cystamine, or reactions run to substrate exhaustion established a ΔA_{300} (ca. 0.78) corresponding to 100% hydrolysis. The equivalence of these calibration methods demonstrated that oxidation of cysteamine was not occurring. The absorption of the pantethine blank against H_2O was ca. 0.35.

Mercaptide Assay

Enzyme preincubation was the same as in previous assays. Three hundred microliters of prereduced enzyme solution was diluted with 2.69 ml of 0.05 M Tris-HCl, pH 8.1, which had been deoxygenated by bubbling N_2 through the solution and warmed to 37°C. The A_{240} was followed for 10 min or until a constant decrease with time could be determined. Pantetheine was added (10 µl of 150 mM) to initiate the reaction and the A_{240} followed. The maximal ΔA_{240} per minute (after linearization and before product inhibition) was proportional to the amount of enzyme present when the rate before substrate addition was taken as baseline. Blanks without enzyme, but with pantetheine or cysteamine, or reactions run to substrate exhaustion established a

ΔA_{240} (ca. 0.73) corresponding to 100% hydrolysis. The equivalence of calibration methods again demonstrated that oxidation of cysteamine was not occurring. The absorption of the pantetheine blank against H_2O was ca. 0.60.

Purification

Fresh pig kidneys (Tri-Miller Packing, Hyrum, UT) were cooled on ice and slices of cortex frozen until use. Five hundred and fifty grams of semi-solid, thawed (4°C, 8 hours) cortex were carried through the purification. Our equipment limited the scale of Steps 1 through 3 to ca. 275 grams of cortex. Therefore, these steps were performed twice per purification to provide a pooled extract from 550 grams of cortex at the end of Step 3 that was used in the remaining purification. All steps were performed at 0-4°C unless indicated.

Step 1: Homogenate

Two hundred and seventy-five grams of cortex were homogenized with 4 volumes of 0.02 M Tris-HCl, pH 8.2 in a Waring blender for 4 min at high speed. The suspension was centrifuged for 10 min at 10,000 g and the supernatant aspirated from pelleted large particulate matter.

Step 2: Microsomal Agglutination

Microsomes in the supernatant were agglutinated at pH 4.2 by the addition of 1 M formic acid and centrifuged for 10 min at 20,000 g. The supernatant was decanted and the pellet resuspended in ca. 400 ml of deionized water by homogenization in a Waring blender at high speed

for 20 sec. The suspension was 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 was added to the microsomal suspension and the mixture homogenized in a Waring blender at low speed for 2 sec. The material was then centrifuged for 10 min at 10,000 g. The aqueous phase containing solubilized enzyme was 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 was brought to pH 5.0 with 1 M formic acid and heated in a 75°C water bath up to 70°C and held at 70°C for 2 min. The suspension was rapidly cooled, centrifuged for 10 min at 20,000 g, and the supernatant aspirated from coagulated proteins.

Step 5: $(NH_4)_2SO_4$ Fractionation

The supernatant from Step 4 was saturated to 55% by adding 32.6 g solid $(NH_4)_2SO_4$ per 100 ml and then centrifuged for 15 min at 20,000 g. The resulting supernatant was saturated to 70% by adding 9.3 g $(NH_4)_2SO_4$ per 100 ml and then centrifuged for 30 min at 20,000 g. The precipitate was dissolved in ca. 20 ml of hydrophobic chromatography "application buffer" (114 g of $(NH_4)_2SO_4$ added to 1 liter of 0.02 M phosphate, pH 7.0).

Step 6: Hydrophobic Chromatography

The protein from Step 5 was 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 was washed with application buffer at a flow rate of ca. 150 ml/hour until the A_{280} of the eluate was less than 0.05. Enzyme was eluted with a solution of 20% ethylene glycol, 80% application buffer (v/v) at the same flow rate until the A_{280} was less than 0.03. The column was regenerated by sequentially applying one column volume each of 50 mM NaOH, 95% ethanol, deionized H_2O, and application buffer.

Step 7: Hydroxyapatite Chromatography

The enzyme eluate from Step 6 was concentrated to ca. 20 ml by ultrafiltration and diafiltered into 0.001 M phosphate, pH 7.0 with an Amicon PM 10 ultrafiltration membrane (Lexington, MA). The solution was applied at ca. 25 ml/hour 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 was eluted at the same rate with 0.02 M phosphate, pH 7.0.

Characterization

Intracellular Distribution

Fresh pig kidney cortex was homogenized and fractionated by the method of Ali and Lack (1965). Glucose-6-phosphatase activity was estimated by the method described by DeDuve et al. (1955) with

inorganic phosphate determined by the method of Fiske and Subbarow (1925). Pantetheine hydrolyzing activity was measured by the cysteamine assay and protein determined according to Lowry et al. (1951).

Substrate Specificity

Analogs of D-pantetheine with modifications in the pantoyl (A series), β-alanyl (B series), and cysteamine (C series) moieties were tested for relative enzymatic hydrolysis with the cysteamine (A and B series) and pantothenate (C series) assays. The initial analog concentration was equivalent to 1 mM D-pantetheine and analog hydrolysis was compared to the hydrolysis of 1 mM D-pantetheine under the same conditions. Enzyme was properly diluted from Step 7 of the purification to give values falling on the linear portion of the relevant hydrolysis curve.

Analogs in the A and B series were often synthesized by lactone fusion of a pantolactone analog and an aletheine analog by a method similar to that of Barnett (1944). The acid salt of the aletheine analog in dry methanol was titrated with sodium methoxide to the color change of cresol red before addition of the lactone. Analogs in the C series were synthesized by the 1-hydroxybenzotriazole dicyclohexyl-carbodiimide method of peptide synthesis of Konig and Geiger (1970), following the synthesis of pantethine.

Cleanup of all neutral analogs consisted of: a) ether extraction (2 x 1 volume), b) passage through an excess of mixed bed ion exchange resin (Amberlite MB-3, Sigma) at 5 ml/min/cm^2, and if necessary, c)

preparative thin layer chromatography (TLC) on Whatman PLK5 plates (Clifton, N.J.) with methyl ethyl ketone saturated with water as solvent. All cleanup was performed at 0-5°C.

Purity of the analogs was demonstrated by analytical TLC on Whatman MK6F plates with methyl ethyl ketone saturated with water as solvent at 0-5°C using iodine vapor staining. Structural verification was by proton magnetic resonance spectroscopy. A JEOL FX90Q Fourier transform NMR spectrometer with proton resonance at 89.60 MHz was used in the homonuclear gated decoupling mode to eliminate solvent resonance. Spectra were taken at ambient probe temperature (ca. 25°C) in 5 mm diameter tubes containing a 100 mM solution of the analog in D_2O with 3-(trimethylsilyl)-tetradeuterosodium propionate (Wilmad, Buena, N.J.) as internal standard. Specific rotations were determined in a 20 cm tube at ca. 10 g/100 ml with methanol as solvent in a Rudolph Model 63 polarimeter.

Analog AI: N-acetylcysteamine. N-Acetylcysteamine was purchased from Vega Biochemicals (Tucson, Ariz.) and used without further purification.

Analog AII: β-alethine diHCl. A modification of the method of Snell and Whittle (1957) was used to prepare β-alethine diHCl. Phthaloyl-β-alanine was synthesized by the method of Nefkens (1960). The final recrystallization of β-alethine diHCl was by solution in a minimal amount of water at 70°C followed by slow addition of an excess of ethanol. m.p. 220-221°C (lit. 221-222°C).

Analog AIII: CoA. Coenzyme A was from P-L Biochemicals (Milwaukee, Wisc.) and was used without further purification.

Analog BI: N,N'-bis[pantoyl] cystamine. Lactone fusion of D-pantolactone (Sigma) and cystamine diHCl gave the desired product (Barnett, 1944). The reaction mixture was purified by ether extraction and mixed bed ion exchange. Yield 46%. m.p. 140-143°C (lit. 141-144°C) Analytical TLC R_f = 0.50.

Analog BII: N,N'-bis[pantoylglycyl] cystamine. Synthesis of N,N'-bis[glycyl] cystamine essentially followed the procedure of Kopelevich et al. (1979) for the synthesis of alethine. Subsequently, fusion with D-pantolactone gave the desired product.

N-t-BOC-glycine was from Vega (Tucson, Ariz.). The succinimide ester of N-t-BOC-glycine required excess ether in the final recrystallization to avoid oil formation. Yield 28%. m.p. 164.5-165.5°C.

After conjugation of the activated and protected amino acid with cystamine, the t-BOC group was removed from N,N'-bis[N-t-BOC-glycyl] cystamine in ethyl acetate solution with excess HCl gas. N,N'-bis [glycyl] cystamine diHCl crystallized on formation and was filtered and washed. Yield 91%. m.p. 110°C (decomposition).

Lactone fusion of D-pantolactone and N,N'-bis[glycyl] cystamine gave the desired product as a viscous oil after cleanup (ether extraction, mixed bed ion exchange, and preparative TLC). Yield 43%. Analytical TLC R_f = 0.42.

Analog BIII: N,N'-bis[pantoyl-γ-aminobutyryl] cystamine. Synthesis essentially followed that of analog BII. N-t-BOC-γ-aminobutyric acid was purchased from Vega Biochemicals (Tucson, Ariz.)

Recrystallization of the succinimide ester of N-t-BOC-γ-aminobutyric acid was done in isopropanol/ether (5/1). Yield 93%. m.p. 116-118°C.

The t-BOC group was removed from N,N'-bis[N-t-BOC-γ-aminobutyryl] cystamine in ethyl acetate solution with excess HCl gas. N,N'-bis [γ-amino-butyryl] cystamine formed an oil which crystallized after two days at 4°C. The crude product was recrystallized from a minimal amount of ethanol with an excess of added isopropanol/ether (5/1). Yield 37%. m.p. 152-155°C.

Lactone fusion of D-pantolactone and N,N'-bis[γ-aminobutyryl] cystamine gave the desired product as a viscous oil after cleanup (ether extraction, mixed bed ion exchange, and preparative TLC). Yield 58%. Analytical TLC R_f = 0.19.

Analog CI: pantothenyl-β-aminoethanol. This analog was synthesized from D-calcium pantothenate and β-aminoethanol (Aldrich, Milwaukee, Wisc.) by carbodiimide coupling. Cleanup by ether extraction and mixed bed ion exchange gave the pure viscous oil. Yield 91%. Analytical TLC R_f = 0.17.

Analog CII: S-ethyl pantetheine. Synthesis by carbodiimide coupling of D-calcium pantothenate and S-ethyl cysteamine (Aldrich) and cleanup by ether extraction and mixed bed ion exchange gave the pure viscous oil. Yield 93%. Analytical TLC R_f = 0.42.

Analog CIII: N,N'-bis[pantothenyl]-1,6-diaminohexane. This analog was synthesized from D-calcium pantothenate and 1,6-diamino-hexane (Aldrich) by carbodiimide coupling. Cleanup by ether extraction and mixed bed ion exchange, and recrystallization

from acetone gave the pure product. Yield 89%. m.p. 74-77°C (decomposition). Analytical TLC R_f = 0.17.

Polyacrylamide Gel Electrophoresis

Nondenaturing polyacrylamide gel electrophoresis was performed after Brewer and Ashworth (1969) and sodium dodecylsulfate polyacrylamide gel electrophoresis after Laemmli (1970). Tube gels of 3 or 6 mm diameter were 10% in acrylamide. Protein and carbohydrate were stained by the method of Phillips (1981) using coomassie blue R-250 and periodic acid-Schiff's (PAS) reagent with 5 and 30 µg of protein, respectively, applied per tube.

Localization of activity on polyacrylamide gels. To stain for enzyme activity, 3 mm gels were immediately stirred after electrophoresis with 500 volumes of deionized water (3 times for 15 min each). The gels were then bathed in 2 volumes of 5 mM pantetheine containing 5 mM dithiothreitol and 250 µM cresol red. The solution surrounding the gels was kept red by adding 0.05 N NaOH as necessary. In ca. 15 min at ambient temperature, bands of activity in the gels turned yellow from local titration of the indicator. An enzyme activity of 100 nmoles of pantothenate produced/min was applied per tube.

Gel scanning. An ISCO UA-5 absorbance monitor with a model 1310 gel scanner and a 0.5 mm slit was used. Coomassie blue and PAS stains were monitored at 580 nm while the activity stain was monitored at 435 nm. The linear transport rate was 30 cm/hour.

Amino Acid Analysis

The amino acid composition was determined on a Beckman Model 120B amino acid analyzer modified to employ a 60 cm column of Durrum DC-1A resin (Palo Alto, Calif.) for single column analysis. Enzyme was hydrolyzed with 4N methanesulfonic acid after Simpson et al. (1976) with subsequent conversion of cysteine and cystine residues to S-sulfocysteine. Serine, threonine, and half-cystine were determined by linear extrapolation of values from 22, 48, and 72 hours of hydrolysis to zero time. Values for isoleucine and valine were the average of data at 48 and 72 hours of hydrolysis. Norleucine was used as an internal standard and tryptophan was determined spectrophotometrically by the method of Bencze and Schmid (1957).

Carbohydrate Analysis

Hexoses by gas chromatography. Hexoses were identified and their quantities estimated by gas chromatography (GC) of their alditol acetates on a 150 cm x 2 mm column of 3% SP-2340 on 100/120 Supelcoport following the directions of the manufacturer (Supelco, Bellefonte, Penn.). Glycoprotein was hydrolyzed and carbohydrate residues derivatized by the method of Yang and Hakomori (1971). A Varian Model 2740 gas chromatograph with flame ionization detection and a nitrogen carrier gas rate of 40 ml/min was used. Column temperature was programmed at 2°C/min from 180°C to 240°C after a 6 min initial hold after injection. Peaks were quantitated using their width at half the peak height.

Quantitation. Total neutral hexoses were determined by the anthrone reaction after Roe (1955) with a mixture of mannose, galactose, fucose, and glucose (in proportion to their relative amounts as estimated by GC) as standard. Sialic acid was determined by the resorcinol reaction after Svennerholm (1957) with N-acetylneuraminic acid as standard. Fucose was determined by the cysteine-sulfuric acid reaction (Dische and Shettles, 1948).

CHAPTER IV

RESULTS

This chapter summarizes results validating the enzymatic assay, purification, and characterization of a pantetheine hydrolyzing enzyme from pig kidney. Discussion of these results follows in Chapter V.

Assay

The effects of enzyme concentration and incubation time on assay results are shown in Table 5 and Fig. 4, respectively. Each assay is independently calibrated: the radiolabeled assay by disulfide analysis of pantethine, the pantothenate assay by dry weight of D-calcium pantothenate, and the cysteamine and mercaptide assays as given on page 35. Pantothenate is quantitatively released at substrate exhaustion (Table 5) and each assay follows a nearly identical time course (Fig. 4).

The apparent activities of the homogenate and the $(NH_4)_2SO_4$ enzyme solution, along with coefficients of variation for the assays, are given in Table 6. A comparison with the enzyme assay of Dupre et al. (1970a) is also included.

A typical 15 min chromotographic separation of pantethine and pantothenic acid as used in the radiolabeled assay is shown in Fig. 5. Substrate and product are well separated with pantethine concentrated at the solvent front.

The effects of dithioerythritol and enzyme prereduction on the mercaptide assay are shown in Fig. 6. Prereduction with

Table 5. The Effect of Enzyme Concentration on Four Independent Assays[a].

	Radiolabeled Pantothenate		Cysteamine Mercaptide	
[Pantetheine] (mM)	5.0	1.0	1.0	0.50
[Dithioerythritol] (mM)	13	10	4.0	1.0
[Enzyme] (%)[b]	Hydrolysis (%)	nmol[c]	A_{300}[d]	ΔA_{240}/min
0.005		0.07		
0.01		0.09		
0.02		0.16		
0.05		0.36		0.0004
0.1		0.84		0.0007
0.2		1.6		0.0014
0.5		3.8		0.0034
1.0		7.9	0.075	0.0072
2.0		17	0.15	0.014
3.0			0.21	0.022
4.0			0.29	0.029
5.0	7.5	40	0.35	0.038
6.0			0.41	0.044
7.0			0.48	0.051
8.0			0.55	0.056
9.0			0.59	
10.0	15	71	0.63	
15	21			
20	27	97		
25	35			
30	39			
35	44			
40	49			
50	60	100		

[a] See Chapter III for assay details. Approximate linear ranges are between horizontal rulings in each column.

[b] Percent volume of enzyme assay incubation mixture that was $(NH_4)_2SO_4$ enzyme solution.

[c] nmol pantothenate/100 µl enzyme assay.

[d] Pantethine blank has been subtracted.

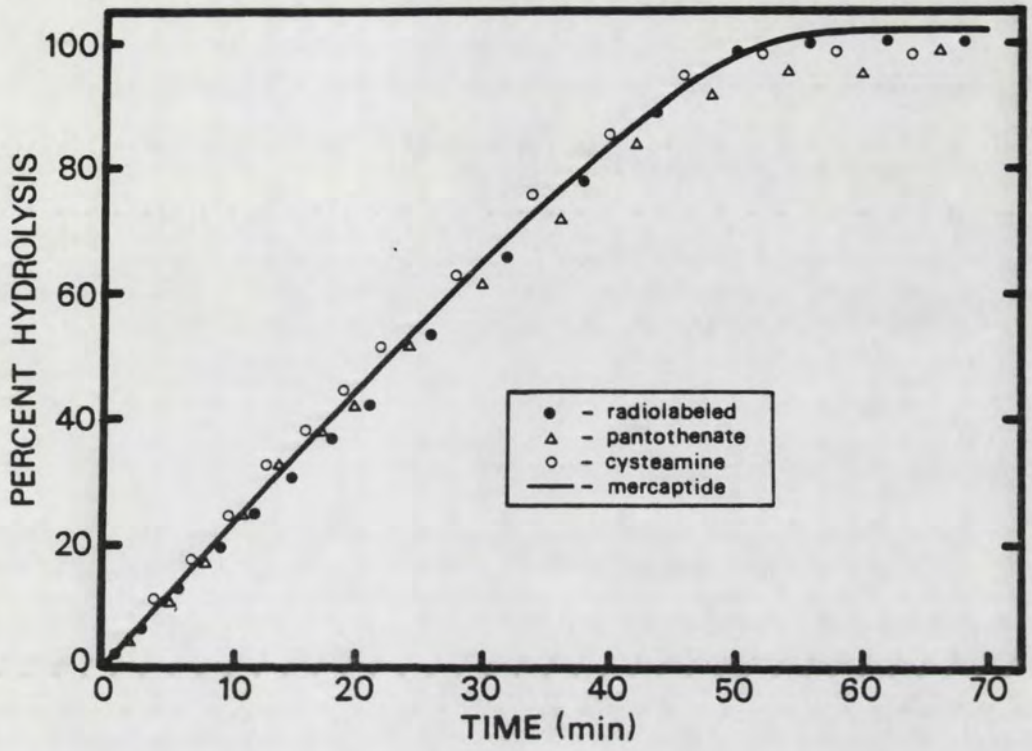

Figure 4. Time course of the enzymatic hydrolysis of pantetheine monitored by four independent assays. In all assays, prereduced enzyme was diluted and the reaction initiated as in the mercaptide assay. The concentration of $(NH_4)_2SO_4$ enzyme solution was 5% of the assay volume, and the initial concentration of D-pantetheine was 1.0 mM. The concentration of dithioerythritol was 1.0 mM, except in the cysteamine assay where it was increased to 4.0 mM to insure complete reduction of cysteamine during analysis. Details of analysis and calibration can be found in Chapter III.

Table 6. Apparent Enzyme Activities and Coefficients of Variation.

	Homogenate[a]	$(NH_4)_2SO_4$ Enzyme Solution[b]		
	Activity[c]	Activity[d]	Coefficient of Variation(%)	
Assay	(µmol/min/ml)	(µmol/min/ml)	Intra-assay[e]	Inter-assay[f]
Radiolabeled	0.060	0.50	2.0	2.5
Pantothenate	0.058	0.60	6.1	10.4
Cysteamine	0.068	0.65	3.7	5.8
Mercaptide	-	0.64	1.6	3.2
Dupre et al. (1970a)[g]	0.051	0.40	-	-

[a]See Chapter III for preparation. The protein concentration was 33 mg/ml by the method of Lowry et al. (1951), and 41 mg/ml by the biuret reaction (Gornall et al. 1949) as used by Dupre and Cavallini (1979). Bovine serum albumin was used as a standard.

[b]The protein concentration was 1.3 mg/ml as measured by either the method of Lowry et al. (1951) or the biuret reaction (Gornall et al. 1949). The $(NH_4)_2SO_4$ enzyme solution was ca. 150-fold purified with respect to the starting homogenate with a total recovery of ca. 37%.

[c]Values are the average of two determinations. The units "µmol/min/ml" refer to µmoles of pantothenate released per min per ml of homogenate.

[d]Values are means of the 7 inter-assay samples, except for the assay of Dupre et al. (1970a), which is the average of two determinations.

[e]n=10

[f]n=7

[g]The substrate used was 2.5 mM D-pantethine instead of 5.0 mM DL-pantethine.

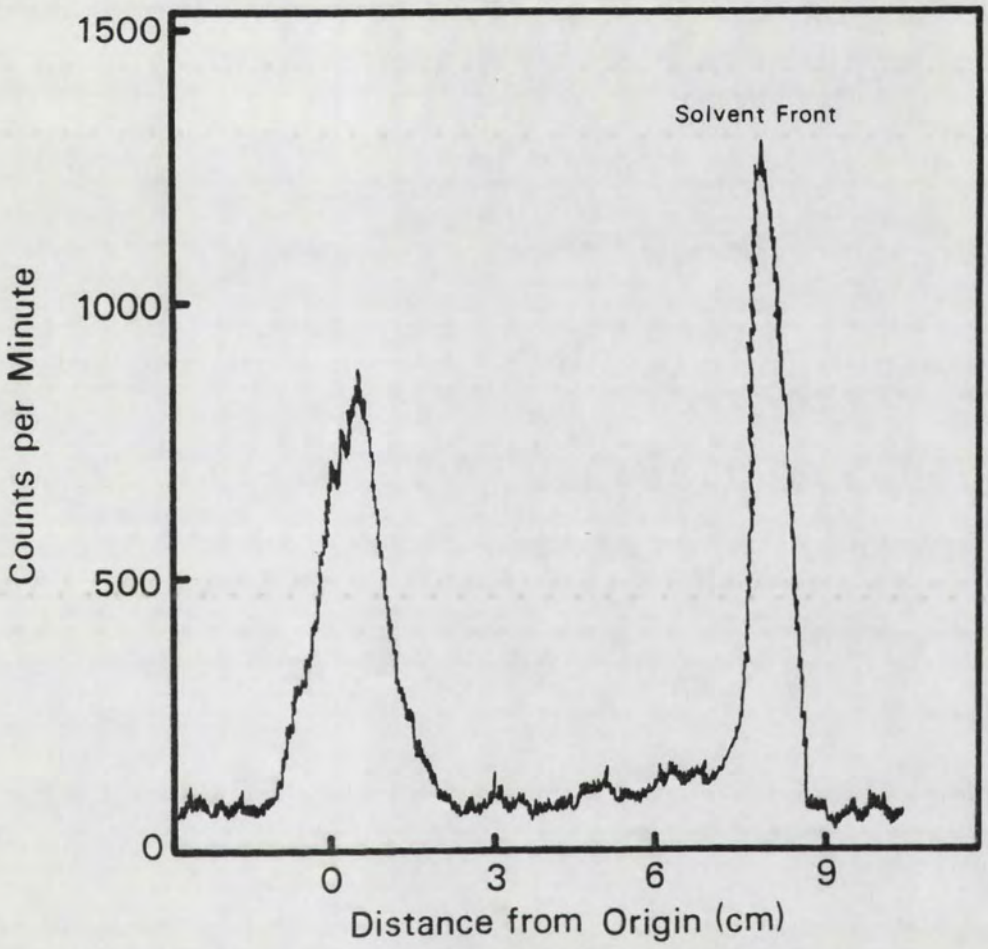

Figure 5. Radiochromatographic scan of the separation between pantethine and pantothenic acid achieved in the radiolabeled assay. Pantetheine was concentrated at the solvent front and pantothenate remained where spotted. For details of the chromatographic separation and its analysis, see page 32.

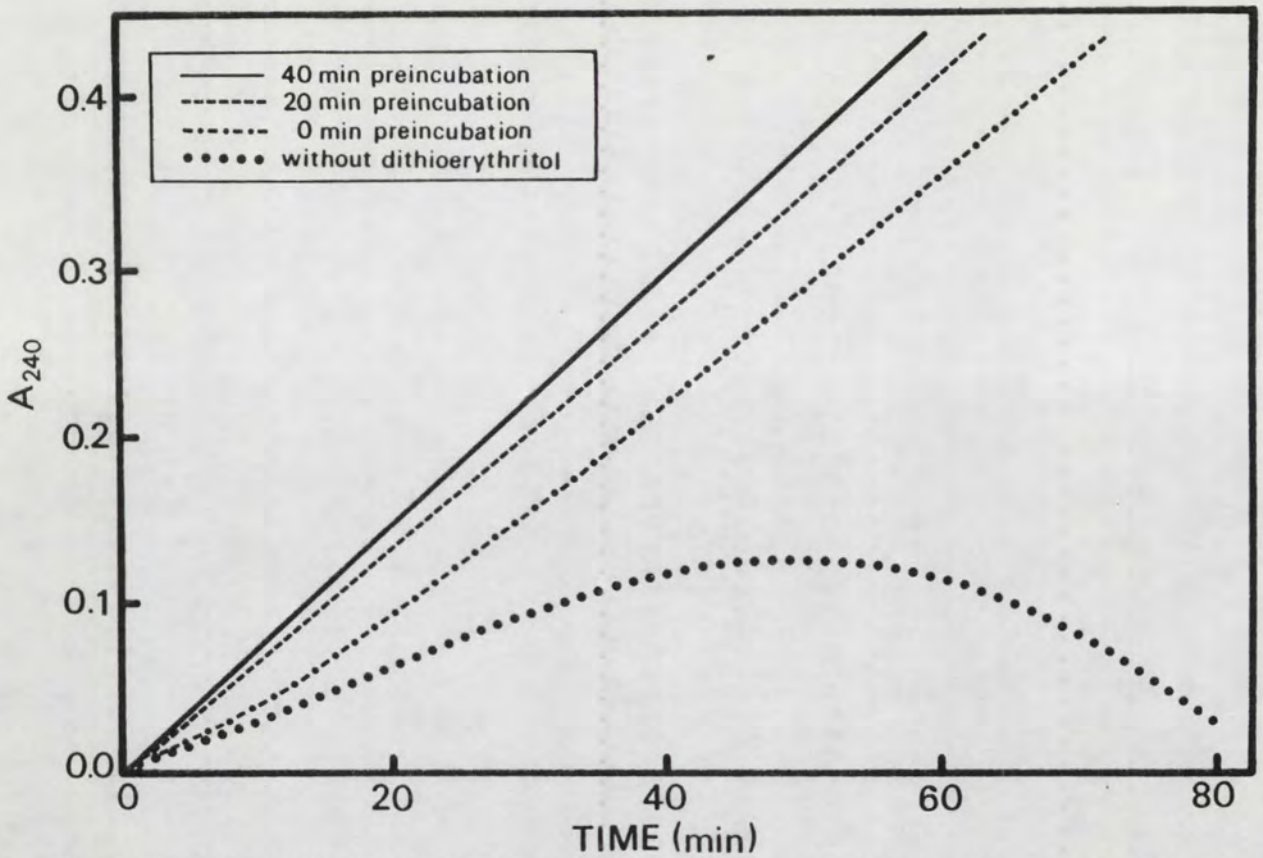

Figure 6. The effects of dithioerythritol and enzyme prereduction on the mercaptide assay. Incubation mixtures contained 1.0 mM D-pantetheine, 1% $(NH_4)_2SO_4$ enzyme solution, and 0.05 M Tris-HCl, pH 8.1, with or without dithioerythritol preincubation as indicated. Enzyme prereduction was with 1.0 mM dithioerythritol at 37°C. Each tracing has the corresponding blank without enzyme subtracted.

dithioerythritol is necessary for valid initial velocities. When pantetheine and dithioerythritol are added simultaneously, the hydrolysis rate increases with time as the enzyme is activated by reduction. When dithioerythritol is not added, the initial rate is the same as when the activator is added simultaneously with substrate. With time, however, the A_{240} decreases when dithioerythritol is omitted, and cysteamine oxidation invalidates the assay.

Purification

The purification of an enzyme from pig kidney hydrolyzing pantetheine to pantothenate and cysteamine is summarized in Table 7. A 5600-fold purification is obtained in 22% yield. The purification, including all assays and calculations can be done in less than 19 hours by one researcher.

Optimization of Individual Steps

In developing the purification scheme, each step was optimized for critical variables using enzyme from the preceeding step.

Microsomal agglutination. The recovery of enzyme activity (radiolabeled assay) and protein (Lowry et al., 1951) on agglutination of microsomes at pH's between 3 and 6 is shown in Fig. 7. A pH of 4.2 was chosen as giving high total and specific activities.

Butanol solubilization. The recovery of enzyme activity (mercaptide assay) and protein (A_{280}) depends on the pH of the butanol solubilization (Fig. 8). An optimal pH of 5.75 was routinely used.

Table 7. Purification of a Pantetheine Hydrolyzing Enzyme from Pig Kidney.

Step	Fraction	Volume(ml)	Activity(μmol/min)[a]	Protein(mg)[b]	Specific Activity (μmol/min/mg)
1	Homogenate	2700	250	99,000	0.0025
2	Microsomal Agglutination	1100	170	43,000	0.0040
3	Butanol Solubilization	990	150	3,900	0.038
4	Heat Treatment	990	130	1,400	0.093
5	$(NH_4)_2SO_4$ Fractionation	20	92	260	0.35
6	Hydrophobic Chromatography	160	68	15	4.5
7	Hydroxyapatite Chromatography	23	56	4.0	14

[a] Expressed in terms of the mercaptide assay in μmoles of pantothenate liberated per minute. Steps 1 and 2 were measured by the radiolabeled assay and corrected for the difference between the radiolabeled and mercaptide assays. This difference was obtained by measuring later steps with both assays.

[b] Protein in steps 1 and 2 was measured by the method of Lowry et al. (1951). Later steps used an $E^{1\%}_{1cm}$ (280) = 11.3.

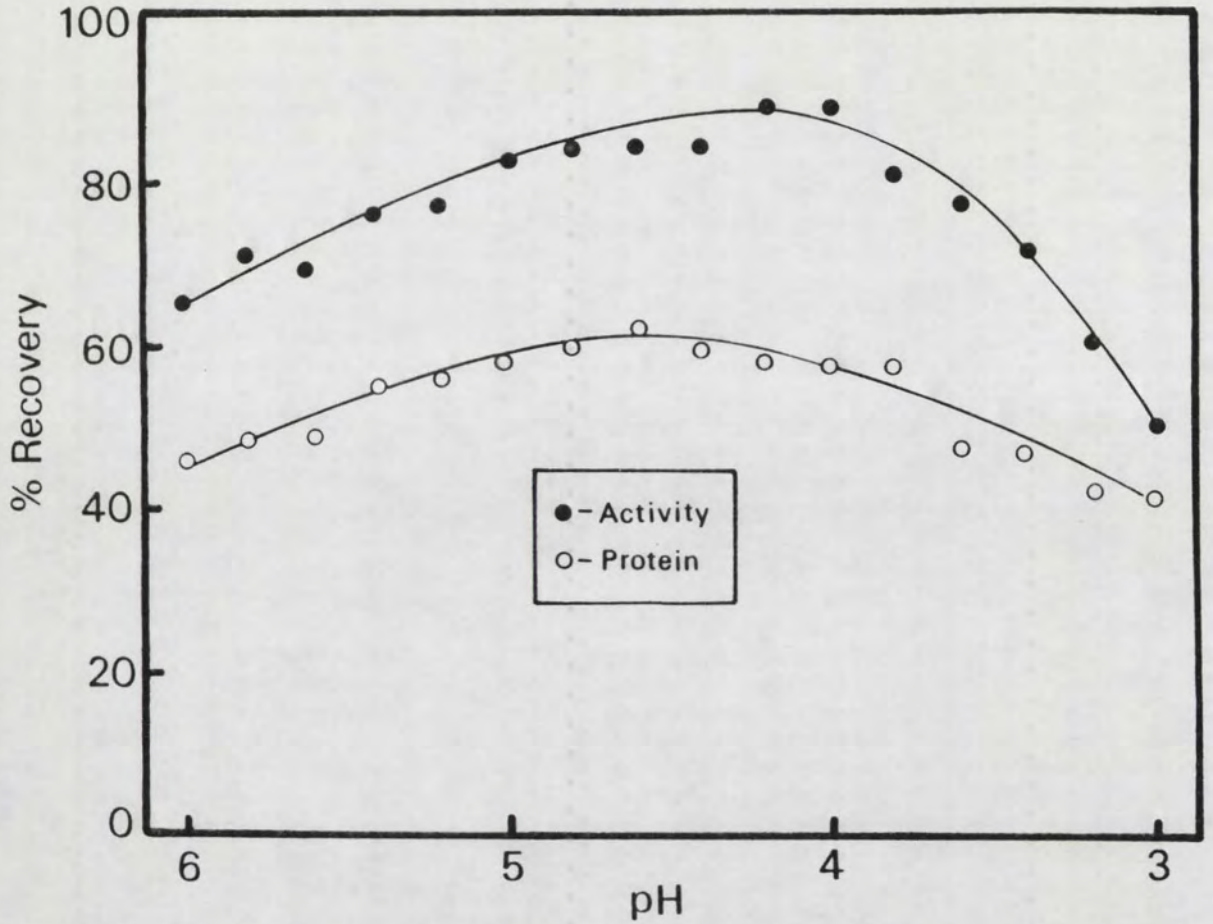

Figure 7. Optimization of microsomal agglutination. The microsomal suspension from Step 1 of the purification (page 36) was agglutinated at a pH between 3 and 6 by the addition of formic acid and centrifuged for 10 min at 20,000 g. The pellet contained various amounts of enzyme activity and protein. Points shown are the average of triplicate determinations.

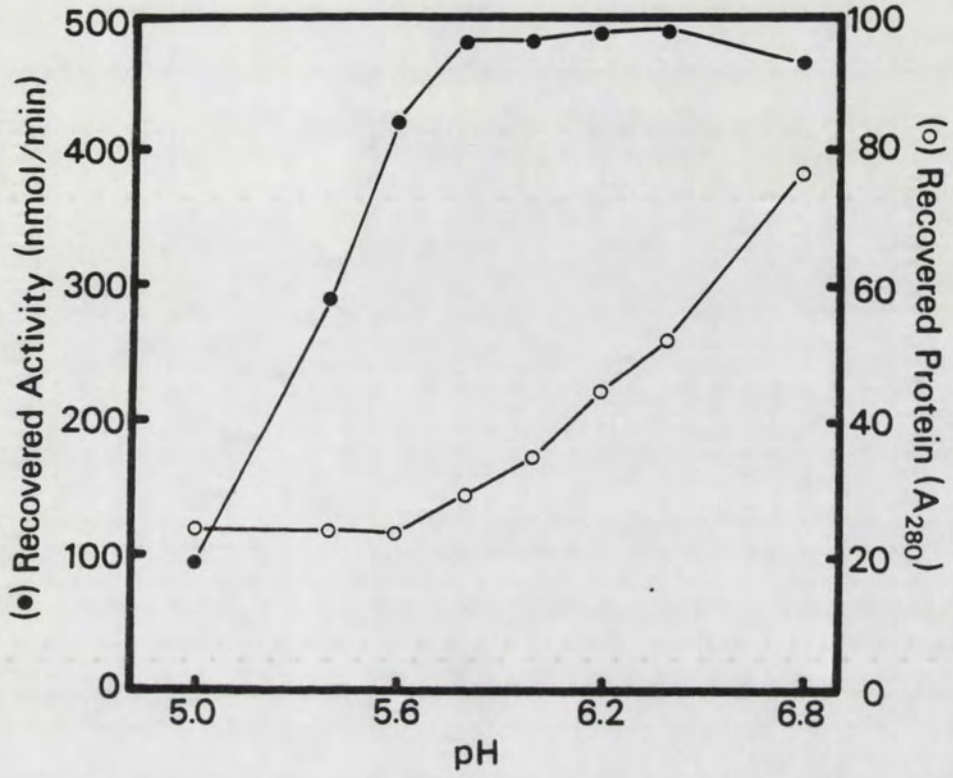

Figure 8. Optimization of butanol solubilization. Microsomes agglutinated at pH 4.2 from Step 2 of the purification (page 36) were resuspended in deionized water by homogenization and aliquots adjusted to various pH's with 0.5 M Tris-HCl, pH 9.0. One and a half volumes of n-butanol were added to each sample, the mixtures homogenized briefly and centrifuged for 10 min at 10,000 g. The aqueous phases were aspirated and immediately assayed for recovery of enzyme activity (mercaptide assay) and protein (A_{280}).

$(NH_4)_2SO_4$ fractionation. Optimization of $(NH_4)_2SO_4$ fractionation using the mercaptide assay is shown in Fig. 9. Routine purification employed the whole 55-70% range.

Hydrophobic chromatography. An elution profile of enzyme activity (mercaptide assay) and protein (A_{280}) is shown in Fig. 10. Almost half of the applied protein washed through the column with the application buffer. Enzyme, along with 5-10% of the protein, is eluted at the breakthrough of the elution buffer.

Hydroxyapatite chromatography. Elution from the hydroxyapatite column is shown in Fig. 11. The mercaptide assay was used to measure enzyme activity. All fractions containing significant protein were pooled as a constant specific activity was observed.

Characterization

Intracellular Distribution

The apparent intracellular distribution of pantetheine hydrolyzing activity and glucose-6-phosphatase, a microsomal marker, are shown in Fig. 12. Data are presented graphically in the manner proposed by DeDuve et al. (1955). The area of each block is proportional to the percentage of activity recovered in the corresponding fraction, and its height to the degree of purification achieved over the homogenate.

Michaelis Constant, pH Profile, and Molecular Weight

Kinetic plots after Hanes (1932) reveal an apparent K_m of 20 μM for D-pantetheine (Fig. 13). A similar K_m was obtained for D-pantetheine with substrate added as DL-pantetheine. Particulate

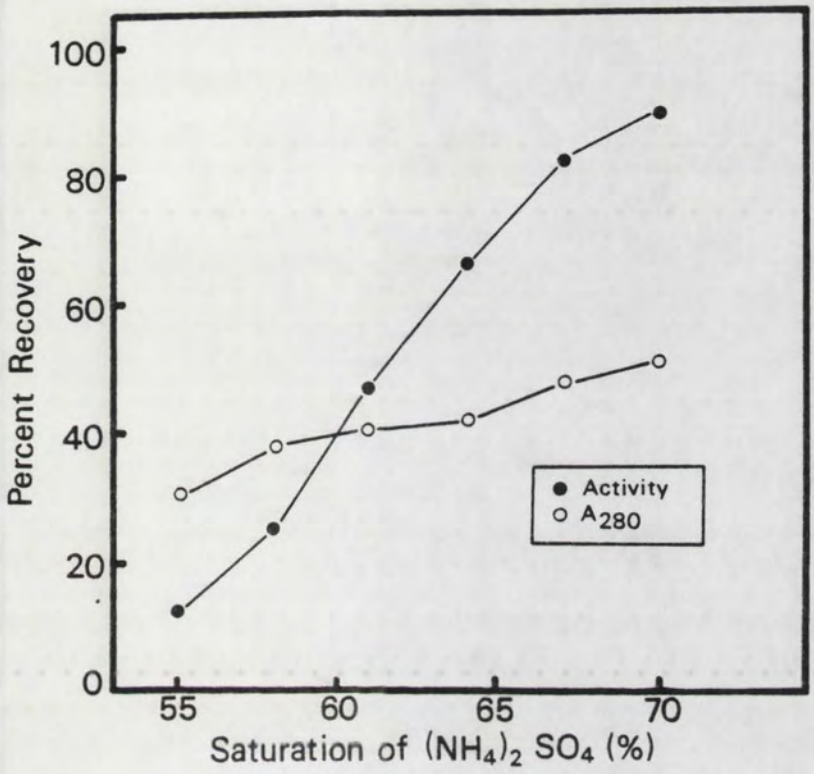

Figure 9. Optimization of $(NH_4)_2SO_4$ fractionation. Aliquots of the supernatant from Step 4 of the purification (page 37) were brought to between 55% and 70% saturated by adding solid $(NH_4)_2SO_4$. The suspensions were centrifuged for 30 min at 20,000 g to recover pellets containing various amounts of enzyme activity and protein.

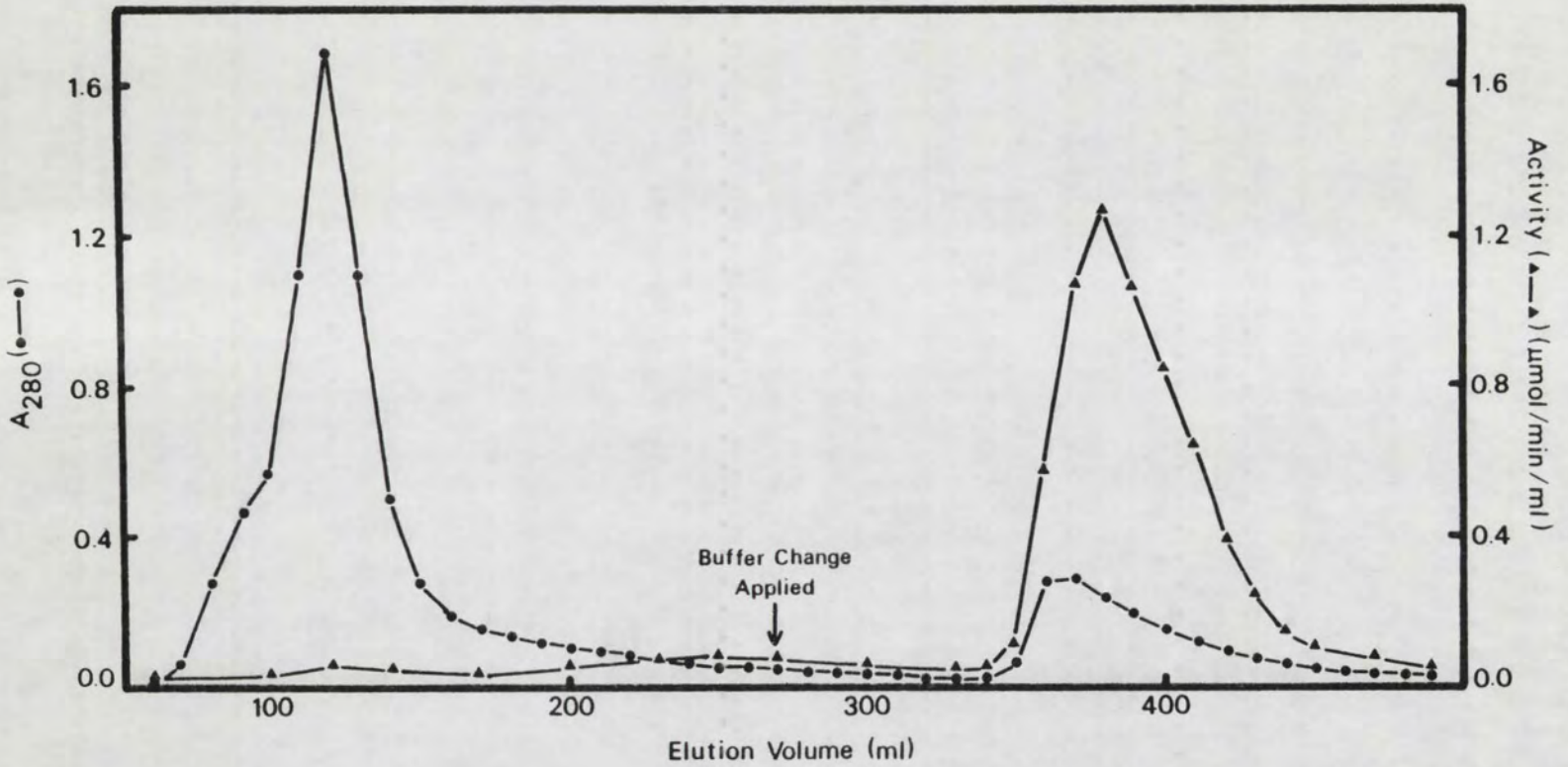

Figure 10. Hydrophobic chromatography elution. Enzyme from Step 5 of the purification (page 37) was absorbed onto a 2.5 x 23 cm Octyl-Sepharose CL-4B column and eluted by adding 20% ethylene glycol to the elution buffer. See page 38 for details.

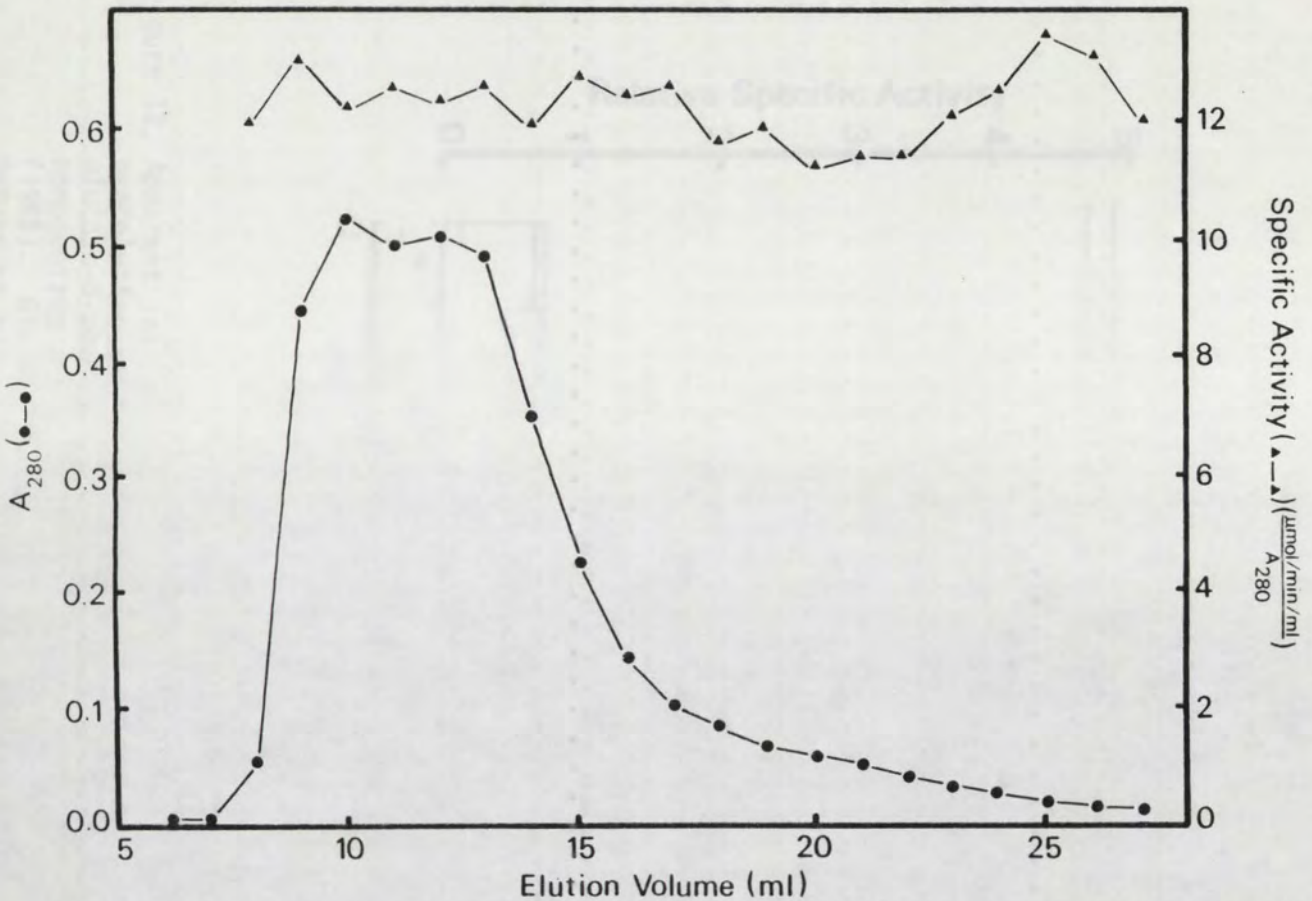

Figure 11. Hydroxyapatite chromatography elution. Protein from Step 6 of the purification (page 38) was concentrated and buffered in 0.001 M phosphate, pH 7.0, and applied to a 1.5 x 5 cm hydroxyapatite column. Absorbed enzyme was eluted with 0.02 M phosphate, pH 7.0. Contaminating protein remained absorbed to the hydroxyapatite.

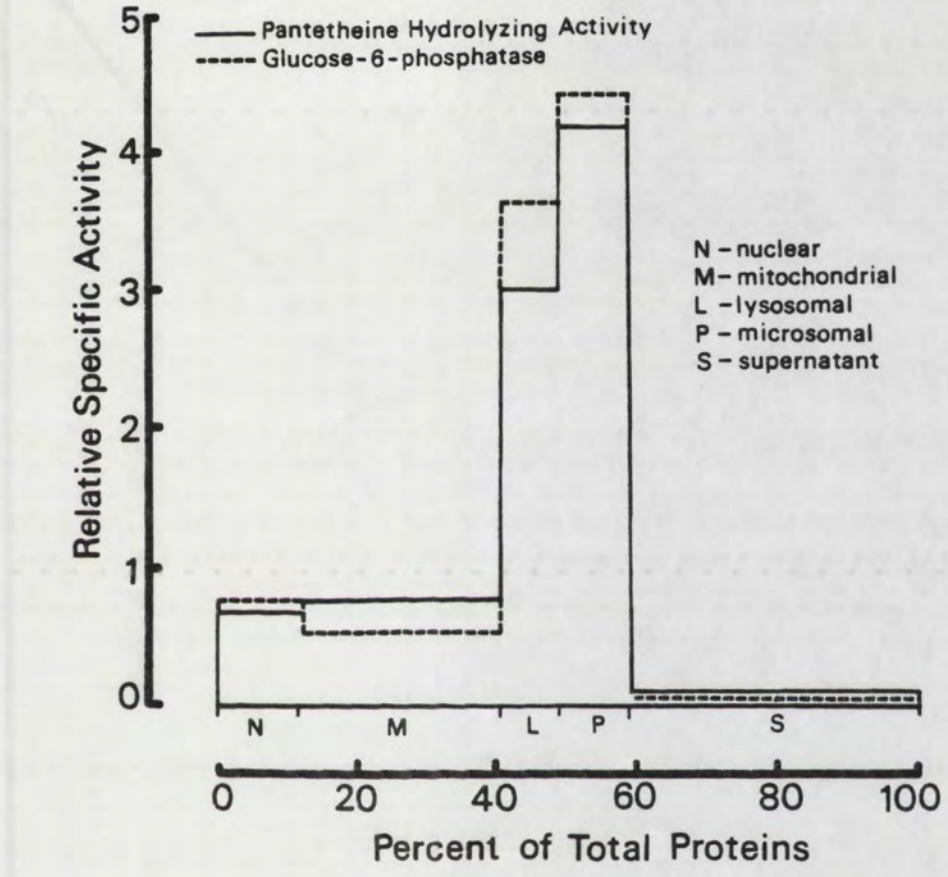

Figure 12. Apparent intracellular distribution of pantetheine hydrolyzing activity and the microsomal marker, glucose-6-phosphatase. Fresh pig kidney cortex was homogenized and fractionated by the method of Ali and Lack (1965). Glucose-6-phosphatase was measured as described by DeDuve et al. (1955) and pantetheine hydrolyzing activity was measured by the cysteamine assay.

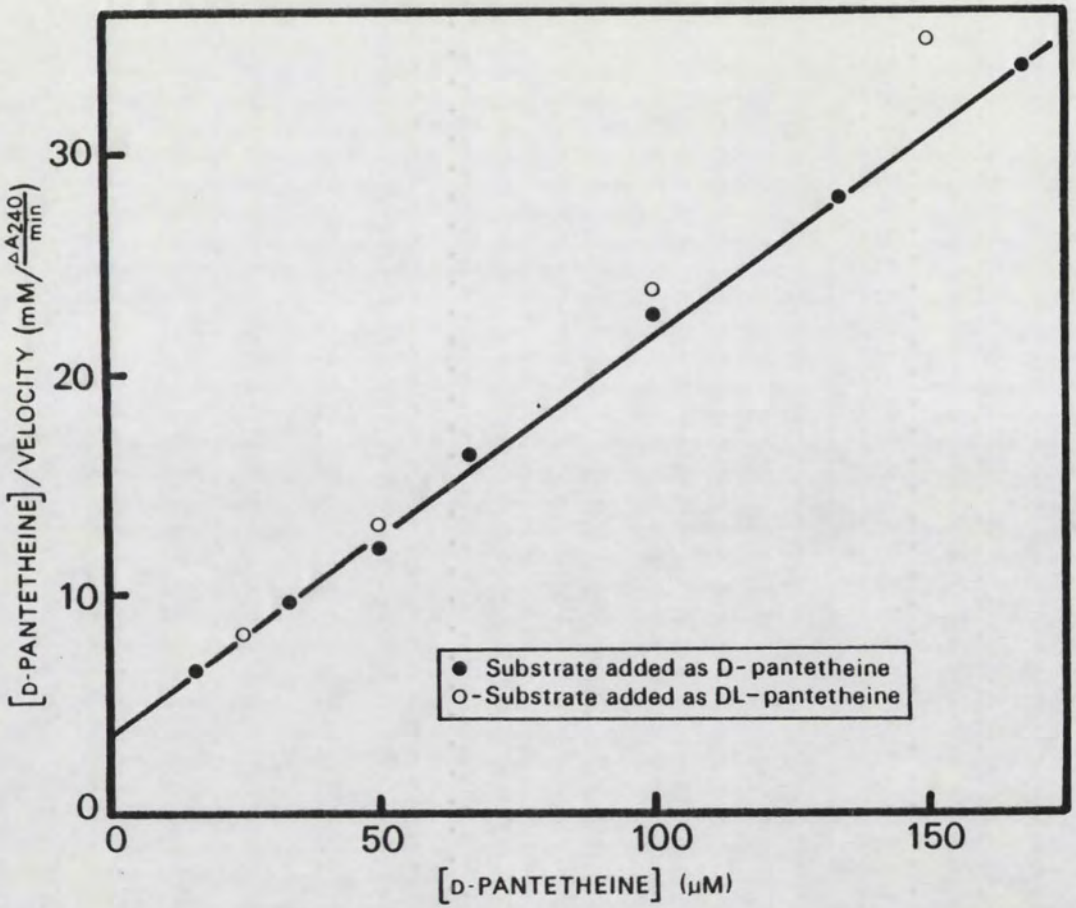

Figure 13. Hanes (1932) plot for K_m determination. The mercaptide assay with 0.75% $(NH_4)_2SO_4$ enzyme solution was used.

enzyme (Step 2 of the purification) had an apparent K_m of 50 μM using the pantothenate assay (data not shown).

The pH profile of enzymatic activity (Fig. 14) shows a broad plateau between pH 3 and 7. Activity increases above pH 7 to a peak at pH 9 to 9.5. The basic optimum was not an artifact of more efficient enzyme activation at higher pH, because enzyme reduced at pH 9 gave similar results. Particulate enzyme (Step 2 of the purification) also showed a similar pH optimum (data not shown).

Molecular weight (MW) estimations of the enzyme by sodium dodecylsulfate (SDS) electrophoresis and gel filtration are shown in Fig. 15. Gel filtration gave an apparent MW of 54,000 while SDS electrophoresis indicated 60,000. The average (57,000) was used as the best estimate of the actual MW.

Substrate Specificity

Verification of analog structure. The proton magnetic resonance (pmr) spectra of pantethine is shown in Fig. 16. Spectra of the analogs varied from pantethine in a predictable and systematic way. Stick pmr spectra of pantethine and some of the more interesting analogs are given in Fig. 17.

Specificity studies. Substrate specificities of the purified pig kidney enzyme (Step 7) and a homogenate of chicken liver are shown in Table 8. Chicken liver homogenates hydrolyze pantetheine 3 times faster than CoA. The pig kidney enzyme requires unchanged D-pantoyl and β-alanyl moieties for significant hydrolysis to occur.

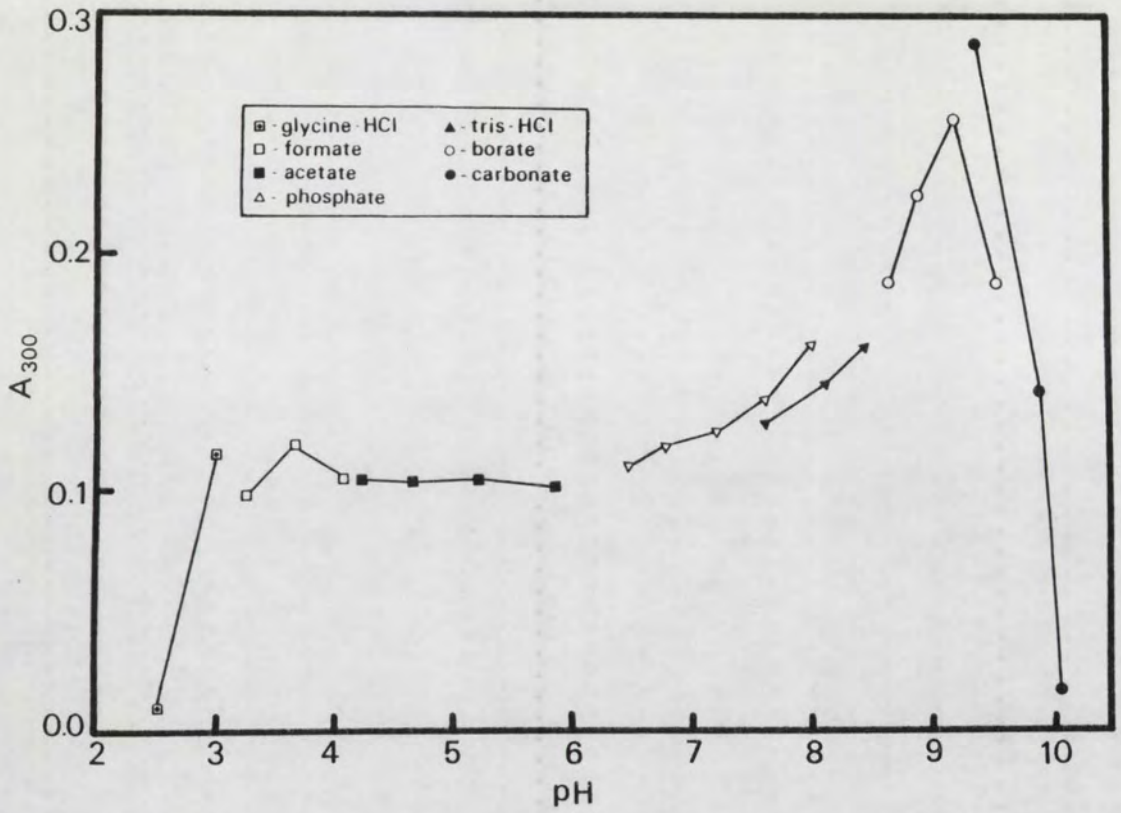

Figure 14. The pH profile of enzymatic activity. $(NH_4)_2SO_4$ enzyme solution (12.5 µl) was prereduced with 10 mM dithioerythritol in 17 mM Tris-HCl, pH 8.1, for 20 min at 37°C in a volume of 150 µl. Fifty microliters of 0.5 M buffer with 295 µl of H_2O was added and the reaction initiated with 5 µl of 100 mM D-pantetheine. Analysis followed the cysteamine assay (page 34). Pantethine blanks have been subtracted.

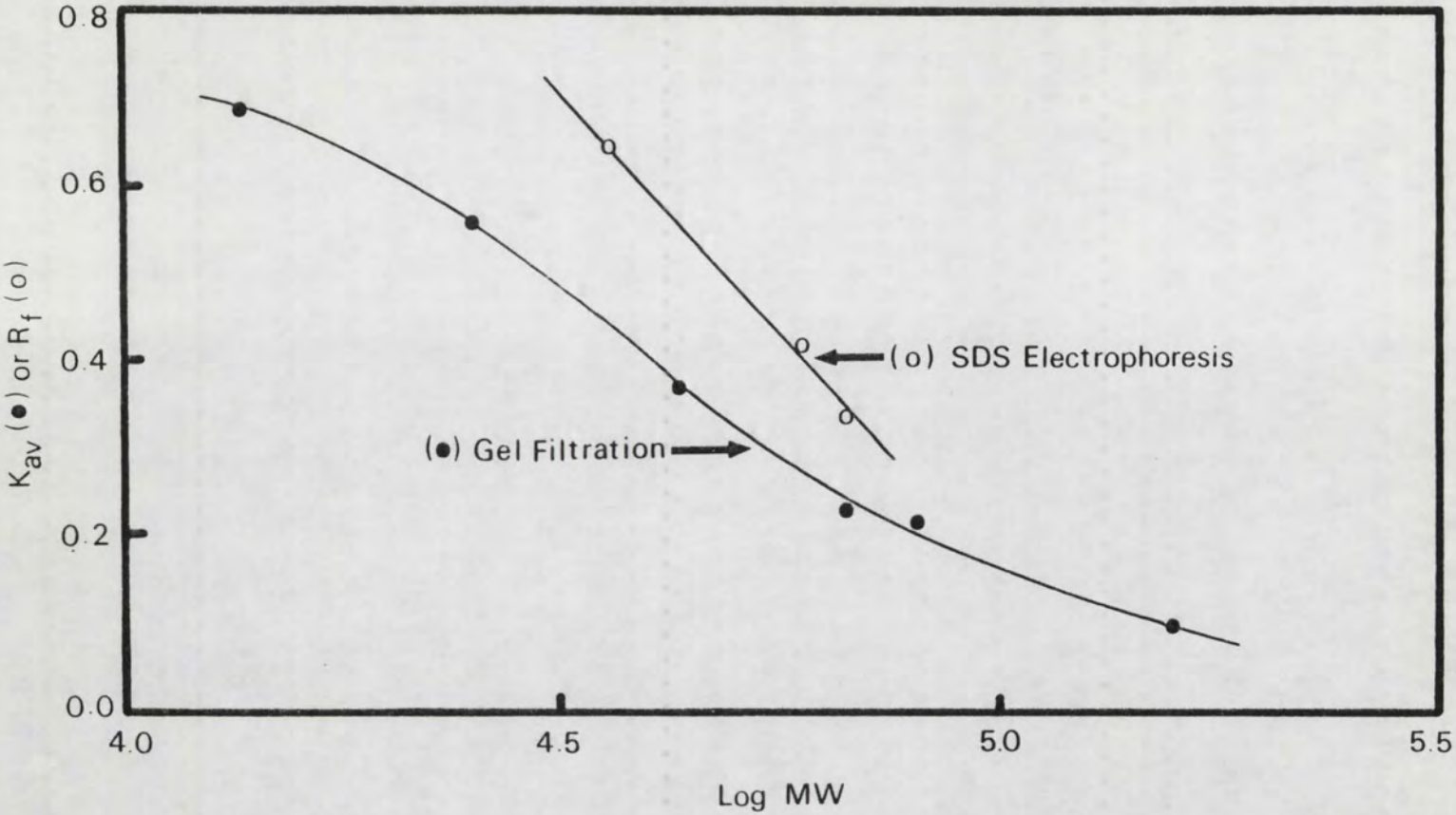

Figure 15. Molecular weight estimation. Gel filtration at 23°C on a 1.5 x 110 cm column of Bio Gel P-300 (BioRad, Richmond, CA) followed the method of Andrews (1965) and SDS electrophoresis was after Laemmli (1970). Molecular weight standards were from Pharmacia (Piscataway, N.J.).

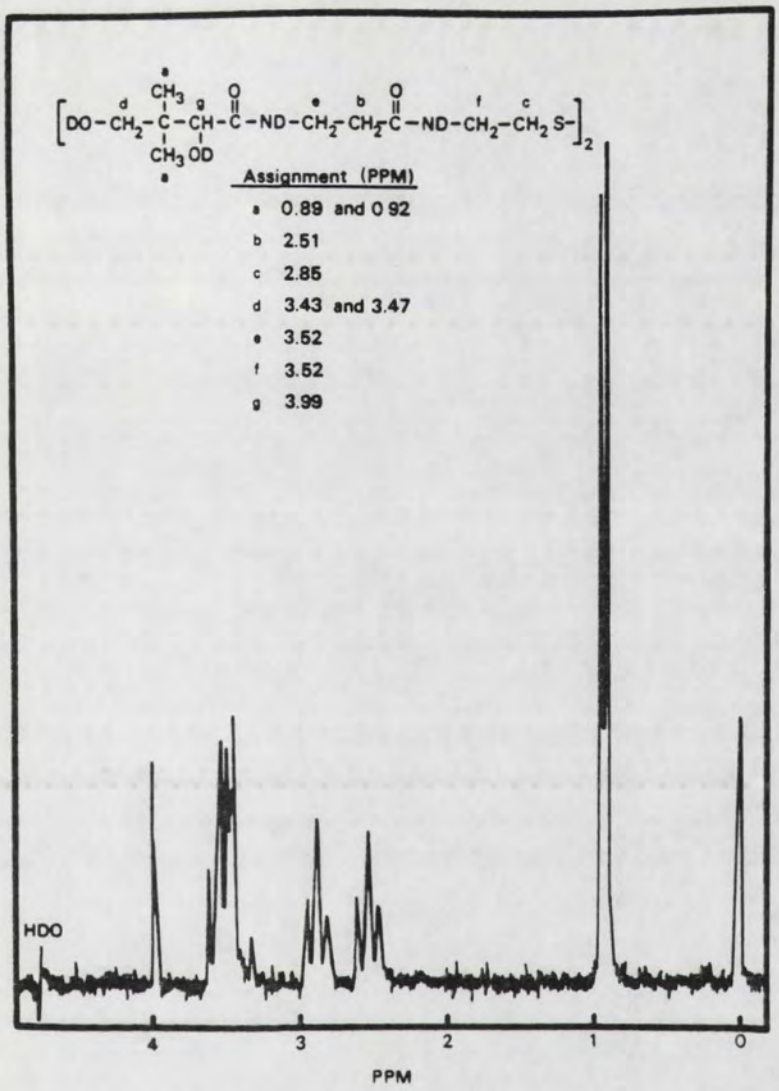

Figure 16. The proton magnetic resonance spectra of pantethine. A JEOL FX90Q Fourier transform NMR spectrometer with proton resonance at 89.60 MHz was used in the mononuclear gated decoupling mode to eliminate HDO resonance. The spectra was taken at ambient probe temperature (ca. 25°C) in a 5 mm diameter tube containing a 100 mM solution of pantethine in D_2O with 3-(trimethylsilyl)-tetradeuterosodium propionate as internal standard.

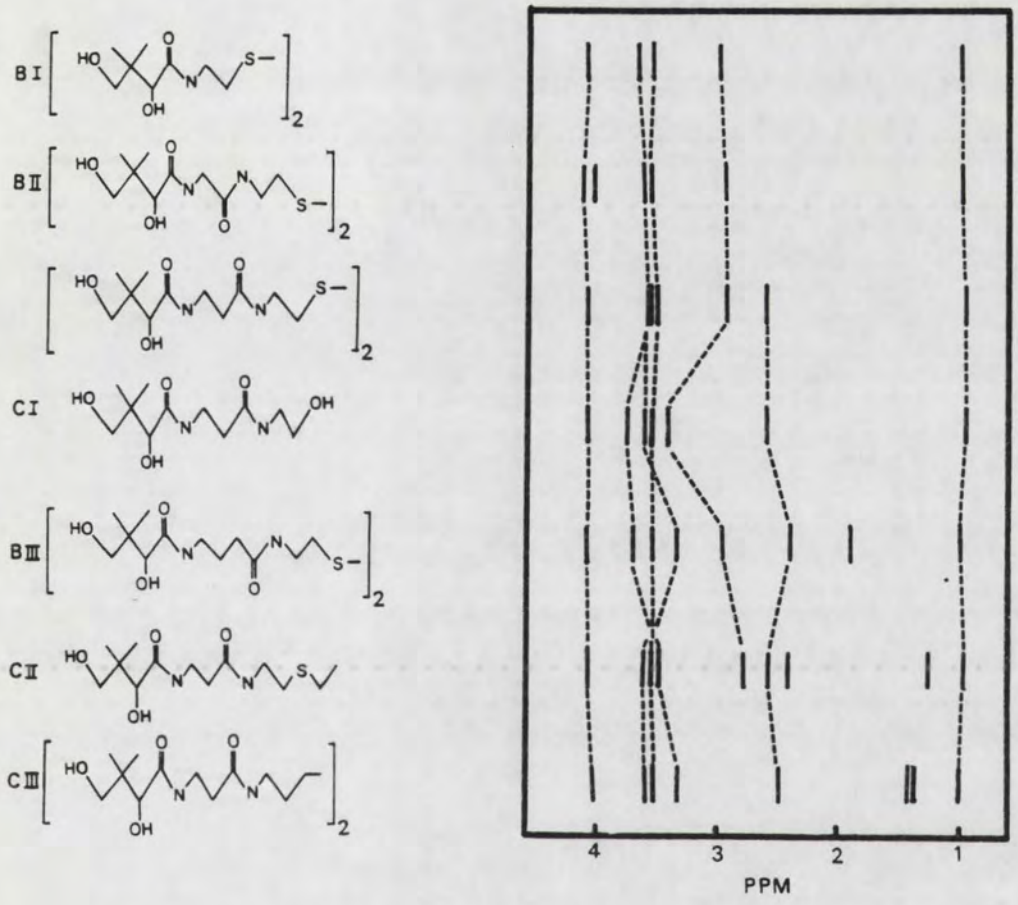

Figure 17. Stick pmr spectra of pantethine and some pantethine analogs. D_2O was used as solvent with a proton resonance at 89.60 MHz and 3-(trimethylsilyl)-tetradeuterosodium propionate as internal standard. Chemical shifts of corresponding protons between analogs (dotted lines) correlate with structural changes made in the analogs.

Table 8. Substrate Specificity.

Substrate	Percent Activity: Chicken liver homogenate[a]	Percent Activity: Pig kidney enzyme step 7
D-Pantetheine	(100)	(100)
Analog[b]		
AI (N-acetylcysteamine)		<5
AII (β-alethine diHCl)		<2
AIII (CoA)	36	<0.5
BI (N,N'-bis[pantoyl] cystamine)		<2
BII (N,N'-bis[pantoylglycyl] cystamine)		<2
BIII (N,N'-bis[pantoyl-γ-aminobutyryl] cystamine)		<1 .
CI (pantothenyl-β-aminoethanol)		75
CII (S-ethyl pantetheine)		80
CIII (N,N'-bis[pantothenyl]-1,6-diaminohexane)		96

[a]Chicken liver (Pel Freeze, Rogers, Ark.) was homogenized with 4 volumes of 0.04 M Tris-HCl, pH 8.2.

[b]Disulfide analogs are routinely reduced during assay prior to exposure to enzyme.

Modification of the cysteamine portion is much less important; analogs in the C series were all hydrolyzed at greater than 50% the rate of D-pantetheine.

Polyacrylamide Gel Electrophoresis

Protein staining of the last 3 enzyme purification steps following nondenaturing electrophoresis is shown in Fig. 18. Enzyme is electrophoresed to a relatively wide band at R_f = 0.3-0.4. Protein, carbohydrate, and activity stains of the purified enzyme after nondenaturing electrophoresis are shown in Fig. 19. Staining occurs at the same R_f by each technique, although the activity stain appears over a wider range. Protein and carbohydrate stains of the purified enzyme after sodium dodecylsulfate electrophoresis are shown in Fig. 20. Protein and carbohydrate co-electrophorese under denaturing conditions.

Amino Acid and Carbohydrate Composition

Typical elution profiles of amino acid and hexose analysis are shown in Fig. 21 and Fig. 22, respectively. The variation in apparent amino acid content with hydrolysis time is shown in Fig. 23. Extrapolation of values in Fig. 23 as indicated in Chapter III, along with average values for the other amino acids, gives a protein absorbancy index for the enzyme, $E^{1\%}_{1cm}$ (A_{280}), of 11.3. The apparent amino acid and carbohydrate composition per mole of enzyme is given in Table 9. Using the average of the two galactosamine estimations, the apparent carbohydrate content of the enzyme is 11.8% by weight, and its turnover number is 12/sec in terms of D-pantetheine.

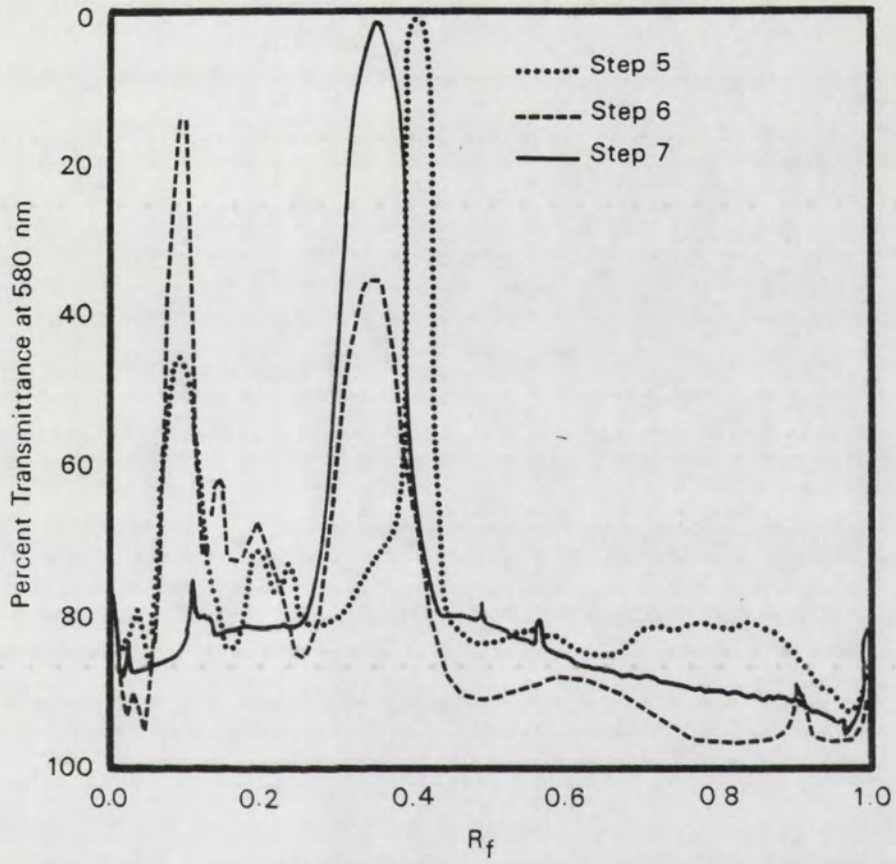

Figure 18. Nondenaturing electrophoresis of the last three steps of enzyme purification stained for protein. Six mm tube gels 10% in acrylamide were electrophoresed after Brewer and Ashworth (1969), stained with coomassie blue R-250, and scanned as detailed on page 43.

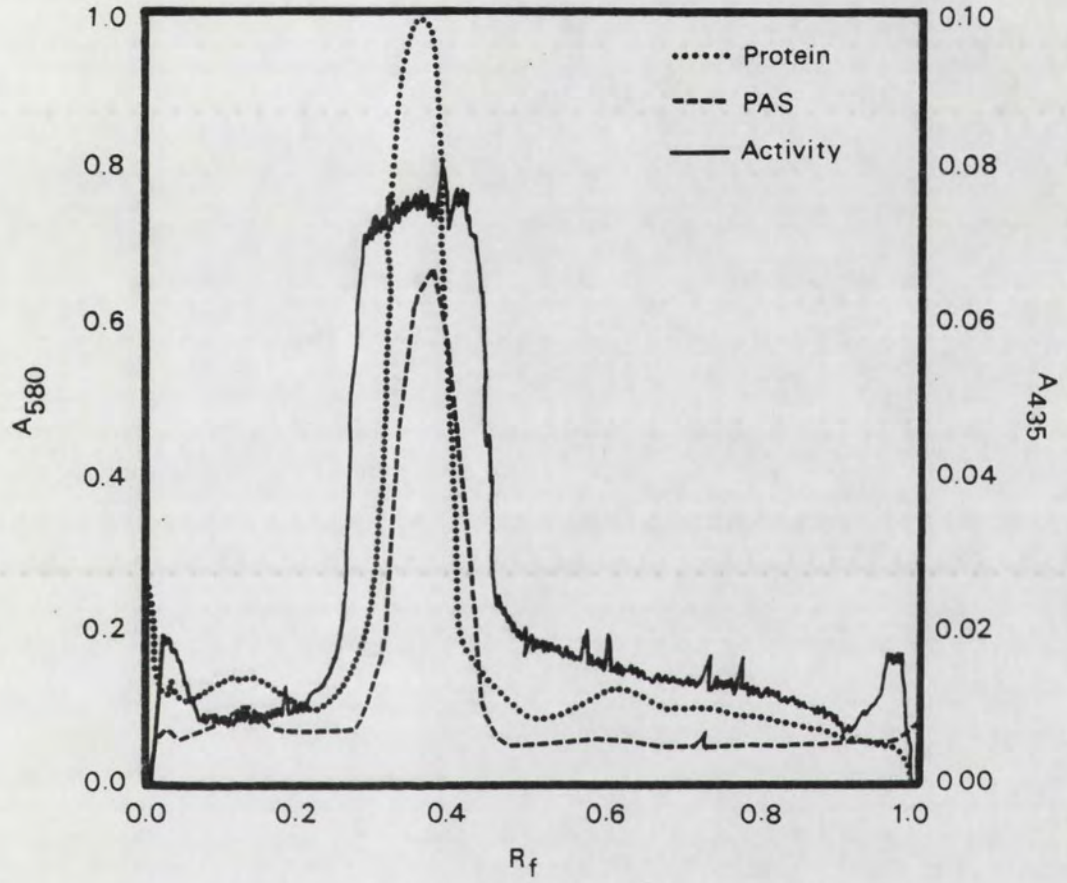

Figure 19. Nondenaturing electrophoresis of the purified enzyme stained for protein, carbohydrate (PAS), and enzyme activity. Three mm tube gels 10% in acrylamide were electrophoresed after Brewer and Ashworth (1969) and stained and scanned as detailed on page 43.

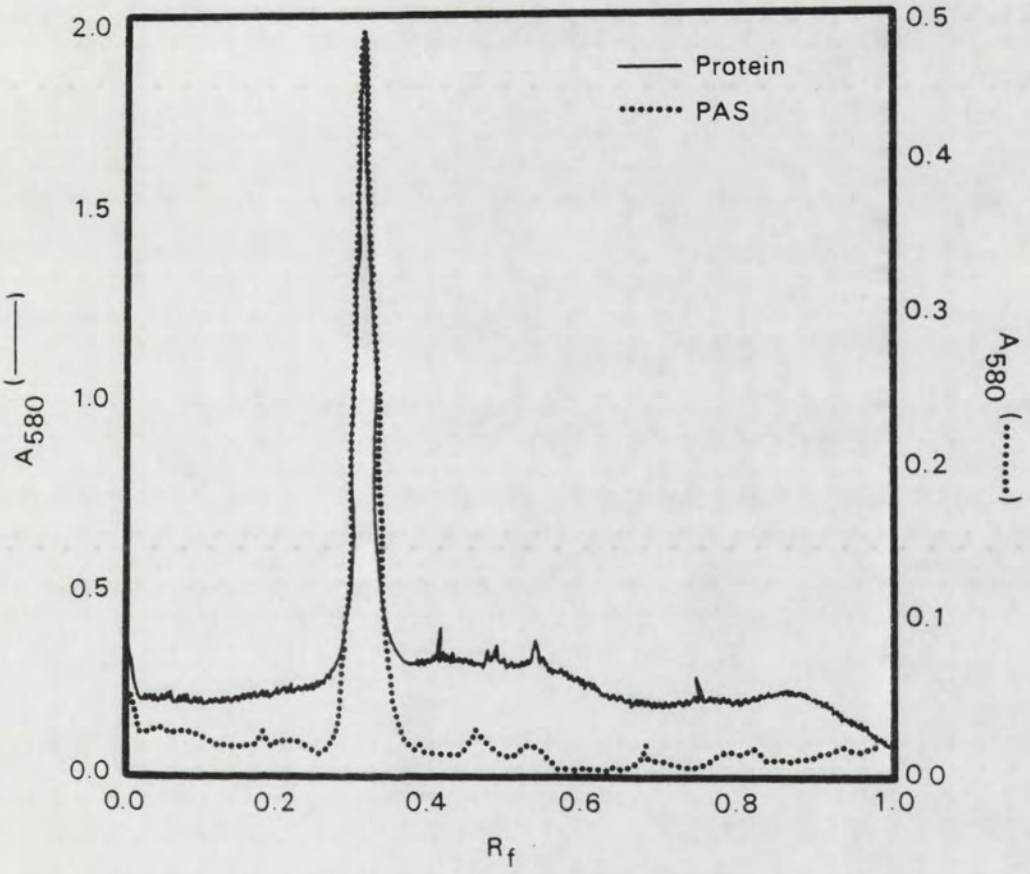

Figure 20. Sodium dodecylsulfate electrophoresis of the purified enzyme stained for protein and carbohydrate (PAS). Six mm tube gels 10% in acrylamide were electrophoresed after Laemmli (1970), stained by methods outlined by Phillips (1981), and scanned as detailed on page 43.

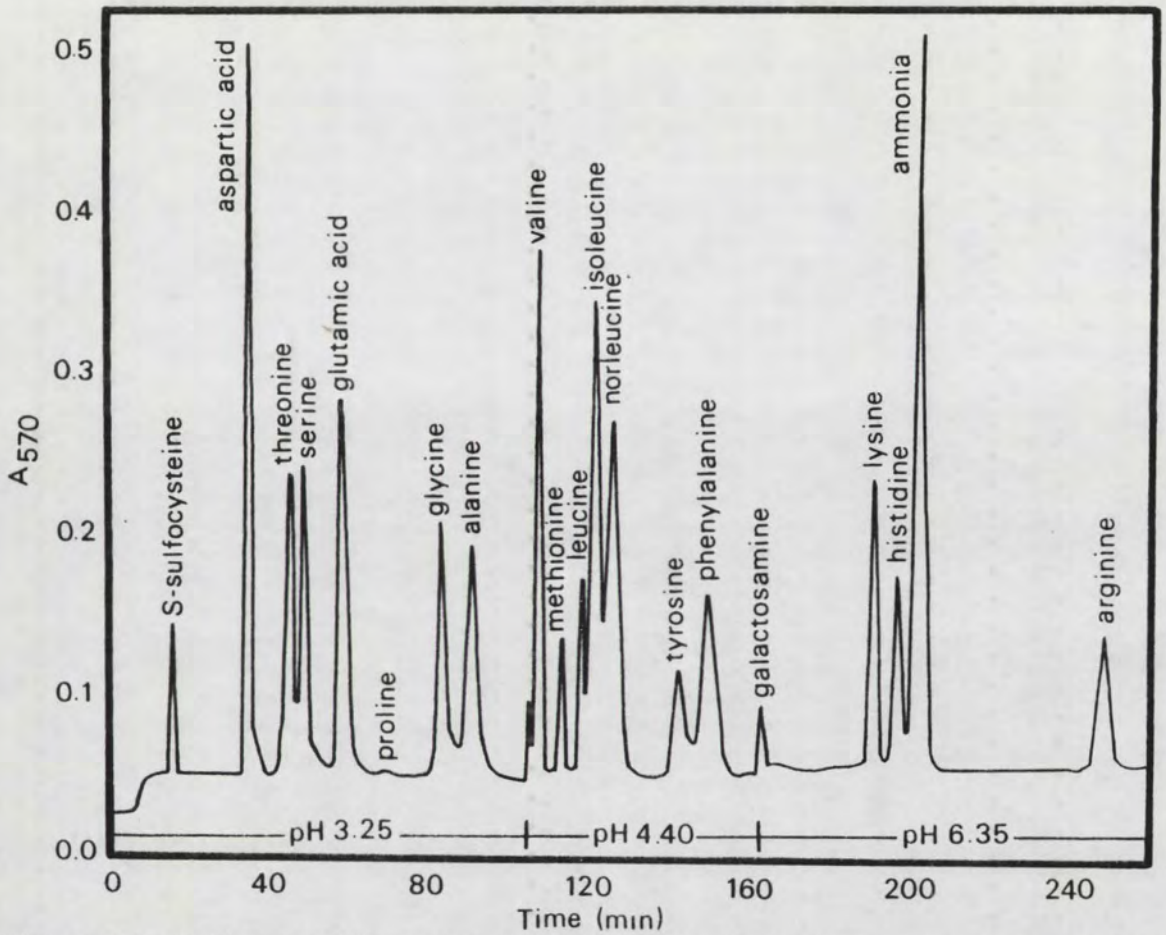

Figure 21. Amino acid elution profile after 22 hours of hydrolysis. Enzyme was hydrolyzed with 4 N methanesulfonic acid after Simpson et al. (1976) and amino acids separated by a 60 cm column of Durrum DC-1A resin with norleucine as internal standard. For details see page 44.

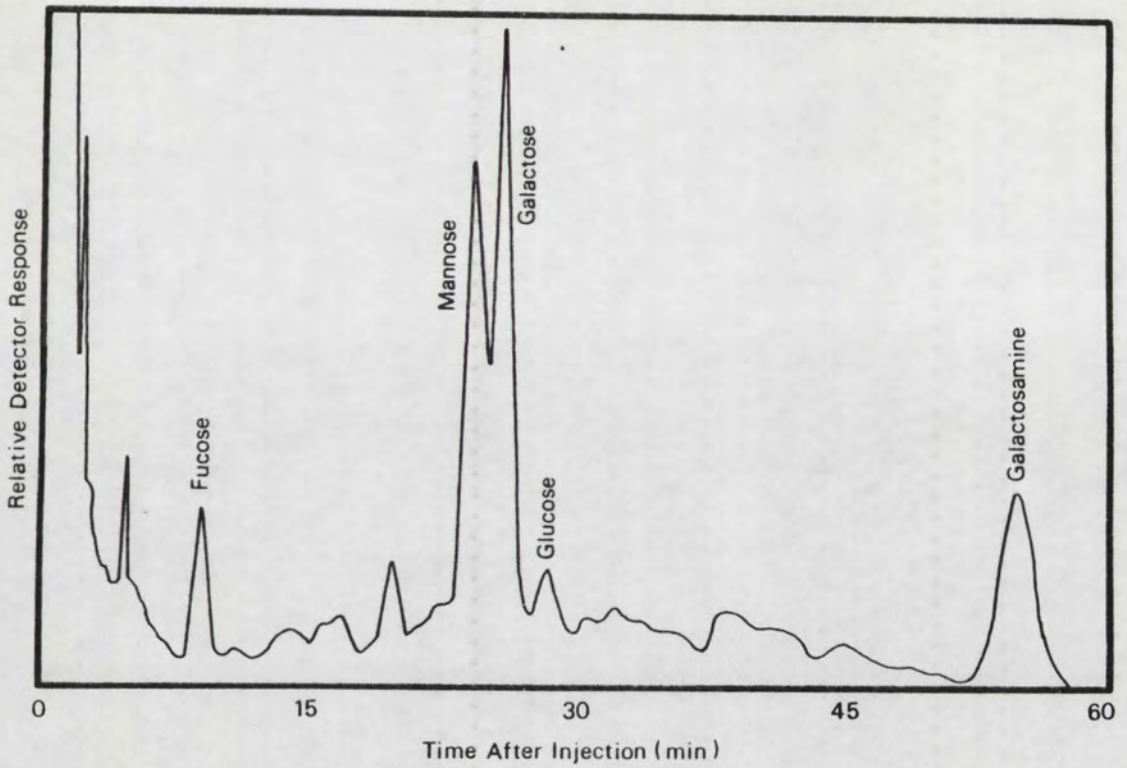

Figure 22. Gas chromatography of hexose alditol acetates following glycoprotein hydrolysis and hexose reduction and acetylation. See page 44 for details of preparation and analysis.

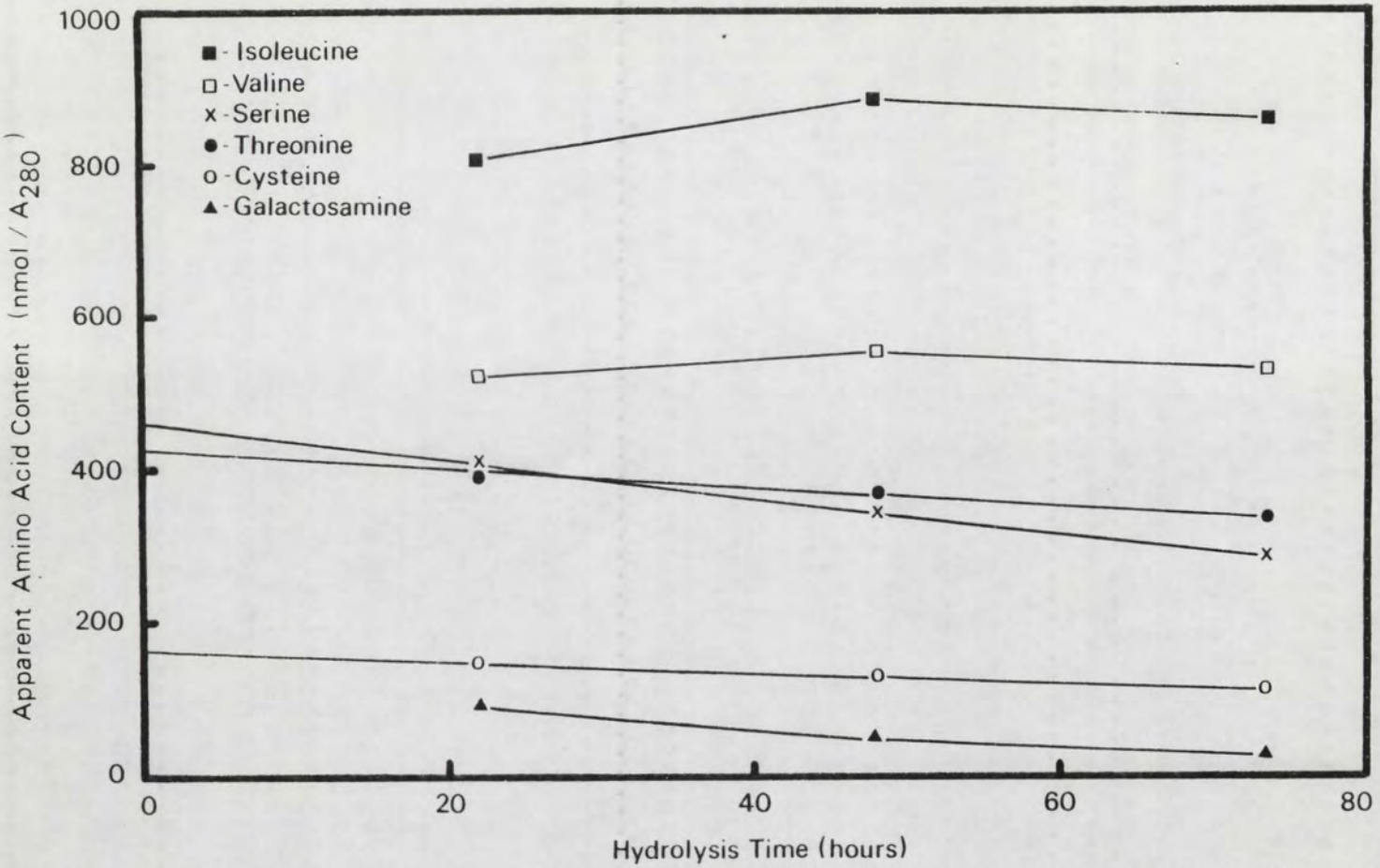

Figure 23. Variation in apparent amino acid content with hydrolysis time. Enzyme was hydrolyzed for varying periods of time with 4 N methanesulfonic acid after Simpson et al. (1976) and amino acids separated on a 60 cm column of Durrum DC-1A resin with norleucine as internal standard. See page 44 for details.

Table 9. Amino Acid and Carbohydrate Composition.

Residue	Apparent moles per mole (57,000 g) of glycoprotein		
	Amino Acid Analysis	GC[a]	Other
Half-cys[b]	9.8		
Asx	42.9		
Thr	24.4		
Ser	26.0		
Glx	37.8		
Pro	24.9		
Gly	26.5		
Ala	28.3		
Val	31.0		
Met	10.4		
Leu	18.7		
Ile	49.9		
Tyr	17.3		
Phe	32.8		
Lys	17.7		
His	16.5		
Arg	19.7		
Trp			7.6
Neutral Hexoses			25.3
Fucose		3.5	3.3
Mannose		9.2	
Galactose		9.5	
Glucose		3.0	
Galactosamine[c]	5.8[d]	6.8	
Sialic Acid[e]			4.5

[a]Calculated by equating the total area of the fucose, mannose, galactose, and glucose peaks with the neutral hexose determination and assuming proportionality between peak areas and the mass of carbohydrate present.

[b]Determined as S-sulfocysteine.

[c]Calculated as N-acetylgalactosamine.

[d]Determined by linear extrapolation of values at 22 and 48 hours of hydrolysis to zero time.

[e]Calculated as N-acetylneuraminic acid.

Comparison of Amidase Preparations

The properties of various amidase preparations are shown in Table 10. Resin treatment successfully removed 80-90% of the pantothenate in pigeon and chicken liver acetone powder. However, hog kidney acetone powder could not be prepared without gross loss of enzyme activity. The $(NH_4)_2SO_4$ enzyme solution of hog kidney has negligible pantothenate content, a specific activity of over 100 times other preparations, and greater than 80% of its activity remains after 8 hours at 37°C.

Table 10. Properties of various Amidase Preparations.

Source	Activity[a] (units/ml)	Pantothenate[b] (ng/unit)	Sp. Act.[c] (units/mg)	Stability[d] (%)
Pigeon Liver				
Acetone powder[e]	28	410	1.7	-
Resin treated	4.8	81	0.80	56
Chicken Liver				
Homogenate[f]	48	240	1.5	75
Acetone powder[g]	63	180	1.9	-
Resin treated	23	19	1.8	57
Hog Kidney				
Homogenate[f]	124	54	4.1	72
Acetone powder[g]	17	470	0.5	83
$(NH_4)_2SO_4$ enzyme solution[4]	600	<0.1	460	82

[a]Enzyme activity was measured by the pantothenate assay. A unit is defined as the amount of enzyme that will liberate 1 nmole of pantothenate per minute.

[b]"Total" pantothenate was liberated by adding an excess of calf intestinal phosphatase (Sigma, Type VII). Pantothenate was measured by radioimmunoassay as in the pantothenate enzyme assay.

[c]Protein was measured by the method of Lowry et al. (1951).

[d]Given as the percent activity remaining after incubation at 37°C for 8 hours.

[e]Purchased commercially (Sigma).

[f]Homogenization was with 4 volumes of 0.02 M Tris, pH 8.2.

[g]Acetone powders of chicken liver (Pel Freeze, Rogers, Ark.) and fresh hog kidney were prepared by the method of Kaplan and Lipmann (1948) and resin treated after Novelli and Schmetz (1951). Commerical chicken liver acetone powder (Sigma) had 43% of the listed activity. Commerical porcine kidney acetone powder (Sigma, Type II) had less than 20% of the listed activity.

CHAPTER V

DISCUSSION

The objectives of this study include development of rapid and reliable assays, routine purification to homogeneity, and characterization of a pantetheine hydrolyzing enzyme. Four rapid, independent assays of pantetheine hydrolysis have been described and compared along with a method for localizing enzymatic activity on polyacrylamide gels. A microsomal enzyme from pig kidney cortex was purified 5600-fold in 22% yield to apparent homogeneity on SDS electrophoresis. Carbohydrate co-electrophoresed with the purified protein and individual amino acid and carbohydrate residues were quantified. The K_m for D-pantetheine was 20 μM and substrate specificity was high for the pantothenate moiety, but not for the cysteamine portion. Properties of the enzyme indicate it should be ideal for liberation of pantothenate from biological tissues.

Assay

Radiolabeled Assay

The radiolabeled assay resembles that previously reported (Dupre et al. 1970a; Dupre and Cavallini, 1979). However, labeled D-pantethine is used as substrate instead of DL-pantethine, a 15-min chromatography step is used instead of 5 hours of electrophoresis to separate labeled substrate and product, and enzyme and substrate are prereduced before assay. Enzyme prereduction is necessary for linear

initial velocities as shown by Fig. 6 and the product vs time curve of Dupre et al. (Dupre et al. 1970a). Preliminary experiments on enzyme prereduction using the radiolabeled assay indicated an optimum concentration of dithioerythritol between ca. 7 and 70 mM when reduction occurred for 20 min at 37°C. Figure 6 shows reduction is still incomplete after 20 min at 37°C with 1 mM dithioerythritol. A concentration of 10 mM dithioerythritol was subsequently used for all enzyme preincubation.

The chromatographic separation in the radiolabeled assay is unusual because of its nonequilibrium nature. Solvent continually evaporates from the solvent front and migrating components are concentrated to a sharp band at the front. Application to other bipartite separations is possible by modifying the solvent system. For example, aletheine can be separated from pantothenic acid and pantetheine by using acetone/0.1 N HCl (90/10). With [^{14}C] pantethine labeled on the β-alanine portion, this separation can be used after exhaustive enzyme hydrolysis to verify which amide bond in pantetheine is hydrolyzed. It is also apparent from Fig. 4 and the specific nature of the pantothenate and cysteamine assays that hydrolysis occurs between pantothenate and cysteamine, and not between pantoate and aletheine. .This specificity has already been demonstrated for the enzyme from horse kidney (Dupre et al. 1970b).

Pantothenate Assay

The pantothenate assay is based on a radioimmunoassay specific for pantothenate (Wyse et al. 1979). Cross-reaction of the antiserum with pantetheine occurs at concentrations of pantetheine ca. 500 times

greater than that of pantothenate. [^{14}C] Pantothenate was used instead of the originally published ^{3}H label because the tritiated form required frequent chromatographic purification and its higher specific activity improved the assay sensitivity only slightly. Antibody-bound and free hapten were simply and rapidly separated by nitrocellulose filters, a method introduced by Gershman et al. (1972). The technique used here is unique because both antibody-bound and free hapten are analyzed. It was necessary to have the solution and wash ice cold for filtration to prevent antigen-antibody dissociation. This may be due to the relatively low association constant of the antisera (ca. 10^{7} M^{-1}). Although the most time-consuming assay presented, 16 samples can be processed in less than 2 hours (excluding counting time). Enzyme blanks must be subtracted if the enzyme contains significant pantothenate.

Cysteamine Assay

The cysteamine assay is based on the observation by Avi-Dor and Mager (1956) that thiols and aminoethanethiols react differently with fluoropyruvate to form products with characteristic uv spectra. Alkylthiols without a free β-amino group form alkylthiopyruvates (Bergmann and Mielewitz, 1963), which absorb maximally around 270 nm (ε = 540 for glutathione). Aminoethanethiols give thiazines with a peak absorption at about 300 nm (ε = 5200 for cysteine). These findings were later applied to quantitation of aminoethanethiols in biologic media (Herrington et al. 1967). This method has been modified for maximal reaction in 10 min by increasing the concentration of fluoropyruvate and performing the reaction at 37°C

instead of at ambient temperature. Enzyme blanks must be subtracted if the enzyme contains significant fluoropyruvate reactive material.

Mercaptide Assay

The mercaptide assay is based on the uv absorption of the mercaptide ion and the different pK values (-SH) of pantetheine and cysteamine. The mercaptide ion has a peak absorption around 235 nm of ca. 5000 M^{-1} cm^{-1} (Benesch and Benesch, 1955). Using the spectrophotometric pK determination of Benesch and Benesch (1955) the pK (-SH) of pantetheine was 9.9, and that of cysteamine 8.1, at 37°C. The cysteamine value is similar to previous reports (Danehy and Noel, 1960). A comprehensive literature search failed to reveal a previous determination for the pK of pantetheine.

The mercaptide ion was monitored at 240 nm because of rapidly increasing end absorption of protein below this value. At 240 nm the absorbance of protein (bovine serum albumin) is 1.6 times that at 280 nm. The highest sensitivity for the mercaptide assay would be obtained at a pH of 9.0, halfway between the pK values (-SH) of pantetheine and cysteamine. However, we used a buffer pH of 8.1 as a compromise between increasing assay sensitivity and enhanced blank absorption with increasing pH. At pH 8.1, over 60% of the maximal sensitivity is obtained, with less than 25% of the blank absorption found at pH 9.0. A lower pH also reduces the rate of thiol oxidation. Dithioerythritol was used instead of dithiothreitol because of its higher pK values (Zahler and Cleland, 1968) and, therefore, lower contribution to blank absorption.

Reduction Requirements

The presence of dithioerythritol in the cysteamine and mercaptide assays is critical. Concentrations were chosen to minimize blank absorption, yet insure complete reduction of cysteamine. In the mercaptide assay, the slow oxidation of dithioerythritol is measured by a downward drift in the A_{240} (less than 0.001/min with adequate deoxygenation) before addition of substrate. On substrate exhaustion, the tracing returns to the same slow downward drift. In the cysteamine assay, the extent of dithioerythritol oxidation is unimportant, as oxidized dithioerythritol absorbs at 300 nm to the same extent as the fluoropyruvate adduct of reduced dithioerythritol (Herrington et al. 1967). The success of these two assays depends on the ability of dithioerythritol to maintain cysteamine in the reduced state (Cleland, 1964). Mercaptoethanol and $NaBH_4$ were not as effective in keeping cysteamine reduced.

Comparison Between Assays

The radiolabeled, pantothenate, and cysteamine assays can be used to monitor enzyme activity at any stage of purification. However, enzyme blanks must be subtracted from the pantothenate and cysteamine assays during initial fractionation when these substances contaminate the enzyme. Comparison between assays for the beginning and ending stages of purification are given in Table 6. The pantothenate and cysteamine assays are particularly useful in the study of substrate specificity. The mercaptide assay is most convenient and precise, but its use is limited to steps after enzyme solubilization. Particulate fractions cause instability in the absorbance reading and high protein

content results in high blank absorption. Assay sensitivity can be inferred from the data in Table 5. The sensitivity of the radiolabeled assay can be varied by using labeled pantethine of different specific radioactivity.

Purification

The purification from pig kidney uses predominantly batch operations with only two chromatographic steps requiring simple step elution. The $(NH_4)_2SO_4$ enzyme solution (Step 5) can be produced in less than 6 hours. The remaining steps take an additional 12 hours. Microsomal pH agglutination was chosen over high speed centrifugation because larger quantities could be produced in the same time with less costly equipment. Butanol treatment (Morton, 1950) effected solubilization, whereas detergent treated microsomes still spun down activity at 100,000 g.

There are only a few critical steps in the purification. The pH of the suspension before butanol treatment is important (Figure 8). Recovered activity drops precipitously below pH 5.75. The temperature of the hydrophobic chromatography column is also critical and must be kept at 0-4°C. At 5-10°C most of the activity will pass through the column during initial washing. Minor variation in other steps makes little difference.

Dupre and Cavalinni (1979) report a 16,000-fold purification to homogeneity of a pantetheine hydrolyzing enzyme from horse kidney. The purification reported here from pig kidney is 5600-fold. Comparisons of specific activities are shown in Table 11. Protein and

Table 11. Specific Activity Comparisons of Horse and Pig Kidney Purifications.

Source	Specific Activity (nmol pantothenate/min/mg)	
	Homogenate	Purified Enzyme
Horse kidney (Dupre and Cavallini, 1979)	0.025	400
Pig Kidney (Table 7)	2.5	14,000

activity assays vary in Table 11, but correction requires less than a factor of 2 and can be calculated from Table 6. The marked apparent difference between horse and pig kidney is suprising. The horse kidney homogenate specific activity may be underestimated as implied in Chapter II (Table 3). A 35-fold difference in the purified enzyme's specific activity is harder to explain.

Characterization

Characteristics of the enzyme purified from pig kidney are compared to previously studied preparations in Table 12. The molecular weight and heat stability of the enzyme are similar to that from horse kidney (Dupre and Cavallini, 1979). However, the pH profile and K_m are more similar to values from rat kidney (Abiko, 1975). All three preparations show similar substrate specificity, but there is a wide variation in maximum specific activities and absorbancy indexes. The presence of carbohydrate has not previously been reported.

Table 12. Comparative Summary of Purified Amidase Preparations.

Parameters[a]	Source: Horse Kidney (Dupre and Cavallini, 1979)	Source: Rat Kidney (Abiko, 1975)	Source: Pig Kidney[a]
Subcellular Localization	-	Microsomal-Lysosomal	Microsomal
K_m (mM D-pantetheine)	5.0	0.04	0.02
pH Optimum	4.0-5.5	7.0	9.0-9.5
Molecular Weight	55,000 (Ultracent.)	-	54,000 (Gel Filt.) 60,000 (SDS Gel Elect.)
Maximum Specific Activity (nmol pantothenate/min/mg protein)	400	19	14,000
Carbohydrate Content (%)	-	-	11.8
$E^{1\%}_{1cm}$(280 nm)	15.2	-	11.3

[a]See Chapter III for methods of determination and purification.

Subcellular Localization

Subcellular fractionation shows the enzyme apparently distributing to both the lysosomal and microsomal fractions (Fig. 12) as concluded by Abiko (1975). However, the microsomal marker, glucose-6-phosphatase, also distributes in the same manner. The fractionation scheme used was originally developed for liver tissue (DeDuve et al. 1955). When applied to kidney tissue, microsomal proteins distribute in the pattern shown in Fig. 12 (Ali and Lack, 1965). Therefore, the enzyme from pig kidney is mainly, if not exclusively, microsomal.

Michaelis Constant, pH Profile, and Molecular Weight Determination

The reported K_m for the horse kidney enzyme is over 100 times other preparations (Table 12). DL-Pantetheine instead of D-pantetheine was used in kinetic studies of the horse kidney enzyme. However, Fig. 13 shows this should not change the K_m determination. Although the presence of the L-isomer inhibits mildly at high concentrations, similar kinetic intercepts are obtained by extrapolation.

The smooth curve of the pH profile by the cysteamine assay is shown in Fig. 14. The pH profile of Dupre et al. (1970b) does not cover the alkaline range. The profile of Dupre et al. (1976) is, a) inaccurate in the neutral range because of the nature of the pH-stat assay, and b) depressed in the alkaline range because of incorrect pK assignments of the functional groups in cysteamine (Benesch and Benesch, 1955).

All molecular weight determinations given in Table 12 are within close limits. However, both the gel filtration (Andrews, 1965) and the SDS gel electrophoresis (Segrest and Jackson, 1972) estimates may be overestimated because of the presence of carbohydrate. Caution in interpretation should await more definitive studies.

Substrate Specificity

Chicken liver preparations prefer pantetheine to CoA (Table 8). This contradicts the impression of Novelli et al. (1954) that avian liver enzymes prefer CoA. The specificity may be even greater than shown in Table 8 as phosphatases endogenous to the homogenate may be converting CoA to pantetheine.

All kidney preparations have shown high specificity (Chapter II and Table 8). However, Table 8 reveals that minor variations in the cysteamine moiety can be made with retention of enzymatic activity. Extended pantetheine molecules, such as S-acyl pantetheine derivatives may be active or even better substrates for the enzyme.

The diaminohexane derivative (Analog CIII) is a very close analog of pantethine. A hydrocarbon chain replaces the disulfide bridge. The ready hydolysis of the molecule (Table 8) suggests that pantethine, as well as pantetheine, is a substrate for the enzyme.

Polyacrylamide Gel Electrophoresis

Co-elution of protein, activity, and carbohydrate, and purity of the glycoprotein are shown under denaturing and nondenaturing conditions in Figs. 18-20. At Step 5 in Fig. 18, only one major protein band is present at an R_f slightly greater than the purified

glycoprotein. It would be easy to think purification nearly complete at this stage. The specific activity of Step 5 is 350 nmol/min/ml, similar to the maximum specific activity of 400 nmol/min/ml of Dupre and Cavallini (1979).

Localization of activity on polyacrylamide gels. Activity electrophoresed on polyacrylamide gels can be visualized by titration of an indicator because the enzymatic reaction produces acid at pH's intermediate to the pK's (-SH) of pantetheine and cysteamine. The Tris gel buffer is first diluted by dialysis against H_2O. Pantetheine and cresol red are added and the solution titrated with NaOH just to the basic side of the indicator. Cresol red and substrate diffuse into the gel and acid produced by the hydrolysis of pantetheine locally titrates the indicator. Resolution is limited by diffusion.

Amino Acid and Carbohydrate Analysis

Carbohydrate analysis of the glycoprotein is consistent with oligosaccharide chains linked O-glycosidically through N-acetyl-galactosamine to the hydroxy group of serine or threonine (Kornfeld and Kornfeld, 1976). Isoleucine and phenylalanine occur in amounts exceeding twice that found in the average protein (Dayhoff, 1978). Increased amounts of these hydrophobic amino acids is suggestive of an integral membrane protein.

The specific values for amino acid and carbohydrate composition in Table 9 should be interpreted cautiously. The whole table depends on the accuracy of the molecular weight determination which is subject to overestimation as previously mentioned. Values for specific sugar

residues additionally depend on several assumptions listed in the footnotes of Table 9. The different absorbancy indexes listed in Table 12, 15.2 and 11.3, were both determined by amino acid analysis and suggest a fundamental difference in the amino acid content of the two preparations.

Use of Various Amidase Preparations in Pantothenate Liberation

The $(NH_4)_2SO_4$ enzyme solution has several properties which make it ideal for use in "total" tissue pantothenate determination (Table 10). It has over 100 times the specific activity and less than 1/100 the pantothenate content of other preparations. It is easily prepared and highly stable. On the other hand, the stability of the avian liver preparations is suprising in view of previous reports of their lability (Chapter I). Avian liver preparations should be adequate for most applications when sensitivity does not require low enzyme pantothenate.

Future Studies

This dissertation provides the methodology necessary to approach the concerns of Chapter I. The enzyme assays should be useful in evaluating the significance of pantetheine hydrolysis in the metabolism of coenzyme A. For example, enzyme activity during the elevated CoA levels of diabetes, fasting, and clofibrate administration could be evaluated. The purified enzyme also provides a well defined "reagent" which could be used in developing schemes of pantothenate liberation from various biologic sources.

Consideration of the specific function of this enzyme in kidney is only speculative. However, delineation of its specificity for S-acyl pantetheine derivatives is an intriguing area for further research.

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