AN ABSTRACT OF THE THESIS OF

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Date Thesis presented May 11, 1949

Title A New Conjugate of Pantothenic Acid

Abstract Approved [redacted] (Major Professor)

Pantothenic acid was discovered 18 years ago, but its function in metabolism is still not clear. However, it seems certain that the functional form of pantothenic acid in organisms is not the free vitamin. Glutamic acid was found in this laboratory to enhance the growth promoting property of pantothenic acid or β-alanine in yeasts. Similar observations were reported from other laboratories.

Therefore it appeared possible that glutamic acid might conjugate with pantothenic acid or β-alanine to produce a substance which, in turn, possesses an important metabolic function. This postulation was partially verified. Glutamic acid was incubated with β-alanine or pantothenic acid in the presence of resting yeast cells. The resulting mixture showed extremely great activity as a growth factor in a pantothenic acid deficient medium of yeast. Unfortunately, the amount of activity so obtained was very variable. It was therefore decided to seek new sources from which the supposed conjugate might be isolated.

As a result of a series of investigations during the past two years, a pantothenic acid conjugate (PAC) was

isolated in relatively pure form from natural materials. By combination of various concentration technique, the best samples with A. suboxydans activity over 10% pantothenic acid were obtained. They contained no free pantothenic acid. After digestion with phosphodiesterase, the pantothenic acid in the conjugate was made avialable to lactic acid bacteria. The best samples also contained 20% glutamic acid. Its probable content of purine(s) was indicated microbiologically and spectrophotometrically.

PAC was more active and showed far faster growth rate than pantothenic acid per se. The ability to reverse the pantothenic acid inhibitors, α-hydroxy-β,β-dimethylbutyryl taurine (in A. suboxydans), and 2-chloro-4-aminobenzoic acid, salicylate or propionic acid (in E. coli), was greater than that of pantothenic acid. In view of these facts, PAC was considered to be involved more directly in metabolism than the free vitamin. The molecule appeared to be fairly large as indicated by its failure to dialyze through cellophane into running distilled water. However, it was not precipitated by the tungstic acid reagent. PAC was a yellow amorphous powder, extremely soluble in water, but insoluble in organic solvents. It was readily adsorbed on charcoal, Lloyd's reagent or superfiltrol and easily eluted from the latter two adsorbents. In dilute solution, PAC gave maximum absorption of light at 260 mμ.

PAC was found to be different from coenzyme A since the former did not acetylate sulfanilamide or choline and

was non-dialyzable. It was not identical with the bound form of pantothenic acid in blood which was unstable at high temperatures and precipitable by the tungstic acid reagent. The alkali stable form of pantothenic acid was considered to be a degradation product of PAC since a small residue of pantothenic acid activity remained after treating PAC with alkali.

In this thesis, the details of concentration of this new pantothenic acid conjugate, its chemical, physical and biological properties and its probable importance in metabolism have been presented.

A NEW CONJUGATE OF PANTOTHENIC ACID

by

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A THESIS

submitted to

OREGON STATE COLLEGE

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

May 1949

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EW 9/6/49

ACKNOWLEDGEMENT

The author wishes to express his sincere appreciation to Professor Vernon H. Cheldelin for his encouragement, advice and direction.

Acknowledgement is made to the National Institute of Health and the Nutrition Foundation who have generously supported the work.

He also wishes to thank Ruth Langdon, Eleanor Parsons, Leanor Lindsay and Marjorie McCanse for their technical assistance with the microbiological assays.

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A NEW CONJUGATE OF PANTOTHENIC ACID

Chapter I.

Introduction

Since the discovery of pantothenic acid eighteen years ago, numerous experiments have been performed to ascertain its function in metabolism. So far, no recognizable conclusion has been reached. However, it seems certain that the functional form of pantothenic acid in living organisms is not the free vitamin.

In addition to R. J. Williams' early work which indicated that pantothenic acid exists in bound form, Wright (34) noted that the vitamin was combined to protein in the blood. The work of Neal and Strong (26) indicated a residual activity of pantothenic acid remaining in liver after alkaline treatment. Unfortunately, apparently neither Wright nor Neal and Strong have continued their studies to characterize these conjugates.

In 1945, using pantoyl taurine, an inhibitory structural analog of pantothenic acid, McIlwain (23) was able to demonstrate that bacterial cells build pantothenic acid into a more complex substance which

presumably then functions in metabolism. However, he did not extend his work to study this complex of pantothenic acid.

Recently Limpann and his group (19, 20) found that coenzyme A, which is involved in the acetylation of aromatic amines in liver preparations and in the acetylation system for choline in brain, was a pantothenic acid derivative. But detailed reports of the metabolic role of the coenzyme, as well as the isolation procedure, are lacking. Whether its function in acetylation could confirm the earlier observation that pantothenic acid permits pyruvate oxidation to proceed to completion (6, 9), as Lipmann has claimed (20), remains to be investigated by direct experiment. The acetylation of aromatic amines is probably not a normal metabolic process. The presence of acetyl choline has not been demonstrable in plants and in most microorganisms. On the other hand, pantothenic acid does occur universally in all animals, plants and microorganism.

Glutamic acid was found in this laboratory to enhance the growth promoting property of pantothenic acid or β-alanine in certain yeasts (13). A related

observation was made by Woolley (33) who found that glutamic acid was very active in reversing the inhibitory effect of ketone analogs of pantothenic acid in organisms which require the preformed vitamin.

From these observations it appeared possible that glutamic acid might conjugate with pantothenic acid or β-alanine (and possibly with pantoic acid in the proper linkage) to produce a substance which, in turn, possesses an important metabolic function. In an attempt to obtain evidence for this (14), glutamic acid was incubated with β-alanine or with pantothenic acid in the presence of resting yeast cells. The resulting mixture showed extremely great activity as a growth factor in a pantothenic acid deficient medium of yeast. However, the amount of activity so obtained was very variable, and it was therefore decided to seek new sources from which the supposed conjugate might be isolated. In the course of a series of investigations dealing was this subject during the past two years, eventually a conjugated form of pantothenic acid has been isolated in relatively pure form, which appears to be different from any of

the pantothenic acid conjugates previously recorded in the literature. It possesses activity for Acetobacter suboxydans greater than that of pantothenic acid per se.

In this thesis, the details of concentration of this new pantothenic acid conjugate (abbreviated PAC), its chemical, physical and biological properties, and its probable importance in metabolism will be presented.

Chapter II.

Experimental Methods

1. Microbiological Assays

Determination of PAC. -- PAC was determined through its growth-promoting activity for Acetobacter suboxydans (ATCC 621). The original method of Sarett and Cheldelin (28) for pantoic acid and pantothenic acid was modified to fit the present purpose (see first two columns, Table I). Since pure PAC was not available, a concentrate (sample # 801) containing about 0.5% (5 mg./g.) pantothenic acid was used as the reference standard in all experiments. This concentrate was employed in preference to pure pantothenic acid, since the nature of the growth response curves (Figure 6) was different for the two preparations (although uniformly reproducible) and assays of PAC made against the free vitamin as a reference were unreliable. Assay values on various PAC preparations have been given in term of pantothenic acid content for the reader's convenience, after assaying them against sample # 801 and converting to pantothenic acid equivalents.

It was found that a 40 - 48 hour growth period was preferable to the 72 hour period previously employed, since free pantothenic acid stimulated growth only slightly during the shorter time. Growth with the conjugate, on the other hand, was nearly maximum after 40 hours. This consideration is of chief importance when crude materials are being assayed; the concentration procedure used herein removes the free vitamin at an early stage. Growth of A. suboxydans was measured with the Pfaltz and Bauer fluorophotometer. Turbidities are recorded as optical density (2 - log per cent transmission).

Rubin and coworkers (28) have modified the medium by the addition of Wilson's 1:20 liver extract. The composition of this modified medium is shown in Table I.

Other microbiological assays. -- The microorganisms used for testing β-alanine and pantothenic acid were Saccharomyces cerevisiae, strains L. M. and G. M. and Lactobacillus arabinosus 17-5. All tests were performed using previously published methods (10, 12, 13, 15). Free pantothenic acid was determined by

direct assay with L. arabinosus. Enzyme digestions were carried out with takadiastase and papain in acetate buffer (10, 15).

The growth of Escherichia coli was carried out under the conditions employed by Harding and Shive (8). Five ml. of double strength glucose-salt medium were introduced into 5 ml. of test solution. After incubation, the growth was measured turbidimetrically as above.

Table I.

Basal Media for A. suboxydans

	Sarett & Cheldelin (30)	Present Medium (King)	Rubin et al (28)
Glycerol	100 g.	100 g.	100 g.
Glucose	5	5	5
Casein*	10	10	20
Peptone**	5	5	5
Liver concentrate, treated****	2	2	2
Tryptophane	200 mg.	200 mg.	200 mg.
Cystine	150	150	150
Adenine	10	10	10
Guanine	10	10	10
Uracil	10	10	10
β-Alanine	2		2
Liver concentrate, untreated***			67
Salt solution A	10 ml.	10 ml.	10 ml.
Salt solution B	10	10	
Salt solution C			10
Nicotinic acid	200 γ	200 γ	200 γ
p-Aminobenzoic acid	200	200	200
Folic acid			20
Biotin			16
Pyridoxine			2000
Thiamine			2000
Riboflavine			2000
Distilled water to 1000 ml.			
pH 6.0			

* Vitamin-free, acid hydrolyzed.
** Norit A treated, cf. (30).
*** Wilson's 1 : 20, untreated.
**** Wilson's 1 : 20, Norit treated (see 30)

2. Acetylation

Acetylation of sulfanilamide. -- The acetylation of sulfanilamide was performed according to Lipmann (17). Coenzyme A or PAC in proper concentrations were placed into the chambers of Warburg flasks, together with sodium citrate, sodium acetate, adenosine triphosphate, sodium bicarbonate buffer, cystein and dialyzed pigeon liver. After shaking 90 - 120 minutes the protein in the flasks was precipitated with trichloroacetic acid. The sulfanilamide concentration after acetylation was determined by the method of Bratton and Marshall (1) in which the supernatant solutions were diazotized and coupled with N-(1-naphthyl)-ethylene-diamine. Color readings were determined in a Klett-Summerson photocolorimeter.

Acetylation of choline. -- The acetylation of choline in the presence of adenosine triphosphate was performed in a Warburg apparatus under an atmosphere of nitrogen with the same technique as used by Nachmansohn and coworkers (24, 25). The method for determining acetyl choline followed essentially Stephenson and Rowatt's modification (31) of Chang's method (2) with frog muscle. The standard acetyl choline solution was

prepared immediately before use. A solution of eserine was likewise prepared before use, made in buffered frog Ringer's solution and then diluted accordingly. The rectus abdominis mucle of a frog was excised and placed in a glass chamber of 30 ml. capacity with buffered Ringer's solution and aerated with a steady stream of oxygen for about one hour. The Ringer's solution was then drained off replaced by eserinized buffered Ringer (to retard the destruction of acetyl choline by the tissue) and aerated for another hour. The various samples were then tested for acetyl choline activity by introducing them into the chamber. The contraction of the muscle was recorded on a smoked kymograph. The general precautions set forth by Chang and by Stephenson and Rowatt were followed.

3. Spectrophotometry

The spectrophotometric behavior of the conjugate was studied with the Beckman DU Photoelectric Quartz Spectrophotometer. A tungsten lamp was used as the light source over the range of 320 to 1,000 mμ and a hydrogen discharge lamp was used from 220 to 350 mμ. Corex viewing cells were used for visible and longer wave lengths, and cells made of silica glass in the ultraviolet region. All cells were of one square centimeter cross section. The Corex cells were matched to within 0.4% transmission at 500 mμ and to within 1% at 320 mμ and the silica cells were matched to within 0.4% transmission at 280 mμ and to within 3% at 220 mμ.

The values for per cent of transmission were plotted against wave length in mμ as shown in Figures 3, 4, 5 and 6.

4. Paper Partition Chromatography

The method used for paper partition chromatography for PAC, and for glutamic acid in hydrolysates of PAC, was essentially that of Consden, Gordon and Martin (4). Paper partition chromatography takes advantage of differences in mobility of structurally related compounds when these are suspended in a solvent which advances along a strip of paper due to capillary action.

The essentials of the apparatus consisted of hanging a filter paper strip (Whatman No. 1), of 2.0 cm. width and about 25 cm. length, on which a drop of test solution was applied about 4 cm. from the upper end. The upper end was immersed in a trough containing 0.3% ammonia in water-saturated phenol. The strip was placed in an airtight chamber which was maintained in an atmosphere saturated with phenol and water. A pair of glass slides were placed along the top of the trough to hold the strip in place. After solvent had run a certain distance, the paper strip was removed and the position of the solvent was marked. The strip was dried and then treated with 0.1% ninhydrin in butanol to develop color.

The chamber was made by cutting the bottom off

a specimen jar (approximately 35 cm. in height) and standing it in a petri dish. Using carborundum the lower end of the jar and the petri dish were ground to form an air tight connection. The upper end of the jar was ground in the same manner. The trough was constructed from 25 mm. glass tubing. Both ends were sealed and a slit approximately four mm. wide was made along the length of the tubing with a suitable drill.

5. Vacuum Concentration

In order to overcome the difficulty of evaporation of large volumes of extract in the laboratory, a special set-up was made. The main feature of the apparatus was the efficient cooling system which consisted of a double-jacketed condenser joined in series to an ordinary coiled condenser. Foaming of solutions was prevented by equipping the condenser with a Kjedahl-like adapter bulb of one liter capacity which also carried an automatic filling device. The evaporator is shown in Figure 1. By means of this apparatus and with an ordinary water aspirator, the evaporation of aqueous solutions could be carried out at 40° C. at a rate of one liter per hour.

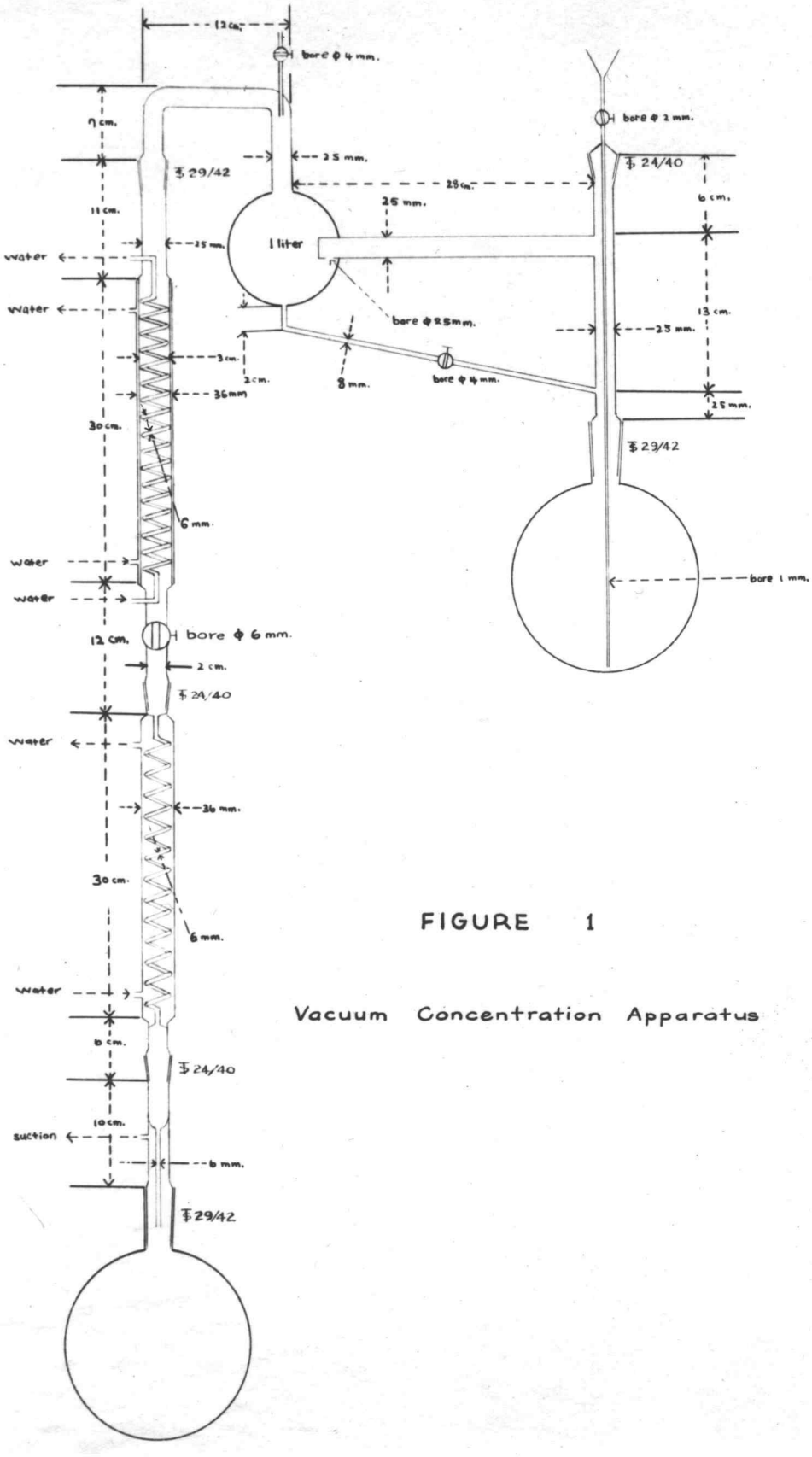

FIGURE 1

Vacuum Concentration Apparatus

Chapter III.

Results and Discussion

1. Enzymic Synthesis of a Growth-promoting Substance from Pantothenic Acid or β-Alanine

Glutamic acid was incubated in buffered saline with β-alanine or with pantothenic acid in the presence of resting L. M. yeast cells (S. cerevisiae L. M. Strain, ATCC No. 9371). The resulting mixture showed extremely great activity, occasionally over a thousand times that of the β-alanine or pantothenic acid present, as measured in a pantothenic acid free medium using L. M. yeast. Some results are outlined in Table II.

The incubated product from β-alanine was always more active than that from pantothenic acid. However, the results were not consistent; in six out of eighteen experiments no increase in activity was obtained, and the trend among the later experiments was toward increases of twofold or less. It was not possible to tell whether this was due to inconsistencies in synthesis of active material or to variable requirements by the assay organism. Therefore it was decided to seek another assay organism for the active principle, as well as new sources from which it might be isolated.

Table II.

Growth Response of Yeast to Pantothenic Acid or β-Alanine

Previously Incubated with Resting L. M. Yeast Cells

Incubation mixture*	Pantothenic acid activity of resulting mixture					
	Experiment 1		Experiment 2		Experiment 3	
	γ	x original	γ	x original	γ	x original
5 mg. pantoic acid 5 mg. glutamic acid	0	0	0	0	0	0
5 mg. pantoic acid 5 mg. glutamic acid 0,1γ pantothenic acid	1.0	10	100	1000	0.5	5
5 mg. pantoic acid 5 mg. glutamic acid 0.2γ β-alanine	1000-2000	5000-10000	400	4000	2	10

*made up to 10 ml. with buffered saline (pH 5.0) in the presence of about 0.2 ml. L. M. yeast cells.

2. Stimulation of Growth in S. cerevisiae 2190

Saccharomyces cerevisiae, strain 2190 (National Collection of Cultures, London) was observed previously to grow very feebly in a pantothenic acid containing medium which was satisfactory for the growth of sixteen other yeasts (29). The addition of glutamic acid improved the growth considerably, although further enhancement was obtained yeast extract. The effects are summarized in Table III.

The glutamic acid effect recalls the similar observation in S. cerevisiae strains L. M. and 2504 (13). However, strain 2190 was not stimulated by the mixture resulting from incubation with resting L. M. yeast cells. The extra stimulatory effect of yeast extract upon 2190 was also manifested by acid hydrolyzed casein, as well as by amino acid mixtures containing no additional glutamic acid.

Table III.

The Influence of Glutamic Acid (GA), Yeast Extract (YE) and Casein Hydrolysate(CH)[a] upon the Growth of Saccharomyces cerevisiae 2190

PA[b] (γ)	O.D.[c]	0.5γ PA		5 mg. YE		5 mg. YE + 2 mg. GA		5 mg. CH	
		GA (mg)	O.D.	PA (γ)	O.D.	PA (γ)	O.D.	PA (γ)	O.D.
0.0	0.000	0.5	0.210	0.1	0.320	0.1	0.320	0.00	0.000
0.1	0.100	1.0	0.240	0.5	0.320	0.5	0.320	0.01	0.020
0.5	0.090	2.0	0.250					0.50	0.580
1.0	0.080							1.00	0.580
2.0	0.100								

[a] Vitamin-free casein was hydrolyzed with 20% hydrochloric acid and the hydrochloric acid was then removed by repeated distillation in vacuo.
[b] Weight of calcium pantothenate (PA).
[c] Growth shown by turbidity in terms of optical density.

3. Response of *A. suboxydans* to Extracts of Natural Materials

Nachmansohn and Berman (25) obtained active preparations of the coenzyme for acetylation of choline from heart tissue. In view of the fact that Lipmann and coworkers (20) showed this coenzyme (coenzyme A) to contain pantothenic acid, and in view of the failure of yeast 2190 to respond to the resting cell preparation, additional experiments were carried out with liver, yeast and heart extracts. The response of a given test organism to these extracts was measured in terms of pantothenic acid. The apparent pantothenic acid contents so obtained were checked by means of other assays using *Lactobacillus arabinosus* 17-5 which utilized the intact vitamin, and by *Saccharomyces cerevisiae* L. M., which after hydrolysis of the preparations with 6 N hydrochloric acid, indicated the total β-alanine content. *Acetobacter suboxydans* was regarded as a preferred organism for study because of its relatively great sensitivity to pantoic acid and the fact that *A. suboxydans* assays (30) of certain tissues frequently gave higher values than those obtained with *L. arabinosus* (30).

In the preliminary experiments when fresh liver

or heart muscle was heated and brought to pH 4.5 - 5.0 to remove most of the proteins, the filtrates from these preparations possessed considerably higher apparent pantothenic acid contents when assayed by A. suboxydans than when L. arabinosus or S. cerevisiae G. M. was used. The activity of these extracts was far higher than could be accounted for by the pantothenic acid present, as measured by L. arabinosus 17-5 after digestion with papain and takadiastase.

4. The Proof of the Active Principle as a Pantothenic Acid Conjugate

That the active material in the extracts of liver and of heart muscle contained pantothenic acid was strongly suggested by the fact that no compound is known to be able to substitute for the vitamin or the pantoic acid moiety in media in which it is lacking, and also by the observation that the β-alanine content, as determined by yeast, increases upon acid hydrolysis. These observations are summarized in Table IV.

Moreover, the pantothenic acid in the active principle was liberated by enzymatic hydrolysis with a pigeon liver enzyme and an intestinal phosphodiesterase*. The preparation thus treated was active as a pantothenic acid source for L. arabinosus, which did not respond to PAC prior to the enzyme treatment. After six hours hydrolysis, the β-alanine content values roughly accounted for half of the activity of the conjugate in A. suboxydans. This suggested that the conjugate may be about twice as active as the free vitamin for this organism.

* Two preparations of phosphodiesterase were generously supplied by Dr. F. Lipmann, of the Medical School, Harvard University, Boston, Mass.

Table IV.

The Apparent Pantothenic Acid Activity of Heart Muscle Concentrates for *L. arabinosus*, L. M. Yeast and *A. suboxydans*[a]

Sample no.	*A. suboxydans*		L. M. Yeast			*L. arabinosus* 17-5	
	None	Enzyme digested[b]	None	Enzyme digested	Acid[c,d] hydrolyzed	None	Enzyme digested
				γ/mg.			
210	2.12		0.44		1.10	0.085	0.05
300	1.93					0.026	0.037
502	2.90	1.1				0.057	0.058
600	1.35		0.11	0.13	0.81	0.053	0.099
601	1.62	1.84	0.073	0.082	0.57	0.076	0.080

[a] In terms of calcium pantothenate.
[b] 2% papain and takadiastase.
[c] Refluxed with 10 volumes of 6 N HCl for 6 hours, then HCl was removed by repeated distillation *in vacuo*.
[d] β-Alanine standard used; pantothenic acid values calculated.

The demonstration of greater activity by a conjugate than by the free vitamin has been observed in Hemophilus influenzae or parainfluenzae with coenzyme I or II, where nicotinic acid or amide has virtually no growth-promoting power (21, 22).

5. Isolation and Concentration Studies

Several kinds of starting materials were tried, including liver, commercial liver extracts, bakers and brewers yeasts, S. cereviasie L. M. cells, heart muscle and others. It was found that heart was a rich source, and in addition was the most suitable for the succeeding manipulations.

When fresh ground pork heart was diluted with about twice its volume of water and boiled for a few minutes, the residue after cooling could be removed readily by filtration. Acidification of the filtrate to pH 4.0 to 4.5 with dilute acetic acid yielded a further (inactive) precipitate which, after standing several hours at 5° C. coalesced and filtered readily. About 30% of the active principle remained after this treatment.

Acetone and barium precipitation. -- Precipitation of PAC from aqueous extracts could be accomplished readily either with twenty to thirty volumes of acetone, or with barium hydroxide until precipitation was complete and a slight excess of alkali remained. The solid from the acetone precipitation

was usually very dark and contained a large percentage of protein. It was hygroscopic but would not redissolve completely in water. Because of the large volume of acetone required, precipitation with barium has usually been preferred to acetone, even though the latter gave almost a quantitative yield of active principle.

The conjugate was only slightly precipitated by barium under the conditions employed by Nachmansohn and Berman (25). However, the precipitation with barium hydroxide was used in spite of the large losses in activity, since it was a very effective agent for removing the impurities associated with the conjugate. Concentrations of over ten-fold in A. suboxydans activity were regularly obtained by this means. Later, it was found that the loss during barium precipitation was due to the considerable solubility of the barium complex of the active principle. The solubility could be reduced either by the addition of excess barium hydroxide or by concentration of the extract to about one-fourth of its volume.

The barium precipitate was separated by centrifugation and washed several times with water (pH 9.5 - 10.0) and finally with small amount of distilled water. It was then decomposed with excess 0.5 or 1 M sodium sulfate solution. The filtrate after removal of barium sulfate was usually golden yellow in color, with pH approximately 10. This preparation, after precipitation with acetone, filtration and drying the precipitate *in vacuo*, contained one to five γ of pantothenic acid activity per mg. of organic solids.

In early experiments fractionation with barium was used. Barium hydroxide solution was added gradually until nearly all of the sulfate from the previous operation was precipitated. This precipitate was discarded, and the addition of barium hydroxide resumed until precipitation was complete. The precipitate was collected, decomposed with sodium sulfate and fractionated with barium hydroxide again. The active principle was retained in the less precipitable fraction. In this way, the potency was increased several fold. However, the barium fractionation was tedious and the losses were great. As soon as other means were developed for concentration, this process

was abandoned.

Removal of proteins and dialysis. -- Although a large part of the protein matter was removed at the outset by boiling and by precipitation with acetic acid at pH 4.5 - 5.0, some protein remained and was carried down during the barium treatment. In addition, the filtrate from the barium precipitation still contained protein. At this step, several protein precipitating agents were tried, and the Folin-Wu tungstic acid reagent was found satisfactory. The precipitation was made in the manner of the original method for the removal of blood protein, except that more concentrated solutions of sodium tungstate and sulfuric acid were employed, due to the low concentration of protein in the extract. Although the tungstic acid filtrate did not interfere with the determination of free and takadiastase-papain digested pantothenic acid by L. arabinosus, it was, however, not suitable for the A. suboxydans assay.

In early experiments, the interference of tungstic acid in the filtrate was roughly estimated through the use of blank tubes containing corresponding amounts of tungstic acid and pantothenic acid. Later,

the tungstic acid filtrates were subjected to A. suboxydans assay after dialysis. This resulted in the removal of inhibition of growth of the test organism. However, it was found that the active principle was practically non-dialyzable. This was somewhat surprising in view of the previous finding that it was not precipitated by tungstic acid, as well as by trichloroacetic acid or the Somogyi reagent (alkaline zinc sulfate).

Dialysis was usually performed in a cellophane tube* against running distilled water. The temperature of the water was below 15° C., and the rate of flow was about 2.5 - 3.0 liters per hour. The recovery of the active principle from dialysis was about 80 - 90 per cent in 15 - 30 hours and 60 - 80 per cent in 60 hours. concentrations of several fold in A. suboxydans activity were regularly obtained by the combined tungstic acid precipitation and dialysis.

These properties recall those of the antitrypsin from soy bean (7), which was also not precipitated by tungstic acid and was non-dialyzable.

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* The Visking Corp., Chicago, Ill. Standard Stretch Lot No. 701 and NoJax Casing Lot No. 701 were used.

Adsorption and chromatography. -- Charcoal (Norit A), which can be used for the adsorption of free pantothenic acid or the diphosphate (32), also adsorbed the conjugate. Adsorption was nearly complete when 10 per cent by weight of charcoal was employed in the olution of pH 2.0 - 2.5. However, attempts at elution have so far been unsuccessful. Whether a suitable eluting agent has not been found or the active principle was decomposed at the surface of charcoal remains to be investigated.

Lloyd's reagent was effective in adsorbing the conjugate at pH 2.0 - 3.0. Elution with five per cent ammonium hydroxide was nearly quantitative. Several of the purest preparations which were adsorbed on columns packed with 1 : 1 by volume of Lloyd's reagent and celite (as diluent) showed a single diffuse yellow band, whereas the filtrates were colorless. However, the rate of flow was slow; passage of 30 ml. through 20 cm. column of Lloyd's reagent-celite required several hours with aid of vacuum from a water aspirator. On the other hand, adsorption by shaking and centrifuging was a very satisfactory process. When the dialysed sample from the tungstic acid filtrate was subjected to Lloyd's reagent adsorption and then

eluted with ammonium hydroxide and dialysed again, the potency could be increased two to four fold. Lloyd's reagent removed much extraneous coloring matter, so that the eluates possessed a light yellow color.

Superfiltrol* was found also to be a good adsorbent for the conjugate. The active principle was nearly quantitatively adsorbed at pH 2.0 - 2.5. Likewise, elution with five per cent ammonium hydroxide was over 90%. Moreover, superfiltrol possessed the added advantage that in the concentration process it did not "overlap" the action of Lloyd's reagent, i. e., when the eluate from Lloyd's reagent was treated with superfiltrol, the potency of the resulting sample increased about two fold. Adsorption on columns of superfiltrol also yielded a single diffuse yellow band. Although the filtration rate was again slow, this process has afforded the best means of concentration yet devised. The purest samples so obtained have not increased further in potency by subsequent adsorption on either superfiltrol-celite or Lloyd's reagent-celite columns. The pantothenic acid content of these

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* Filtrol Corp., Los Angeles, Calif. In the present experiment grade IX-202 was used. The author wishes to thank Dr. L. D. Wright, of Sharp and Dohme, Glenolden, Pa., for this helpful suggestion.

preparations (A. suboxydans) was approximately seven to ten per cent.

Ion exchange resins* were also fairly effective in both acid and alkaline solutions but weaker adsorbents such as corn starch, potato starch and freshly precipitated barium sulfate were of little value. Florex** adsorbed about 80 - 90% of the active principle in acid medium (pH 2.0 - 2.5) and released it readily into five per cent ammonium hydroxide. However, the potency was not increased after Lloyd's reagent adsorption. The results of recovery from these adsorbents are summarized in Table V.

One of the purest PAC samples was subjected to one-dimensional paper partition chromatography. Water saturated phenol was used as the solvent. Since the conjugate was colored, it was possible to directly observe its progress along the strip. At all times

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* Supplied by The Resinous Products and Chemical Co., West Washington Square, Philadelphia, Pa. Amberlite IR-4-B, A. G. and Amberlite IR-100 H. A. G. were used.

** Floridin Co., Warren, Pa. In the present experiments, an adsorbent of 60/100 mesh activated at 900° F. was used.

Table V.

Adsorption of PAC by Various Adsorbents

Adsorbents	Fraction tested	Per cent of Original Activity Observed
Charcoal	filtrate	0 - 8
	eluate	0 - 4
Lloyd's reagent	filtrate	20 - 40
	eluate	50 - 80
Florex	filtrate	10 - 30
	eluate	40 - 80
Superfiltrol	filtrate	0 - 20
	eluate	80 - 90
Anion ion exchange resin (Amberlite IR-4-B,A.G.) at pH 10	filtrate	15
Amberlite IR-4-B, A. G. at pH 2.5	filtrate	25 - 50
Cation exchange resin (Amberlite IR-100-H,A.G.) at pH 10	filtrate	25
Amberlite IR-100-H, A.G., at pH 2.5	filtrate	30 - 40
Starch	filtrate	80 - 100
Barium sulfate	filtrate	80 - 100

only a single yellow band was observed. After developing with ninhydrin in butanol, a single purple band at the same position was noted. Both the purple and the yellow band, however, were very faint.

The chromatographic behavior on the superfiltrol column and on filter paper suggested that either the preparation was approaching purity, or that the impurities present were colorless, both *per se* and after treatment with ninhydrin. Due to the fact that maximum potency was reached by means of chromatography on Lloyd's reagent or superfiltrol columns, the first explanation may be favored.

Solubility behavior; counter-current extraction. -- One of the classical approaches to purification of organic compounds has been through solvent extraction. Dry samples of PAC were treated in micro-Soxhlet extractors with various solvents, including absolute ethanol, methanol, dioxane and ether. After 16 - 24 hours extraction, the residues were brought into water, the solvents in the extracts were completely removed *in vacuo* and then dissolved into water. Both fractions were assayed microbiologically. It was found that none of the solvents were able to dissolve the active principle,

for the extracts were inactive, and the recovery of the residues in the Soxhlet thimbles was practically quantitative.

Another effective approach to purification of large-molecular weight compounds has been made through counter-current extraction. Craig (5) has discussed this subject fully. In view of the fact that methyl isobutyl ketone and methy ethyl ketone were used in the liquid-liquid extraction for pantoyl lactone (28), the aqueous solution of PAC was counter-currently extracted with these two ketones respectively for 16 - 24 hours. However, the extraction by these ketones was only approximately five per cent, and the potency of the aqueous phase was not increased. Similar results were obtained in extraction with butanol-water and iso-butanol-water mixtures.

Since phenol is a good solvent for many organic compounds, extraction of PAC was also attempted with this solvent. An aqueous solution of PAC was extracted three times with one-half its volume of phenol. The phenol extract was then extracted three times with dilute hydrochloric acid. The hydrochloric acid extract was in turn extracted with ether three times to remove residual phenol, as was the aqueous layer from

the phenol extraction. All the aqueous fractions were evaporated to dryness *in vacuo* and then dissolved in water. The results from the microbial test showed that most of the active principle had not been extracted with dilute hydrochloric acid. Since it was not possible to assay the phenol fraction directly, it was steam distilled under reduced pressure for approximately 30 hours and then assayed. The resulting activity of this fraction was only about ten per cent of the original, and the sum of the activities in all fractions was only about 30 per cent of that originally extracted with phenol. It seems likely that the PAC may have undergone partial destruction during steam distillation of the phenol extract.

Reactions with heavy metals. -- Concentration by heavy metal precipitation was also attempted. The active principle was treated with lead acetate, mercuric chloride, silver nitrate and cadmium chloride respectively in alkaline solution. The precipitates formed were filtered, washed and decomposed with hydrogen sulfide. The filtrates were also decomposed with hydrogen sulfide. After removal of H_2S, all fractions were tested for microbiologic activity.

These attempts at concentration were generally unsuccessful. The recovery of activity was usually far from quantitative, and it was felt that the amount of active material was so small in relation to the quantity of the hydroxides or sulfides produced that adsorption or mechanical occlusion of the PAC may have taken place. This technique was therefore discarded.

Outline of concentration procedure. -- On the basis of information obtained through various experiments the following general method has been devised for concentrating the conjugate.

Ten kilograms of fresh pork heart were freed from blood and connective tissues, passed several times through a fine meat grinder and diluted with 20 liters of water. The mixture was stirred well for a few minutes. Live steam was introduced with stirring and the mixture was maintained at boiling temperature for three to five minutes. After cooling in running water, the residue was removed by filtration in a large Büchner funnel. The filtrate was brought to pH 4.5 with acetic acid.

After standing in a refrigerator overnight the acidified mixture was filtered through Whatman No.

42 filter paper. The filtrate was concentrated *in vacuo* to about 5 liters. Ten N sodium hydroxide solution was added to pH 8.5 (about 20 ml.) and 400 ml. of a saturated solution of barium hydroxide was added. The precipitate was removed immediately after 30 minutes by centrifugation, washed at pH 8.5 with three 200 ml. portions of water (at pH 8.5), and finally with 150 ml. distilled water.

The barium precipitate was shaken with five or six successive 100 ml. portions of 1 M sodium sulfate solution, and filtered after each addition. To the combined filtrates was added 25 -50 ml. of 40% per cent sodium tungstate (or a corresponding amount of 10 per cent solution). The pH was adjusted with sulfuric acid to 2.8. The mixture was stirred with care being taken to prevent foaming, allowed to stand for 15 minutes, and filtered without washing. The pH of the filtrate was checked (to pH 2.8) and the completion of protein precipitation was tested with a few drops of the reagent.

One hundred and fifty grams of Lloyd's reagent was added to the tungstic acid filtrate and shaken for one hour. The adsorbent was separated by centrifuging, washed twice with 150 ml. of water at

pH 2.8 and subsequently eluted by repeated shaking or stirring with successive 100 ml. portions of five per cent ammonium hydroxide until the eluates were nearly colorless. The final volume was usually about 400 ml. This solution was dialysed against running distilled water for 16 hours. The material remaining in the dialysis bag showed 3 - 5% of pantothenic acid activity based on the organic solids content. Further dialysis could slightly increase the potency.

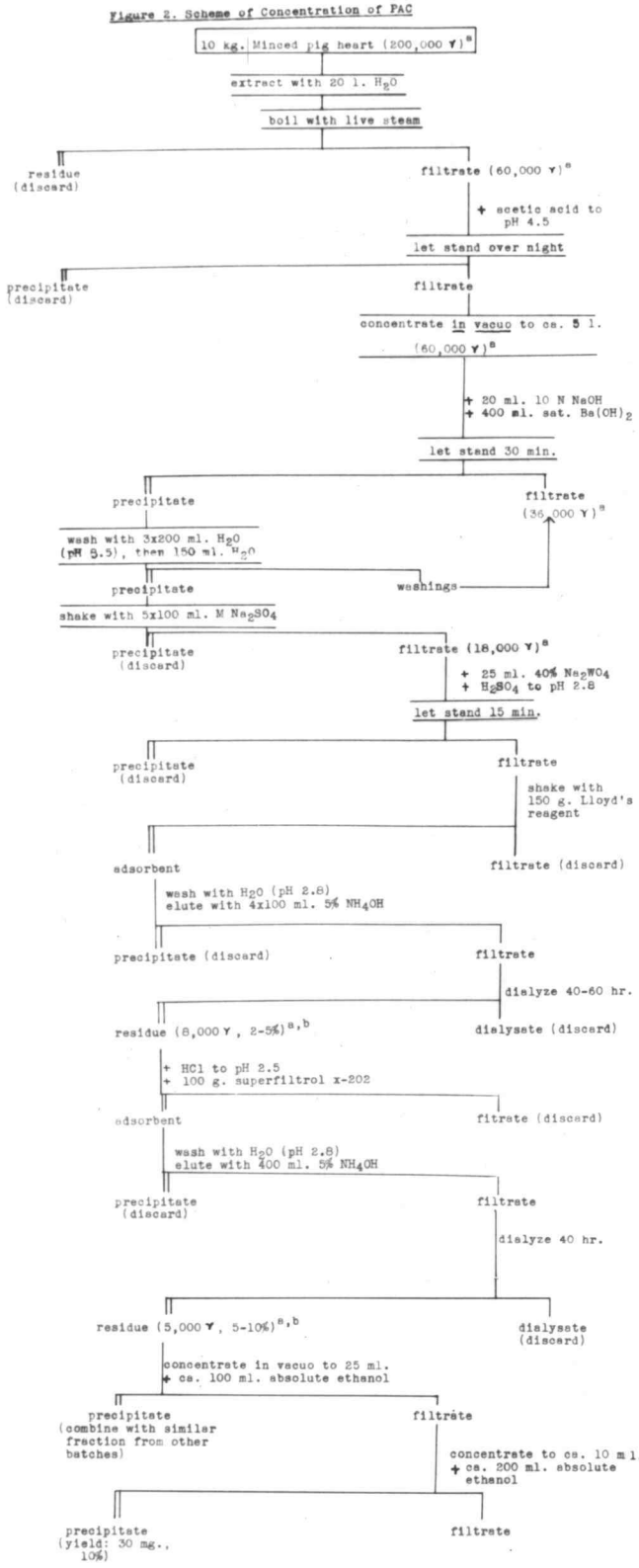
For subsequent superfiltrol adsorption, the solution was adjusted to pH 2.5 with concentrated hydrochloric acid. Fifty to one hundred grams of superfiltrol X-202 were added and the mixture was shaken for 30 minutes. The superfiltrol was removed by centrifuging, washed with water (pH 2.5) and eluted with approximately 200 ml. of five per cent ammonium hydroxide. The elution was repeated three or four times with 50 - 100 ml. portions of ammonium hydroxide. The combined eluates were then dialysed against running distilled water for 40 - 60 hours. Potencies, calculated from the total organic solids, usually showed 5 - 10% apparent pantothenic acid content at this point.

The solution was then carefully concentrated

in vacuo to about 25 ml. About 100 ml. absolute ethanol were added up to about 75% of alcohol content in the mixture. The precipitate thus formed was removed by suction. The filtrate was further concentrated to 10 ml. Two hundred ml. of absolute ethanol were added. The mixture was allowed to stand for about 30 minutes and then filtered. After drying, about 30 mg. of a yellow solid was obtained with over 10% pantothenic acid activity for *A. suboxydans*, but with none for *L. arabinosus* 17-5.

The scheme of the method is outlined in Figure 2. The potencies of some fractions were not calculated because they contained large amounts of inorganic salts, especially the ammonium salt. Moreover, the crude extracts before dialysis always contained free pantothenic acid and other bound forms of the vitamin. Therefore they were somewhat unreliable.

Figure 2. Scheme of Concentration of PAC



[a] Total activity in γ, measured with A. suboxydans.
[b] Pantothenic acid concentration in per cent.

6. Chemical and Physical Properties

Certain important properties of the conjugate have been observed in connection with concentration studies.

Although the molecule appeared to be fairly large as indicated by its failure to dialyze through cellophane, it was not precipitated by the Folin-Wu tungstic acid reagent. A possible explanation this behavior may be found in the fact that the conjugate contains glutamic acid. In addition, a preparation of intestinal phosphodiesterase was found to liberate pantothenic acid from the conjugate. This indicates the presence of phosphoric acid linkages in the conjugate, which could also be expected to resist precipitation by tungstic acid.

The conjugate contained glutamic acid in the ratio about 1 : 2 for pantothenic acid activity to glutamic acid values, as obtained from microbiological assay. The presence of glutamic acid in the acid hydrolysed samples was further confirmed by paper partition chromatography. The existence of purine or purines in the conjugate was indicated by microbiological

assay* and was supported by the spectrophotometric behavior in the ultraviolet region.

The sphectrophotometric properties** of the conjugate were studied in the visible and in the ultraviolet regions. There was no characteristic peak in the visible region in spite of the yellow color. In the ultraviolet region, on the other hand, absorption in dilute solutions (25 to 150 γ per ml., containing approximately ten per cent apparent pantothenic acid activity) increased rapidly as the wave length of the incident light was decreased below 300 mμ, reaching a minimum near 260 mμ and decreasing rapidly again to complete transmission at 230 mμ in very dilute solutions. Some characteristic absorption spectra are shown in Figures 3, 4, 5 and 6. In Figures 3 and 4 are listed curves in the visible region for a relatively crude preparation (# 28-1) containing 0.2 γ apparent pantothenic acid per mg., and others (# 4-2, 4-6 and 114-2), each with over five per cent pantothenic acid activity

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* This was done by Leanor Lindsay.

** The author wishes to thank Dr. M. B. Williams for his guidance in performing the spectrophotometric measurements.

for A. suboxydans. The irregularities in the curve for sample # 28-1 were apparently due to impurities which were removed in the more concentrated samples. Since the absorption decreased rapidly as the ultraviolet range was reached, it was found necessary to employ more dilute solutions for the ultraviolet region, as shown in Figures 5 and 6. PAC exhibited a very different absorption spectrum from that of the free vitamin (Figure 4).

Treatment with nitrous acid for 15 minutes did not appreciably reduce the activity of PAC for A. suboxydans. The conjugate must therefore either possess no free amino groups, or, if they are present, they could not be essential for biological activity.

Figure 3. Absorption Spectra of PAC Samples

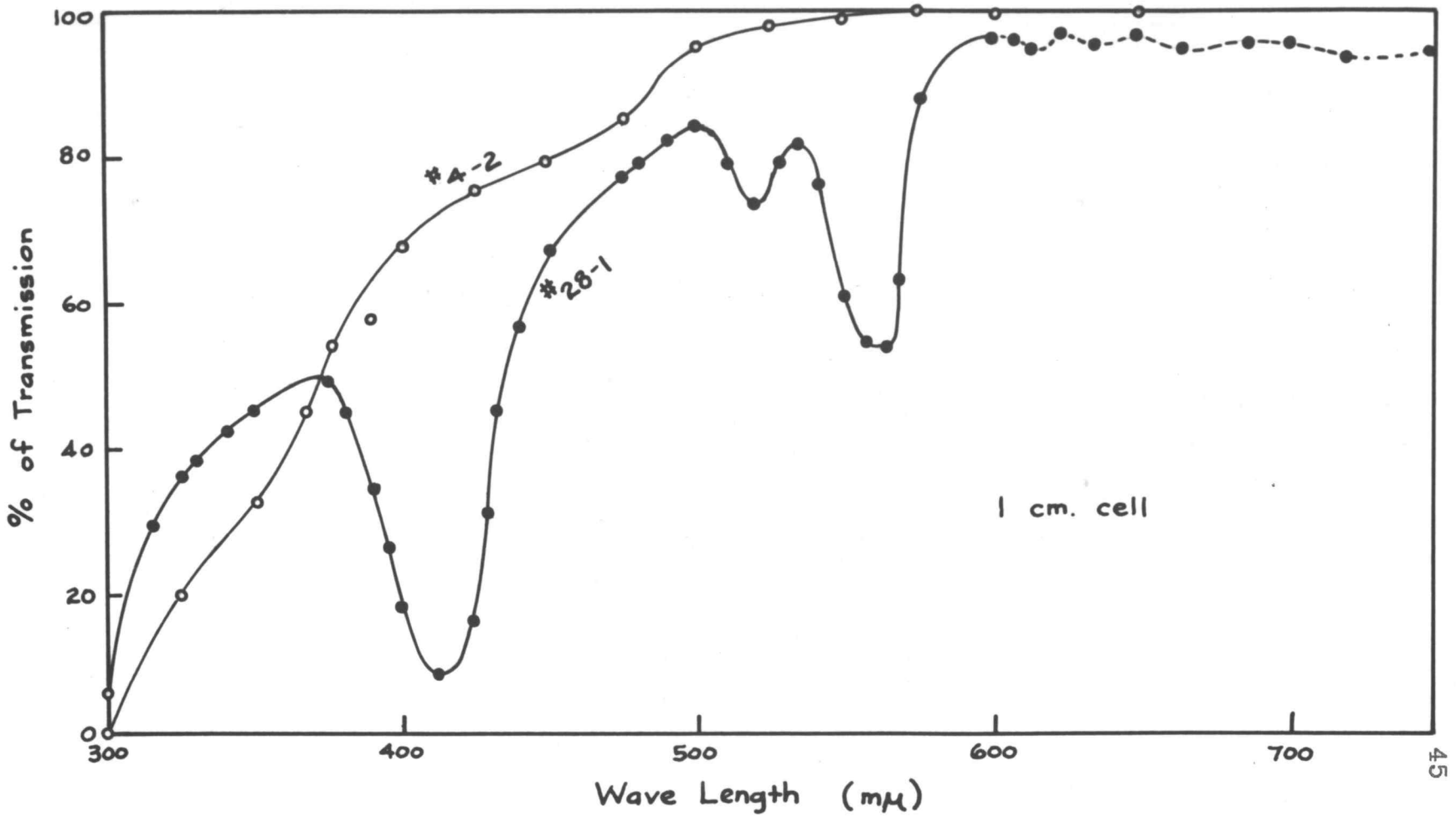


Figure 4. Absorption Spectra of Pantothenic Acid & PAC

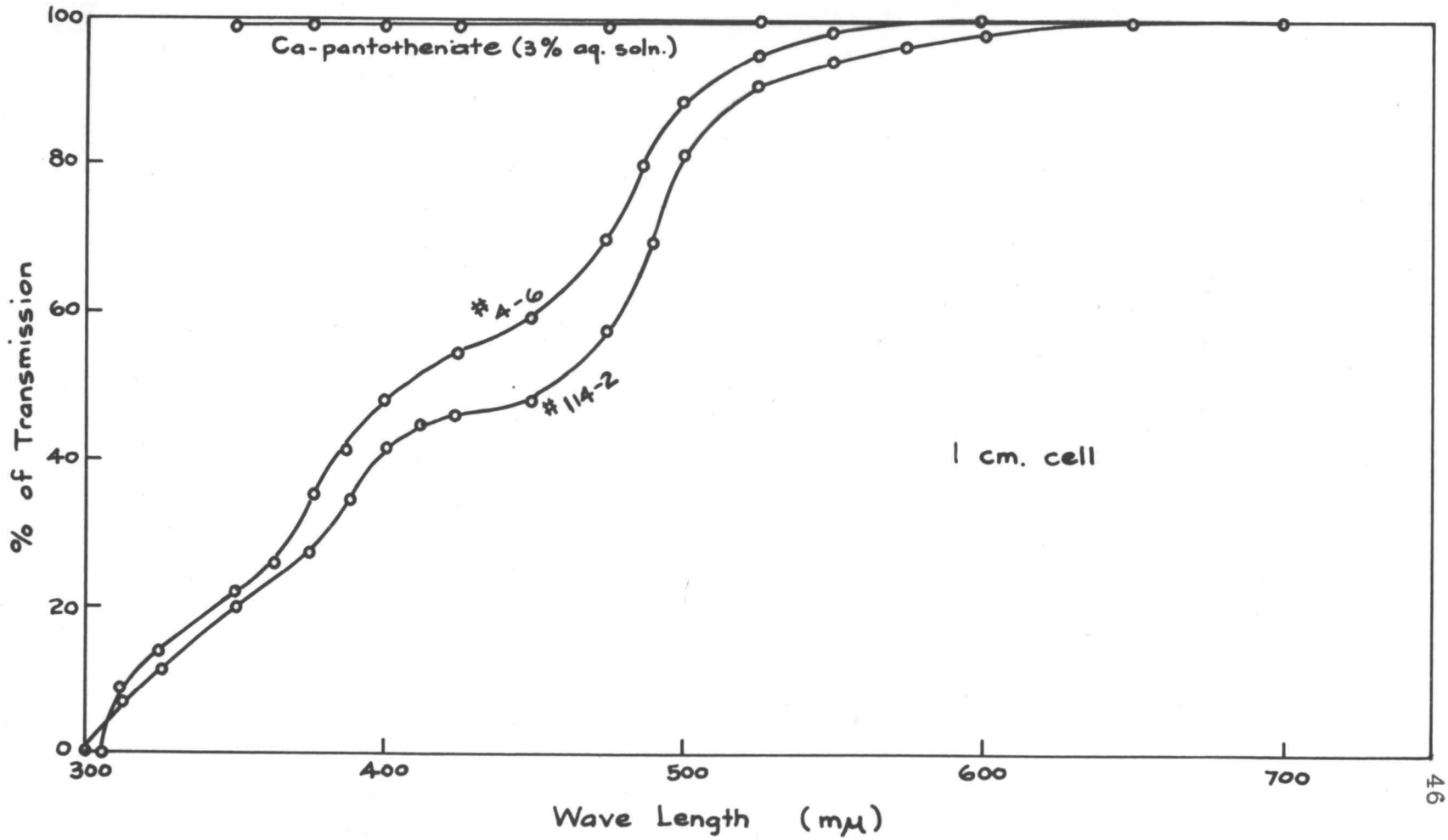


Figure 5. Ultraviolet Absorption Spectra of PAC #34-5

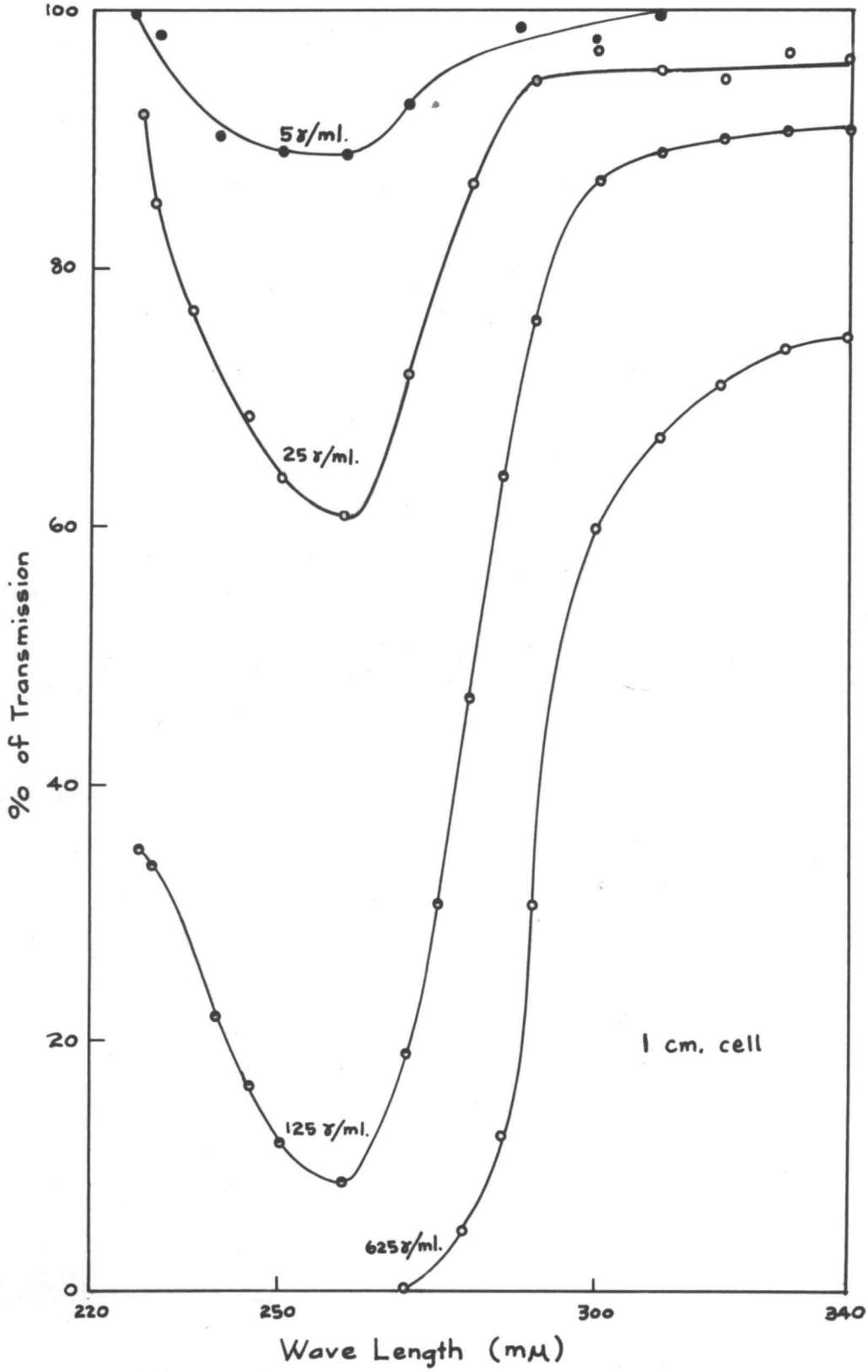
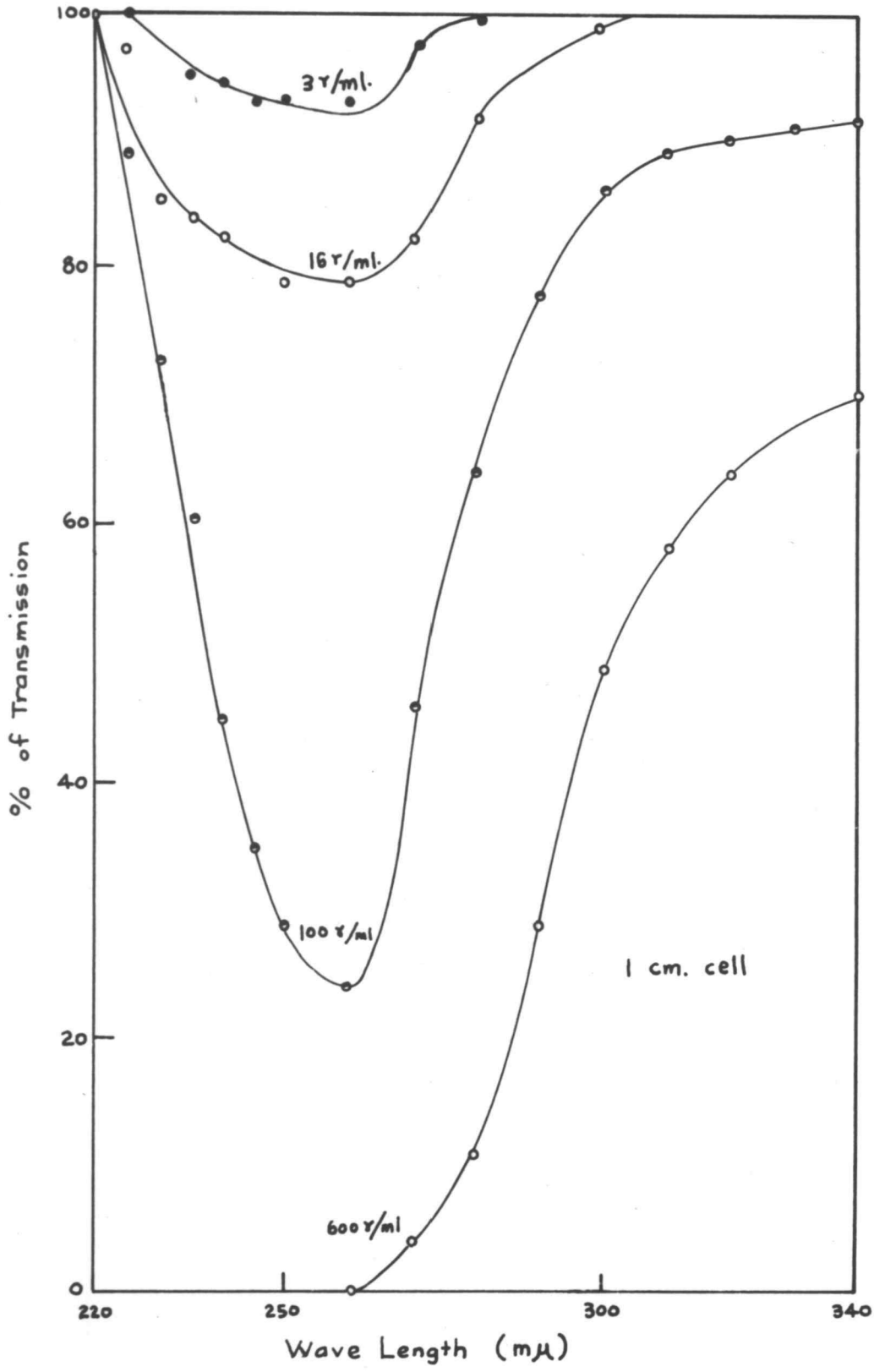


Figure 6. Ultraviolet Absorption Spectra of PAC # 114-1

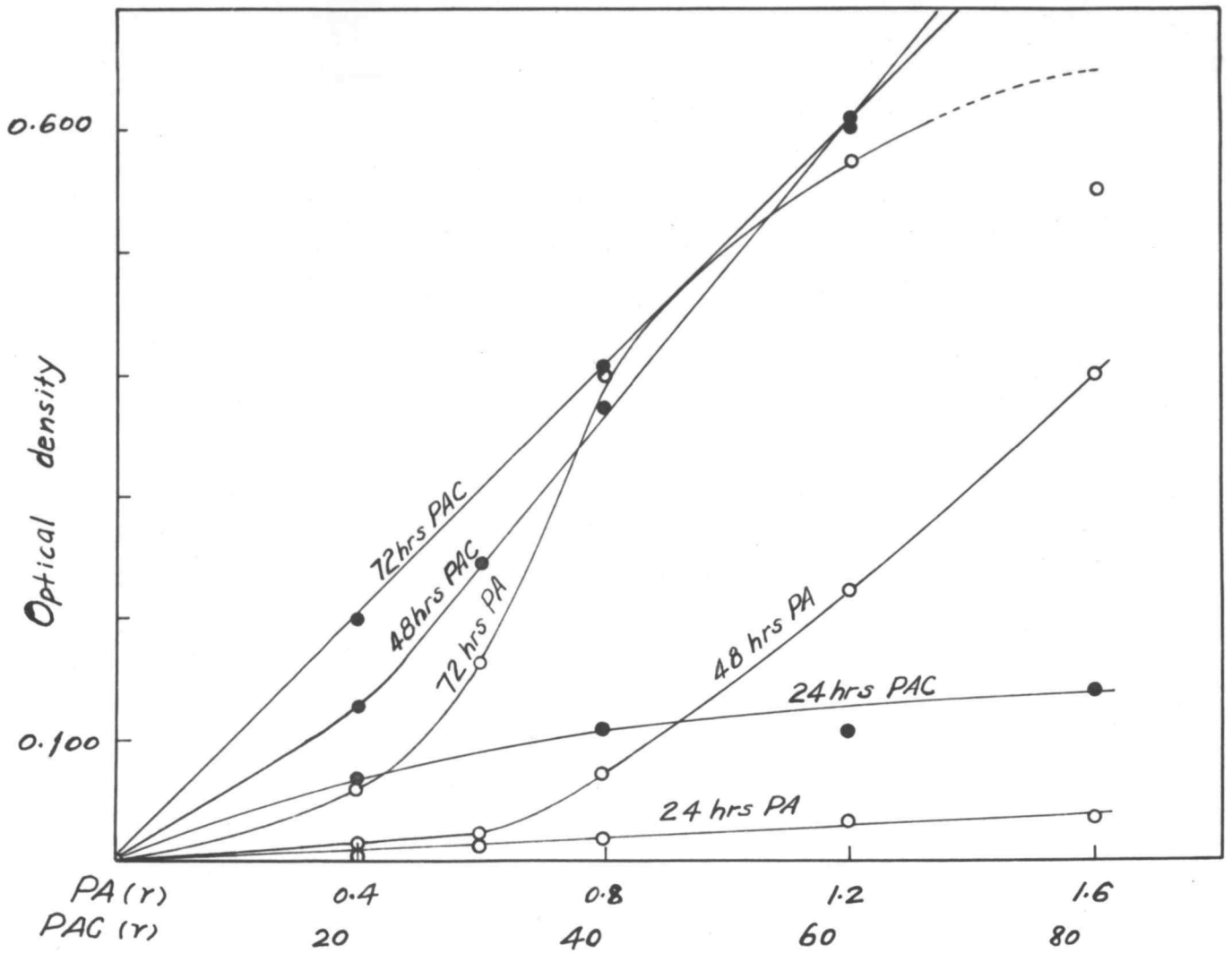


7. Biological Properties

Growth response. -- The conjugate was more active than pantothenic acid *per se* for the growth of *A. suboxydans* when pantothenic acid or pantoic acid was the limiting factor. The cultures containing adequate amounts of the vitamin grew rather slowly, requiring 60 to 70 hours to reach optical density values of 0.5 or greater. By contrast, the curves obtained with the conjugate were nearly straight and the rate of growth was much more rapid than in those containing pantothenic acid. As may be seen in Figure 6, flasks containing pantothenic acid showed very little growth after twenty-four hours of incubation, whereas those containing the conjugate had already exhibited perceptible turbidity.

Rubin and his coworkers (28) showed that the growth of *A. suboxydans* in the medium with additional Wilson's 1 : 20 liver extract was faster than in the medium of Sarett and Cheldelin (29). PAC was therefore also tested in Rubin's modified medium (see Table I). The results showed that the conjugate gave the same superior response over that of pantothenic acid which was noted above. The results for several samples of the conjugate were practically the same from the original medium and from Rubin's modified medium.

Figure 7. Growth Response of *A. suboxydans* to PA & PAC



In contrast to A. suboxydans, the conjugate was not utilized at all as a pantothenic acid source for the lactic acid bacteria, e.g. Leuconostoc mesenteroides 694, 8042 or P-60 (Dunn); Lactobacillus gayoni 8289; L. delbruckii 3; L. arabinosus 8014 or 17-5; L. acidophilus (two unidentified strains); L. brevis 118-8 or L 35; and Streptococcus fecalis R. This non-utilization may likely be due to the inability of the large molecule of the conjugate to penetrate into the microoganisms. Coenzyme A, which is presumably a smaller molecule (27a) is also non-utilizable as a pantothenic acid source by the lactic acid bacteria.

Reversing ability of the conjugate for pantothenic acid inhibitors. -- α-Hydroxy-β,β-dimethylbutyryl taurine is an inhibitor for A. suboxydans when pantothenic acid or pantoic acid is used as the limiting growth factor. The analog/growth factor ratio at 50% inhibition was reported as 800 for pantoic acid and 2,000 for pantothenic acid (3). The conjugate was used to reverse the inhibition from this taurine derivative, and the results are summarized in Table VI. Two mg. of the conjugate contained about 0.6 γ pantothenic acid.

Table VI.

Reversal by PAC and Pantothenic acid (PA)
of α-Hydroxy-β,β-dimethylbutyryl Taurine (HT)
Inhibition in A. suboxydans (72 hours incubation)

Constituents	Optical density
PA (γ)	
0.0	0.000
.4	.043
.8	.160
1.2	.310
PAC (mg.)	
0.5	0.073
1.0	.108
2.0	.178
PA 1.2 γ + HT (mg.)	
2	0.270
4	.193
8	.072
16	.032
PAC 2mg.* + HT (mg.)	
2	0.153
4	.146
8	.149
16	.150

* 2 mg. PAC contained about 0.6 γ pantothenic acid.

It is clearly shown that the analog of pantothenic acid even at high level inhibited to only a very small degree when the conjugate was used as the limiting growth factor.

Shive and coworkers (8) from numerous inhibition studies proposed a theory that the ability of the product of a metabolite to reverse the inhibition from an analog of the metabolite is always greater than that of metabolite itself. Since the reversing ability for α-hydroyx-β,β-dimethylbutyryl taurine is in the order: conjugate > pantothenic acid > pantoic acid, it is reasonable to conclude that pantothenic acid normally produces PAC, which, in turn, functions in metabolism. The fact that the slight inhibition from the analog was not reversed even at high levels of PAC may likely indicate that the function of pantothenic acid is not only for the formation of PAC but for some other purpose, although to a smaller extent in this organism.

It was reported (16) that 2-chloro-4-aminobenzoic acid* is a good inhibitor for E. coli. Panto-

*2-chloro-4-aminobenzoic acid was kindly supplied by Dr. F. B. Strandskov, of Wallace & Tiernan Products Inc., Belleville, N. J.

thenic acid was found to reverse the inhibition effectively. However, the effect of the conjugate was again somewhat greater than that of pantothenic acid. The results are outlined in Table VII.

A similar result was obtained when salicylate was used as the inhibitor; reversal could be brought about by pantothenic acid (11), but the crude PAC sample (# 701) was more effective than pantothenic acid *per se*.

The growth of *E. coli* in a salt-glucose medium (in the absence of inhibitors) was stimulated by the conjugate, since the tube with PAC were turbid after 12 hours, even though no growth was observed in the tube containing the free vitamin.

Experiments with highly purified samples of the conjugate have yielded a slightly different picture regarding the reversal of 2-chloro-4-aminobenzoic acid. Although PAC was very active against low concentrations of chloroaminobenzoic acid, its reversing effect became relatively smaller at high levels of the inhibitor, so that the free vitamin gradually became a superior agent. These results indicated again that PAC is probably only one of the products of pantothenic acid, and that it is not readily converted into the free

vitamin or other functional forms of pantothenic acid. Of course, the extent of the different functions of pantothenic acid is probably not the same in different organisms, and the reversal of inhibition in different organisms would therefore be different.

PAC content in the tissues of pantothenic acid deficient rats*. -- Further information on the metabolic importance of PAC may be gained from a study of its distribution in the tissues of animal deficient in dietary pantothenic acid. Preliminary experiments were reported (27). Extended experiments were conducted in the present study.

Tissues from pantothenic acid deficient rats (maintained on the diet listed in Table VIII) were extracted with hot water, and the extracts were treated with tungstic acid reagent, filtered and assayed for PAC. Other aliquots were extracted with hot water, dialyzed and tested for their ability to acetylate sulfanilamide. It was found that the difference in the PAC content of normal and deficient tissues was not as

* The author wishes to thank Dr. J. S. Butts for his advice on animal experiments, the supply of rats and the use of animal room facilities.

Table VII.

Reversal by PAC and Pantothenic Acid (PA) of 2-Chloro-4-aminobenzoic Acid Inhibition in E. coli. (40 hours Incubation)

CAB (mg.)	PA (γ)					PAC # 701 (mg)*			
	0	0.2	0.5	2.5	10	0.05	0.5	1.0	5.0
	Optical density								
0.00	0.760	0.760	0.740		0.760	0.760	0.760	0.780	0.800
.10	.710	.720	.720	.720		.740	.740	.760	.790
.25	.700	.700	.700	.710	.710	.600	.710	.740	.780
1.0	.120	.700	.700	.700	.700	.360	.700	.700	.780
3.0	.050	.660	.670		.680	.360	.650	.650	.760

* PAC # 701 contains 0.2 - 0.5 γ pantothenic acid per mg.

great as that of free pantothenic acid. Also, after dialysis, over half of the PAC activity was still retained, whereas the acetylating power (presumably due to coenzyme A) was lost during dialysis. Since PAC is retained by the rat during pantothenic acid starvation at the expense of the free vitamin, it appears likely that the growth of the animal is dependent upon the formation of the conjugate within its tissues, and that the conjugate represents a form of the vitamin which is of more direct functional importance.

Table VIII.

Composition of Pantothenic Acid Deficient Diet

	kg.
Cerelose	3.2
Casein (vitamin-free)	1.8
Corn oil	0.4
Salt mixture (Foster and Jones)	.4
Cod liver oil	.1
Sulfaguanidine	.1
	mg.
Thiamin	40
Riboflavin	80
Nicotinic acid	500
Pyridoxine hydrochloride	40
Biotin	0.2
p-Aminobenzoic acid	250
Choline chloride	5000
Inositol	2000
Menadione (2-Methyl-1,4-naphthoquinone)	50

8. Comparison of PAC with other Bound Forms of Pantothenic Acid

Three forms of bound pantothenic acid have been reported in the literature: viz., coenzyme A (20), a blood pantothenic acid conjugate (34) and an alkali stable form of pantothenic acid (26). The present conjugate is evidently not identical with any of these, as may be seen from the following observations.

Comparison of PAC with coenzyme A. Acetylation of sulfanilamide. -- Lipmann and his group, while working with a preparation of minced pigeon liver found an acetylating coenzyme (later called coenzyme A) which was able to promote acetylation of sulfanilamide in the presence of adenosine triphosphate (20). The acetylating system retained its activity upon freezing but was destroyed on standing or upon dialysis. They observed further that the dialyzed material could be reversibly reactivated by the addition of extracts of liver or kidney (17). This indicated that the enzymatic condensation of the sulfanilamide and acetate was

due to a dialyzable coenzyme (18)*. which was later found to contain pantothenic acid (20).

The question of possible identity of PAC and coenzyme A can be answered by comparing the acetylating power of the two preparations. The results in Table IX indicate that PAC was not active in the acetylation of sulfanilamide, in contrast to coenzyme A. The sample of Lipmann's preparation used contained about 11γ of pantothenic acid, while 15 mg. PAC contained about 10 γ of pantothenic acid activity for A. suboxydans.

Acetylation of choline. -- Since Lipmann and Kaplan have shown that coenzyme A is also active in the acetylation of choline and is therefore presumably identical with Nachmansohn and Berman's coenzyme (25), PAC was also examined for ability to acetylate choline under the conditions described by Nachmansohn and his coworkers (24, 25). The results are summarized in

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* Very recently Lipmann and his coworkers (27a) reported that the dializability of coenzyme A was irregular, with 30 - 60 per cent sometimes being retained after 18 hours of dialysis.

Table IX.

The Acetylation of Sulfanilamide by Coenzyme A and PAC*

	Optical density
Sulfanilamide, 20 γ	0.039
50 γ	.070
100 γ	.110
Lipmann's coenzyme A (1.1 mg.)** + 100 γ sulfanilamide	.082
Duplicate	.081
PAC (15 mg.) + 100 γ sulfanilamide	.110
Duplicate	.110

* This experiment was generously performed by Mr. I. G. Fels.

** Coenzyme A was kindly supplied by Drs. G. D. Novelli and F. Lipmann, of the Medical School, Harvard University, Boston, Mass.

Table X, where the samples of PAC used contained approximately 58 γ, 21 γ, and 15 γ of pantothenic acid activity respectively. From this table, it is apparent that PAC possessed no activity in the acetylation of choline. The slight response in samples #300 was probably not significant.

From the acetylation studies with sulfanilamide and choline, together with the differences in dialyzability and barium precipitation previously discussed, it is obvious that PAC is not identical with coenzyme A.

The behavior of alkali toward the pantothenic acid conjugate. -- Neal and Strong (26) reported the existence of an alkali stable form of pantothenic acid which could be utilized with greater efficiency by lactic acid bacteria after takadiastase and papain digestion. Since preliminary experiments had indicated that PAC was somewhat more resistant to alkali than the free vitamin, the possible similarity of PAC and the alkali stable factor was tested. This was accomplished in two ways: by determinating the A. suboxydans activity of liver autoclaved with alkali, and by noting the extent of degradation of PAC concentrates by alkali and

Table X.

The Acetylation of Choline by Coenzyme A and PAC*

Material added	Height of contraction curve (mm.)	
Acetyl choline (6.5 γ)	50	
Acetyl choline (3 γ)		40
Control without ATP**	3	
Apoenzyme from rat brain without dialysis	11	18
Coenzyme A (3 mg.)***	11	
PAC # 300 (30 mg.)	6	4
PAC # 700 (30 mg.)	0	0
PAC #701 (30 mg.)	0	0

* The author wishes to acknowledge with thanks to Dr. Rosalind Wulzen her helpful suggestions in this study and the use of her laboratory facilities.

** ATP (adenosine triphosphate).

*** Coenzyme A was kindly supplied by Drs. G. D. Novelli and F. Lipmann, of the Medical School, Harvard University, Boston, Mass.

and their possible conversion into alkali stable residues.

The results in Table XI indicate that the conjugate is different from the alkali stable factor, since heating of sample # 701 with alkali destroyed virtually all the activity for A. suboxydans. On the other hand, a small residue of activity remained both in sample # 701 and in fresh pork liver. This could be due either to the presence of the alkali stable form in these preparations or to its formation from PAC during hydrolysis. That this conversion actually took place was indicated by the increased activity of sample # 701 for L. arabinosus after treatment with alkali (cf. samples 3 and 4; 6 and 7 of Table XI). The Neal and Strong factor (or something biologically indistinguishable from it) may thus be considered as one of the degradation products of the present pantothenic acid conjugate.

Comparison of the conjugate with blood pantothenic acid conjugate. -- Wright (34) noted that pantothenic acid existed in bound form in blood. The vitamin appeared to be combined to protein, since

Table XI.

The Behavior of Alkali Toward PAC and Pantothenic Acid Activity in Pork Liver

No.	Samp.	wt.	Total vol. of sample ml.	Subst.	Time of standing at room temp., hr.	Time of autoclaving (15#) hr.	mM of NaCl added[b]	Pantothenic acid activity (γ/g.) L. arabinosus Hot water extract[a]	L. arabinosus Enzyme digest[c]	A. suboxydans, hot water extract
		g.								
1.	Pork liver	100	600	H_2O	0	2	600	33	33-40	30-60
2.	" "	100	600	.1 N NaOH	0	2	0	.5	6.7-7.8	5.2
3.	PAC#701	0.2	20	H_2O	0	2	10	.7	3	500
4.	" "	0.2	20	.5 N NaOH	0	2	0	1.0	10	12
5.	" "	0.25	5	H_2O	48	0	0[d]		10	500
6.	" "	0.25	5	.5 N NaOH	48	0	0		10	300
7.	" "	0.25	5	.5 N NaOH	48	1.5	0		80	0

[a] Samples treated under stated conditions, filtered; filtrates assayed for PA with L. arabinosus. [b] Since A. suboxydans is influenced by moderate concentrations of NaCl, a sufficient amount was added to equal that present after alkali hydrolysis and neutralization. [c] Samples treated under stated conditions, then digested with takadiastase and papain, filtered and assayed for PA. [d] The amount of NaCl present in (6) and (7) would not influence the growth of A. suboxydans.

it could be precipitated by tungstic acid and could be released from combination by takadiastase digestion or by autoclaving for short periods. In contrast, PAC is not precipitated by tungstic acid, and is not destroyed by heat or enzymes under above conditions. Therefore PAC is not identical with blood pantothenic acid conjugate.

Chapter IV.

Summary

1. A pantothenic acid conjugate (PAC) has been prepared from natural materials in relatively pure form. It was a yellow amophorus powder, extremely soluble in water, but insoluble in organic solvents such as acetone, ether, absolute alcohol, methyl alcohol, dioxane, etc.

2. Although the molecule appeared to fairly large as indicated by its failure to dialyze through cellophane into running distilled water, it was not precipitated by the Folin-Wu tungstic acid reagent.

3. The conjugate was only slightly precipitated by barium hydroxide. However, barium precipitation was used for concentration because it was a very effective agent for removing the impurities associated with the conjugate.

4. PAC was adsorbed readily on charcoal, Lloyd's reagent or superfiltrol and eluted from the latter two adsorbents.

5. By combination of various technique, the best samples with A. suboxydans activity of over 10%

pantothenic acid were obtained. They contained no free pantothenic acid. However, after digestion with intestinal phosphodiesterase and a pigeon liver enzyme, the pantothenic acid in the conjugate was made available to L. arabinosus. The best samples contained about 20% glutamic acid which was determined by microbiological assay. Its presence was confirmed by paper partition chromatography. Its probable content of purines was indicated by a microbiological method and supported by the spectrophotometric behavior in the ultraviolet.

6. PAC was more active than pantothenic acid per se in A. suboxydans. The cultures containing amounts of free pantothenic acid grew rather slowly, requiring more than 60 hours to reach the maximum. By contrast, the curves obtained with the conjugate were nearly striaght and the rate of growth was much more rapid.

7. α-Hydroxy-β,β-dimethylbutyryl taurine, which was an inhibitor for A. suboxydans when pantothenic acid or pantoic acid was used as the limiting growth factor, was much less effective in preventing growth when PAC was used. The superiority of PAC over the free vitamin was similarly demonstrated in the reversal of the inhibition of E. coli in a salt-glucose

medium by 2-chloro-4-aminobenzoic acid, sodium salicylate or propionic acid. Moreover, the growth of *E. coli* in a salt-glucose medium was stimulated by the conjugate to a much greater extent than by the free vitamin. In view of these facts, PAC was considered to be involved more directly in metabolism than the free vitamin.

8. PAC was found to be different from coenzyme A since the former did not acetylate sulfanilamide or choline and was non-dialyzable. It was not identical with the bound form of pantothenic acid in blood which was unstable at high temperatures and precipitable by the tungstic acid reagent. The alkali stable form of pantothenic acid was considered to be a degradation product of PAC since a small residue of pantothenic acid activity remained after treating PAC with alkali.

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