than are the reducing dextrins formed from the linear components. Marked differences were also found in the relative concentrations of the products formed by pancreatic amylase from unfractionated potato starch and from the linear substrate. These differences were observed whether the comparisons were made for the same time intervals with the same concentrations of amylase or at equivalent stages in the hydrolysis of the two substrates.

Maltose was present in measurable concentrations from the very early stages of the hydrolysis of unfractionated potato starch and of the linear substrate. However, this sugar was present in considerably higher concentrations in the hydrolyzates from the linear substrate than in those from potato starch whether comparisons are made at the same time intervals with equal concentrations of amylase or at equivalent stages in the hydrolysis of the two substrates.

Glucose was liberated by maltase-free pancreatic amylase from unfractionated potato starch and from the linear substrate although this sugar did not appear in the very early stages of the hydrolyses of these substrates. Comparisons made at the same time intervals with equal concentrations of amylase and also at equivalent stages in the hydrolyses of the two substrates show that glucose was liberated somewhat earlier and at equivalent hydrolysis was present in slightly larger concentrations in the hydrolyzates of potato starch than in those of the linear substrate.

A study of the results as a whole indicates that purified pancreatic amylase causes the random hydrolysis of both the straight and the branched chain components of starch.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, OREGON STATE COLLEGE]

Pantothenic Acid Studies. VI. A Biologically Active Conjugate of Pantothenic Acid[1]

BY TSOO E. KING, I. GORDON FELS AND VERNON H. CHELDELIN

In a previous paper from this Laboratory[2] glutamic acid was reported to enhance the growth promoting property of pantothenic acid in certain strains of yeast. A related observation was made by Woolley,[3] 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 in the medium. Although the mechanism of these actions is at present obscure, 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 is more active for the growth of microörganisms. Since numerous possibilities exist for combinations of pantothenic acid, glutamic acid and β-alanine, their direct preparation was deferred in favor of biosynthesis of active materials by resting cells.

Glutamic acid was incubated in buffered saline with β-alanine or with pantothenic acid in the presence of resting yeast cells (*S. cerevisiae* L.M. strain, A.T.C.C. 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. The incubated product from β-alanine was always more active than that from pantothenic acid. However, the results were not consistent; in six 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 organisms. It was therefore decided to seek another assay organism for the active principle, as well as new sources from which it might be isolated.

Stimulation of Growth in *S. cerevisiae* 2190

Strain 2190 (National Collection of Cultures, London) was observed previously[4] to grow very feebly in a pantothenic acid containing medium which was satisfactory for the growth of sixteen other yeasts. The addition of glutamic acid improved the growth considerably, although further enhancement was obtained with yeast extract. The effects are summarized in Table I. The glutamic acid effect recalls the similar observation in strains L.M. and 2504.[2] 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.

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(2) T. E. King and V. H. Cheldelin, *Arch. Biochem.*, 16, 231 (1948).

(3) D. W. Woolley, *J. Biol. Chem.*, 168, 481 (1946).

(4) H. P. Sarett and V. H. Cheldelin, *J. Bact.*, 49, 81 (1945).

TABLE I

THE INFLUENCE OF GLUTAMIC ACID (GA), YEAST EXTRACT (YE) AND CASEIN HYDROLYSATE (CH)[a] UPON THE GROWTH OF *S. cerevisiae* 2190

Added PA[b]	Optical[c] density	0.5 γ PA Added GA, mg.	Optical density	5 mg. YE Added PA, γ	Optical density	5 mg. YE + 2 mg. GA Added PA, γ	Optical density	5 mg. CH Added PA, γ	Optical density
0.0	0.000	0.5	0.210	0.1	0.320	0.1	0.320	0.00	0.000
.1	.100	1.0	.240	.5	.320	.5	.320	.01	.020
.5	.090	2.0	.250					0.50	.580
1.0	.080							1.00	.580
2.0	.100								

[a] Vitamin-free casein was hydrolyzed with 20% hydrochloric acid and the hydrochloric acid was removed by repeated distillation *in vacuo*. [b] Weight of calcium pantothenate (PA). [c] Growth shown by turbidity in terms of optical density (= 2 − log per cent. transmission).

The Response of Acetobacter suboxydans to Extracts of Natural Materials

Nachmansohn and Berman[5] have obtained active preparations of the coenzyme for acetylation of choline from heart tissue. In view of the fact that Lipmann and co-workers[6,7] 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 *L. arabinosus*, which utilized the intact vitamin, and by L.M. yeast, which, after hydrolysis of the preparations with 6 *N* hydrochloric acid for one hour, indicated the total β-alanine content. *A. suboxydans* was regarded as a preferred organism for study because of its relatively great sensitivity to pantoic acid[8] and the fact that *A. suboxydans* assays of certain tissues[8] frequently gave higher values than those obtained with *L. arabinosus*.

It was reported in a preliminary note[9] that when fresh liver or heart muscle was heated and brought to *p*H 4.5–5.0 to remove most of the protein, the filtrates from these preparations possessed considerably higher apparent pantothenic acid contents when assayed by *A. suboxydans* than when *L. arabinosus* or G.M. yeast 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* after digestion with takadiastase and papain.[10] That the active material in these extracts 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 increased upon hydrolysis. After six hours hydrolysis, the β-alanine values accounted for roughly half of the activity exhibited in *A. suboxydans*, and it was felt that the active principle was therefore about twice as effective as the free vitamin for this organism. The activity was thought to be due to a conjugated form of pantothenic acid which for convenience will be abbreviated PAC in the present paper.

When pantothenic acid (or pantoic acid[8]) was the limiting factor for growth of *A. suboxydans*, growth curves were sigmoidal in shape. The cultures containing adequate amounts of the vitamin grew rather slowly, requiring sixty to seventy 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 Fig. 1, flasks containing pantothenic acid showed very little growth after twenty-four hours of incubation, whereas those containing the conjugate had already exhibited perceptible turbidity.

Comparison of the Pantothenic Acid Conjugate with Coenzyme A. Acetylation Studies

Sulfanilamide.—Lipmann and co-workers[6] reported that coenzyme A possessed the ability to acetylate sulfanilamide in the presence of adenosine triphosphate (ATP) and an apoenzyme from pigeon liver. The question whether PAC is identical with coenzyme A can be answered by comparing the acetylating power of the

TABLE II

THE ACETYLATION OF SULFANILAMIDE BY COENZYME A AND THE PRESENT PANTOTHENIC ACID CONJUGATE (PAC)

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

(5) D. Nachmansohn and M. Berman, *J. Biol. Chem.*, **165**, 55 (1946).

(6) F. Lipmann, N. D. Kaplan, G. D. Novelli, L. C. Tuttle and B. M. Guirard, *J. Biol. Chem.*, **167**, 869 (1947).

(7) F. Lipmann and N. O. Kaplan, *ibid.*, **162**, 743 (1946).

(8) H. P. Sarett and V. H. Cheldelin, *J. Biol. Chem.*, **159**, 311 (1945).

(9) T. E. King, L. M. Locher and V. H. Cheldelin, *Arch. Biochem.*, **17**, 483 (1948).

(10) E. H. Hoag, H. P. Sarett and V. H. Cheldelin, *Ind. Eng. Chem., Anal. Ed.*, **17**, 60 (1945).

two preparations. The results in Table II show that the present conjugate is not active in the acetylation of sulfanilamide. The sample of Lipmann's preparation used contained about 11γ of pantothenic acid, while 15 mg. of PAC contained about 10 γ of pantothenic acid activity for *A. suboxydans*.

Choline.—Since Lipmann and Kaplan have shown[7] that coenzyme A is also active in the acetylation of choline (and is therefore presumably identical with Nachmansohn and Berman's coenzyme[5]), the PAC preparation was also examined for ability to acetylate choline under the conditions described by Nachmansohn and co-workers.[5,11] The results are summarized in Table III, 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 sample No. 300 was probably not significant.

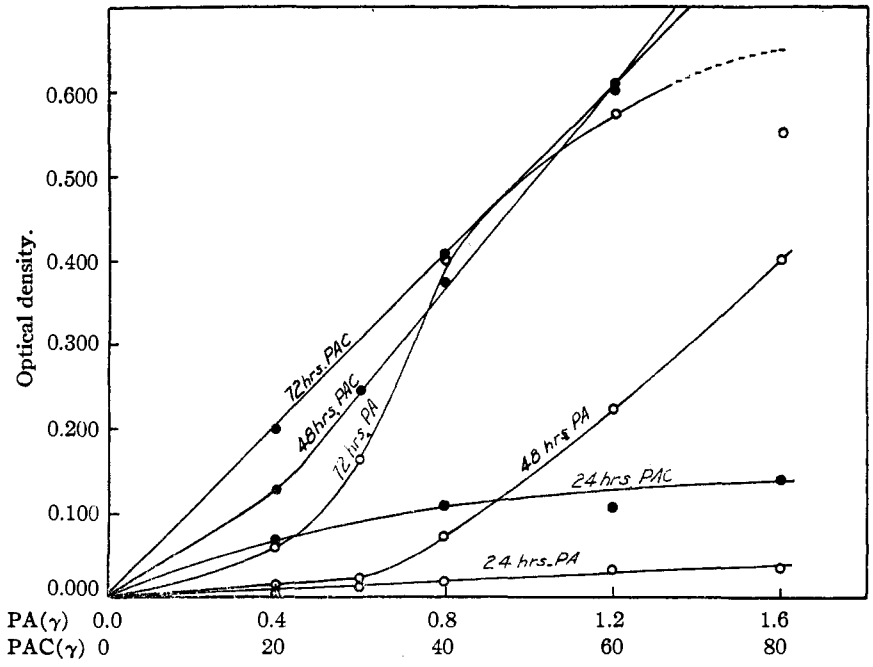

Fig. 1.—The growth response of PA and PAC in *A. suboxydans*.

TABLE III

THE ACETYLATION OF CHOLINE BY COENZYME A AND PAC

Material added	Height of contraction curve, mm.	
Acetylcholine (6.5γ)	50	
Acetylcholine (3γ)		40
Control without ATP	3	
Apoenzyme from rat brain without dialysis	11	18
Coenzyme A (3 mg.)	11	
PAC, no. 300 (30 mg.)	6	4
PAC, no. 700 (30 mg.)	0	0
PAC, no. 701 (30 mg.)	0	0

The Behavior of Alkali toward the Pantothenic Acid Conjugate

Neal and Strong[12] reported the existence of an alkali-stable form of pantothenic acid which remained in pork liver after alkaline hydrolysis and 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 determining the *A. suboxydans* activity of liver autoclaved with alkali, and by noting the extent of degradation of PAC concentrates by alkali and their possible conversion into alkali-stable residues.

The results in Table IV indicate that the conjugate is different from the alkali-stable factor, since heating of sample 701 with alkali destroyed virtually all of 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 takes place is 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 IV). The Neal–Strong factor (or something biologically indistinguishable from it) may thus be considered as one of the degradation products of PAC.

Comparison of the Conjugate with Blood Pantothenic Acid

Wright[13] noted that pantothenic acid existed in bound form in blood. The vitamin appeared to be combined to protein, since 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 the above conditions.

(11) D. Nachmansohn and A. L. Machado, *J. Neurophysiol.*, **6**, 397 (1943).

(12) A. L. Neal and F. M. Strong, THIS JOURNAL, **65**, 1659 (1943)

(13) L. D. Wright, *J. Biol. Chem.*, **141**, 261 (1943).

TABLE IV

THE BEHAVIOR OF ALKALI TOWARD THE PANTOTHENIC ACID CONJUGATE

Sample no.	Sample	Weight	Total volume of sample Ml.	Subst.	Time of standing at room temp., hr.	Time of autoclaving (15#), hr.	Millimoles of NaCl added[a]	Pantothenic acid activity, γ/g. *L. arabinosus* Hot water extract[b]	*L. arabinosus* Enzyme digest[c]	*A. suboxydans* Hot water extract
1	Pork liver	100 g.	600	H_2O	0	2	600	33	33–40	30–60
2	Pork liver	100 g.	600	0.1 *N* NaOH	0	2	0	0.5	6.7–7.8	5.2
3	PAC No. 701	200 mg.	20	H_2O	0	2	10	0.7	3	500
4	PAC No. 701	200 mg.	20	0.5 *N* NaOH	0	2	0	1.0	10	12
5	PAC No. 701	250 mg.	5	H_2O	48	0	0[d]		10	500
6	PAC No. 701	250 mg.	5	0.5 *N* NaOH	48	0	0		10	400
7	PAC No. 701	250 mg.	5	0.5 *N* NaOH	48	1.5	0		80	0

[a] Since *A. suboxydans* is influenced by moderate concentrations of sodium chloride, a sufficient amount was added to equal that present after alkali hydrolysis and neutralization. [b] Samples treated under stated conditions, filtered; filtrates assayed for PA with *L. arabinosus* 17-5(10). [c] Samples treated under stated conditions, then digested with takadiastase and papain, filtered and assayed for PA (10). [d] The amount of NaCl present in samples (6) and (7) would not influence the growth of *A. suboxydans*.

Properties of the Conjugate

Certain important properties of the conjugate have been observed in connection with concentration studies. Although the molecule appears to be fairly large as indicated by its failure to dialyze through cellophane into running distilled water over a sixty-hour period, it is not precipitated at all by the Folin–Wu tungstic acid reagent. The latter behavior suggests that it may be a polypeptide containing several acidic groups. The previous statement[9] that the conjugate appears to contain glutamic acid is in line with these observations. 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.

The conjugate is only slightly precipitated by barium hydroxide under the conditions employed by Nachmansohn and Berman.[5] This is in contrast to their results with the coenzyme for choline acetylation. The precipitation with barium hydroxide has been used in spite of the large losses in activity, however, since it is a very effective agent for removing the impurities associated with the conjugate. Concentrations of over ten-fold in *A. suboxydans* activity are regularly obtained by this means.

Charcoal (Norite A), which can be used for the adsorption of free pantothenic acid or the diphosphate,[14] also adsorbs the conjugate. Adsorption is nearly complete when 10% by weight of charcoal is employed. On the other hand, attempts at elution have so far been unsuccessful. Lloyd's reagent is effective (60–80% adsorbed when 200% by weight is used), and elution with dilute ammonia is nearly quantitative. Combination of this treatment with tungstic acid precipitation regularly results in concentrations in activity of over twenty fold. Lloyd's reagent removes much extraneous coloring matter, so that the eluates possess a light yellow color. The ion exchange resins are also fairly effective in both acid and alkaline solutions (equal weights adsorb 50–80%), but weaker adsorbents such as corn starch and barium sulfate are of little value.

(14) D. W. Woolley, *J. Biol. Chem.*, **134**, 461 (1940).

Experimental Methods

The organisms used for testing β-alanine, pantothenic acid and PAC were *Saccharomyces cerevisiae*, strains L.M. and G.M., *Lactobacillus arabinosus* 17-5 and *Acetobacter suboxydans*, A.T.C.C. No. 621, respectively. All tests were performed using previously published methods.[2,8,10] "Free" pantothenic acid was determined by direct assay with *L. arabinosus*. Enzyme digestions were carried out with takadiastase and papain.[15]

The acetylation of sulfanilamide was performed according to Lipmann.[16]

Coenzyme A or PAC in proper concentrations were placed into the chambers of Warburg flasks, together with a salt mixture, sodium acetate, ATP, sulfanilamide and dialyzed pigeon liver. After shaking ninety minutes the residual sulfanilamide concentrations were determined by the method of Bratton and Marshall[17] in an electrophotometer.

The acetylation of choline in the presence of ATP was performed in a Warburg apparatus under an atmosphere of nitrogen with the same technique as used by Nachmansohn and co-workers.[5,11] The method for determining acetylcholine followed essentially Stephenson and Rowatt's modification[18] of Chang's method.[19] The standard acetylcholine solution was prepared immediately before use. A solution of eserine was likewise prepared in buffered frog Ringer's solution. The rectus abdominis muscle (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 bubbles for about one hour. The Ringer's solution was then drained off and replaced by eserinized buffered Ringer's solution and aerated for another hour. The muscle was then used for testing acetylcholine activity.

Concentration Studies

Fresh pork heart was freed as much as possible from blood and connective tissue, passed several times through a fine meat grinder, and diluted with about twice its volume of water. Live steam was introduced and the mixture

(15) V. H. Cheldelin, M. A. Eppright, E. E. Snell and B. M. Guirard, Univ. Texas Publ. No. 4237, 15 (1942).

(16) F. Lipmann, *J. Biol. Chem.*, **160**, 173 (1945).

(17) A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, **128**, 537 (1939).

(18) M. Stephenson and E. Rowatt, *J. Gen. Microbiol.*, **1**, 279 (1947).

(19) H. C. Chang and J. H. Gaddum, *J. Physiol.*, **79**, 255 (1933).

was boiled for a few minutes. After cooling, the residue was removed by filtration, washed with water and discarded. The filtrate was brought to *p*H 4.0–4.5 with acetic acid, and after standing several hours at 5° was filtered again. The resulting filtrate was then precipitated either with 20–30 volumes of acetone, or with barium hydroxide until precipitation was complete and a slight excess of the alkali remained. The solid from the acetone precipitation was usually of a very dark color and contained a large percentage of protein. It was hygroscopic but would not redissolve completely in water. Since the volume of acetone required was so great, barium precipitation was usually employed.

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

By combining the above treatments with tungstic acid precipitation, adsorption with Lloyd reagent, and dialysis, concentrates containing up to 5% pantothenic acid activity have been obtained, based upon the total solid matter present. Further studies are in progress to accomplish the isolation of the pure conjugate.

Acknowledgment.—The authors wish to acknowledge with thanks, gifts of coenzyme A and alkaline phosphodiesterase from Drs. G. D. Novelli and F. Lippman, to Ruth S. Langdon and Eleanor C. Parsons for technical assistance, and to Dr. Rosalind Wulzen for helpful suggestions and the use of laboratory facilities for the testing of acetylcholine activity.

Summary

A conjugate of pantothenic acid (abbr. PAC) has been obtained in partially purified form from pork heart. It appears to be roughly twice as active for the growth of *A. suboxydans* as is the free vitamin. Growth of this organism is also more rapid with the conjugate than with pantothenic acid. The present conjugate is different from coenzyme A, the alkali-stable form of pantothenic acid or the protein conjugate found in blood.

From the behavior toward tungstic acid and dialysis, PAC appears to be an acidic molecule of molecular size considerably greater than pantothenic acid. Studies are in progress to accomplish the isolation of the pure material.

CORVALLIS, OREGON RECEIVED JULY 6, 1948

[CONTRIBUTION FROM THE DIVISION OF ORGANIC CHEMISTRY, THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH]

Streptomycin. IX.[1] The Stepwise Degradation of Mannosidostreptomycin

BY HOMER E. STAVELY[2] AND JOSEF FRIED

Streptomycin concentrates have been shown to contain in addition to streptomycin a second antibiotic substance which has been termed streptomycin B or mannosidostreptomycin.[1] The degradation of this substance to derivatives of streptidine, streptobiosamine and D-mannose has been the subject of a preliminary communication.[3] The present paper gives a detailed account of those findings and, moreover, presents experimental data showing that in mannosidostreptomycin D-mannose is attached glycosidically to the streptobiosamine moiety through one of the three free hydroxyl groups of N-methyl-L-glucosamine.

Mannosidostreptomycin was isolated in the pure state as the crystalline reineckate which was converted to an amorphous hydrochloride.[1] Analyses of these two salts indicated that this antibiotic is a triacidic base of the composition $C_{27}H_{49}O_{17}N_7$. The difference in composition, $C_6H_{10}O_5$, between this substance and streptomycin appeared to be due to one hexose unit attached to the latter with the loss of one molecule of water. This assumption was substantiated[4] by the following degradation reactions.

When a solution of mannosidostreptomycin in 1.3 *N* methanolic hydrogen chloride was allowed to stand at room temperature for two days and the residue from the methanol solution, after careful neutralization with silver carbonate, was acetylated, an amorphous material was obtained, which upon chromatography on alumina afforded the two anomers of methyl tetraacetyl-D-mannopyranoside, as well as methyl tetraacetyl streptobiosaminide dimethyl acetal[5] and octaacetyl streptidine,[6] the latter two substances identical with the products obtained by similar degradation of streptomycin.

Confirmatory evidence as to the presence of D-mannose, streptobiosamine and streptidine in

(1) Paper VIII of this series: J. Fried and E. Titus, THIS JOURNAL, **70**, 3615 (1948).

(2) Present address: Commercial Solvents Corporation, Terre Haute, Indiana.

(3) J. Fried and H. E. Stavely, THIS JOURNAL, **69**, 1549 (1947).

(4) The experimental evidence adduced in this paper does not permit any conclusions as to the position of attachment of the streptobiosamine moiety to streptidine in mannosidostreptomycin. It is most likely, however, that this linkage involves the 4-hydroxyl group of streptidine as has been shown to be the case in streptomycin by Kuehl, Peck, Hoffhine, Peel and Folkers, *ibid.*, **69**, 1234 (1947).

(5) N. G. Brink, F. A. Kuehl, Jr., E. H. Flynn and K. Folkers, *ibid.*, **68**, 2557 (1946).

(6) R. L. Peck, R. P. Graber, A. Walti, E. W. Peel, C. E. Hoffhine, Jr., and K. Folkers, *ibid.*, **68**, 29 (1946).