THE LIBERATION OF PANTOTHENIC ACID FROM COENZYME A*

By G. DAVID NOVELLI, NATHAN O. KAPLAN, AND FRITZ LIPMANN

(From the Biochemical Research Laboratory, Massachusetts General Hospital, and the Department of Biological Chemistry, Harvard Medical School, Boston)

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It was reported previously that coenzyme A contained pantothenic acid bound in such a manner as to make it unavailable in microbiological tests (1). A liberation of β-alanine on acid hydrolysis indicated early the presence of pantothenic acid, which was confirmed by chick assay (2). While the chick assay for pantothenic acid and the β-alanine assay by the yeast growth test yielded equivalent amounts of pantothenic acid, the microbiological assay for pantothenic acid with *Lactobacillus arabinosus* was practically negative with the intact coenzyme. The problem arose, therefore, how to liberate pantothenic acid from the coenzyme.

Since pantothenic acid itself is very sensitive to any severe treatment with strong acid or alkali, the use of enzymatic methods to free the vitamin seemed most hopeful. It had been observed variously that the coenzyme activity was destroyed enzymatically. It appeared probable that such destruction may be due to the removal from pantothenic acid of attached groups which were essential for coenzyme activity, but, on the other hand, inhibitory to the utilization of the vitamin by intact microorganisms. A very rapid inactivation had been found with preparations of intestinal phosphatase (3) as well as with the pigeon liver extract used in acetylation experiments (4). Inactivation by intestinal phosphatase was accompanied by a complete liberation of organically bound phosphate. Inactivation by the pigeon liver extract, however, proved not to be due to a removal of phosphate but to a so far undetermined reaction. A combination of the two agents eventually proved necessary for a complete removal from pantothenic acid of all attached groups. After the incubation of coenzyme A with intestinal phosphatase and liver extract, the microbiological assay of the coenzyme checked well with the results obtained by chick assay and by the β-alanine test with acid-hydrolyzed coenzyme. Only little capacity to liberate pantothenic acid in coenzyme was found with clarase, mylase, and prostate phosphatase.

Enzyme Preparations

Liver Extract—The extract is routinely prepared from acetone powder of pigeon liver as described by Kaplan and Lipmann (5). Chicken liver may

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be used equally well, but very little activity was found with rabbit liver extract. The enzyme responsible for the liberation of pantothenic acid is relatively unstable. Cooling is, therefore, necessary during the extraction and centrifugation. The inactivation of coenzyme as described in the assay method has to be omitted because standing at room temperature destroys the enzyme rapidly. The enzyme is, however, perfectly stable in the frozen state and is preserved in a deep freeze for continued use. The extracts used in these experiments were prepared by rubbing acetone powder into 10 times its weight of 0.02 M bicarbonate solution with outside cooling. The final extract is obtained by centrifugation for 15 minutes at high speed in the cold.

Intestinal Phosphatase—The procedure of Schmidt and Thannhauser (6) is followed essentially. Dr. Gerhard Schmidt has been kind enough to make available to us such modifications as were applied more recently: Digestion of the intestinal extract with trypsin and toluene at 37° is now continued for 1 to 2 weeks, instead of 24 hours, the toluene being renewed frequently. Ammonium sulfate precipitation is then carried out as described, followed by overnight dialysis against ammonium acetate-ammonia buffer. This enzyme solution contains very little pantothenic acid and was found suitable for our experiments. Trypsin does not interfere, and it is, therefore, unnecessary to remove it for our purpose.

A number of experiments were carried out with a highly purified preparation kindly given to us by Dr. Gerhard Schmidt.

Recently a dry preparation of intestinal phosphatase was prepared at Armour and Company in Chicago, which corresponds in activity to Schmidt and Thannhauser's crude phosphatase. It was made available to us in generous quantities and has been used successfully in our tests. It contains only negligible amounts of pantothenic acid. A 2 per cent solution of the dry powder was used, containing 20 to 30 units (6) per ml.

Intestinal phosphatase is strongly inhibited by phosphate (6). Therefore, phosphate may not be used as buffer, and the concentration of phosphate in the sample should be kept as low as possible. We buffer our solutions with bicarbonate, adding enough to bring the pH to 8 to 8.5, which is near enough to the somewhat more alkaline pH optimum of the intestinal phosphatase.

Enzymatic Inactivation of Coenzyme A and Liberation of Pantothenic Acid

Preliminary experiments had shown that neither intestinal phosphatase nor liver extract alone would satisfactorily liberate pantothenic acid, although both agents completely inactivated the coenzyme. This finding complicated the problem but offered, on the other hand, some clues to the constitution of the coenzyme. It appeared that at least two different

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linkages had to be broken to free pantothenic acid of attached groups which prevented independently its utilization as a vitamin in microbiological tests. The chemical aspects of the problem will be discussed elsewhere.

Separate and Combined Action of Two Enzymatic Principles—Details of the pantothenic acid assay of enzymatically treated coenzyme A will follow in the paragraph describing the method of pantothenic acid liberation even-

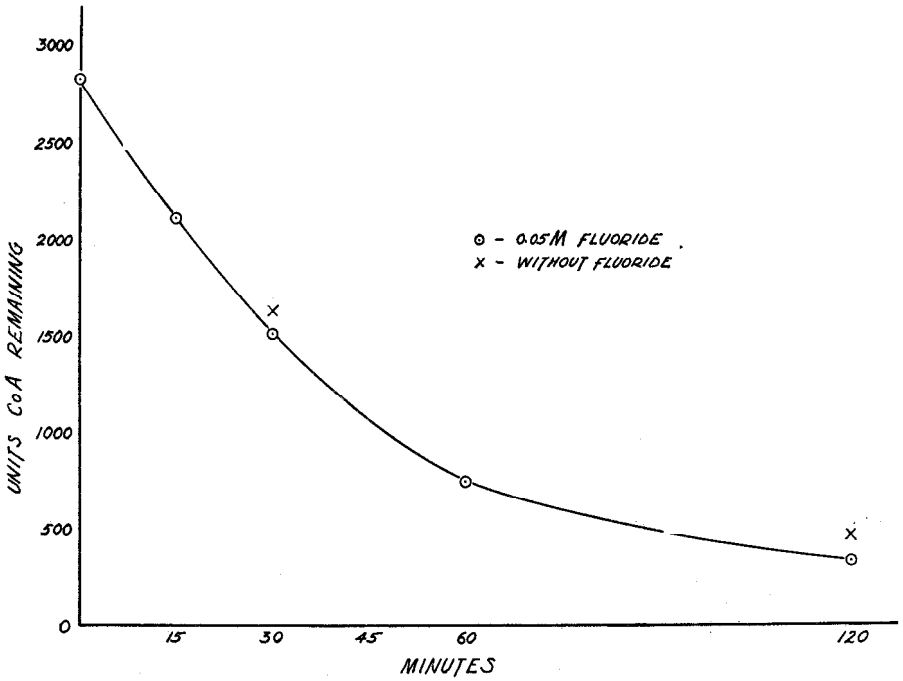

FIG. 1. Destruction of coenzyme A by fresh pigeon liver extract. The reaction mixture contained 10 ml. of liver extract and 2800 units of coenzyme A in a final volume of 67 ml.; incubated at 37°. A relatively large amount of liver extract was used in this experiment to match the amount of coenzyme A used. Aliquots were taken for assay.

tually adopted. The microbiological response is used in the following experiments as a measure of free pantothenic acid.

Effect of Liver Extract—The autolytic inactivation by liver extract of a heat-stable factor necessary for sulfanilamide acetylation was observed early in the study of enzymatic acetylation (4). This observation led eventually to the isolation and partial identification of coenzyme A. Some data on the inactivation were included in earlier publications, particularly in the recent description of the method for coenzyme A estimation (5). The gradual destruction of added coenzyme A with time is shown in Fig. 1,

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from which it appears that it is a relatively slow process. In our routine procedure, a 3 hour period of incubation was found necessary. The data of Fig. 1 show, further, that the addition of fluoride does not prevent coenzyme A inactivation. Fluoride, however, prevents practically completely the liberation of phosphate by the liver extract. In Table I liberation of phosphate and of pantothenic acid in the presence and absence of fluoride is compared with inactivation with variously treated enzyme preparations.

TABLE I

Effect of Pigeon Liver Extract

Coenzyme A preparations of about 100 units per mg. were used, containing approximately 9 per cent phosphorus and 8 to 10 per cent pantothenic acid.

	Coenzyme A inactivation	Phosphate* release	Pantothenic acid liberation
	per cent	*per cent*	*per cent*
Fresh extract	90–100	25–50	30–50
" " + 0.05 M NaF	90–100	0	0
Dialyzed extract	25	25	25–30
" " + 0.05 M NaF	0	0	0
Aged extract	25–50		15–20

* Phosphate was measured by the method of Fiske and Subbarow (13).

TABLE II

Effect of Intestinal Phosphatase on Release of Inorganic Phosphate and Pantothenic Acid from Coenzyme A

The composition of the coenzyme A preparations was similar to those in Table I.

Phosphatase* added	Phosphate liberated	Coenzyme A inactivation	Pantothenic acid liberated
ml.	*per cent*	*per cent*	*per cent*
0.05	90	100	25
0.10	98	100	28
0.15	98	100	28
0.20	100	100	30

* The highly purified preparation of Dr. Schmidt was used. It contained 50 units per ml. The samples were incubated for 3 hours at 37°.

A considerable loss of activity with dialysis and aging indicates the instability of the inactivating liver enzyme. This emphasizes its non-identity with phosphatase, which is quite stable.

Effect of Intestinal Phosphatase—In a manner analogous to that with the liver enzyme, the functional inactivation with phosphatase is compared with liberation of phosphate and pantothenic acid in Table II. The data show that increase of enzyme concentration above a certain level does not

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increase markedly the liberation of pantothenic acid; even at the highest level not more than 30 per cent of the total pantothenic acid becomes available by dephosphorylation of coenzyme A. The phosphatase used in these experiments was a highly purified preparation, and could not have contained more than traces of other enzymes. It is suggested by this observation that in the microbiological assay the product of dephosphorylation of coenzyme A is only one-third as active as free pantothenic acid.

Combined Action of Two Enzymes—The data with the two enzymes separately indicated that a mixture of phosphatase and liver might liberate pantothenic acid completely. Liver extract alone had given up to 50 per

TABLE III

Comparison of Various Preparations of Liver Enzyme for Release of Pantothenic Acid

Intestinal phosphatase 0.25 ml.; other preparations 0.05 ml.; pH 8.5. Total volume 1.5 ml. 3 hours incubation at 37°.

	Liberation of pantothenate	
	Total	Excess due to liver enzyme
	per cent	*per cent*
Intestinal phosphatase alone	20	
+ fresh pigeon liver enzyme	90	70
+ aged pigeon liver extract (4 hrs., room temperature)	20	0
+ dialyzed pigeon liver extract (24 hrs., 5°)	30	10
+ 70% ammonium sulfate fraction of fresh pigeon liver extract	39	19
+ 42% ammonium sulfate fraction of fresh pigeon liver extract	35	15
+ rabbit liver extract	25	5
+ chicken " "	92	72

cent liberation. Such liberation of pantothenic acid was, however, abolished by the addition of fluoride which suppressed phosphate liberation. Therefore, combinations of intestinal phosphatase and liver enzyme were tested systematically. The results of such experiments are shown in Table III. The excess liberation due to liver extract measures its activity towards the residual linkage in the coenzyme. After inactivation of the liver extract by aging or dialysis, neither addition of boiled fresh extract nor of cysteine restored activity. Some fractionation with ammonium sulfate was tried but not very successfully. It appears, furthermore, from Table III that pigeon and chicken liver are about equally active; rabbit, hog, and beef liver were only slightly active.

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The results of the experiments discussed are summarized in Table IV which shows that, with suitable amounts of the two enzymes, the full equivalent of pantothenic acid appears after treatment. This observation suggested a method for pantothenic acid liberation in coenzyme-containing material.

Procedure

In mapping out the present procedure, consideration had to be given to the relatively large pantothenic acid blank introduced by one of the enzyme solutions. It has been mentioned that the intestinal phosphatase preparation, fortunately, contained only negligible amounts of pantothenic acid. This is not true, however, for the liver enzyme. The 0.05 ml. of fresh pigeon liver routinely used has a content of 0.5 to 1.5 γ of pantothenic

TABLE IV

Summary of Action of Liver Extracts and Intestinal Phosphatase

	Coenzyme A inactivation	Phosphate liberated	Pantothenic acid liberated
	per cent	*per cent*	*per cent*
Fresh liver extract	90–100	25–50	30–50
" " " + 0.05 M NaF	90–100	0	0
Intestinal phosphatase	100	100	20–30
Fresh liver extract + intestinal phosphatase	100	100	85–95

acid, mostly in the bound form. The optimum region for the microbiological assay is 0.02 to 0.2 γ.

With a base-line of about 1 γ, the sample to be assayed should contain not much less than 5 γ of total pantothenic acid. In an emergency it was found possible, however, to determine 1 to 2 γ with reasonable accuracy. The final dilution for the microbiological assay, then, has to be 100- to 250-fold. The high dilution of the sample for the eventual microbiological test has the advantage of minimizing turbidities in tissue extracts which otherwise might interfere with the nephelometric determination of the growth response.

Pantothenic Acid Assay of Coenzyme A-Containing Material—For the liberation of bound pantothenic acid, 0.05 ml. of fresh pigeon liver extract, 0.2 ml. of intestinal phosphatase (= 4 to 6 units (6)), and 0.05 ml. of 0.12 M sodium bicarbonate were added to the sample. 5 ml. volumetric flasks were used. The volume was uniformly adjusted with water to 1.3 (1 to 1.5) ml., and the flasks, including the blank with enzymes, were incubated

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for 3 hours in a water bath at 37°. At the end of the incubation period, water was added to the 5 ml. mark. To bring the pantothenic acid content within the range of the microbiological assay the samples were then further diluted (see the third column, Table V). The diluted samples were tested at two levels, 1.0 ml. and 2.0 ml., and the results of the two levels were averaged. Table V gives a detailed example of the results of such an experiment.

The pantothenic acid is measured by the method of Skeggs and Wright (7), with the medium of Cheldelin *et al.* (8), with *Lactobacillus arabinosus* 17-5 at two levels in duplicate. The individual samples for the microbiological assay were prepared as described by the authors and autoclaved for 10 minutes at 120° before inoculation with the organism. We found that coenzyme A is not destroyed by autoclaving at neutral

TABLE V

Assay of Total and Free Pantothenic Acid

Assay No.	Boiled rabbit liver extract	Dilution for microbiological test	Pantothenic acid			
			Per ml. diluted sample*	Dilution factor	Per extract sample	Per ml. liver extract†
	ml.		γ	γ	γ	γ
1	0.2	1:12.5	0.065	66.5	4.3	21.5
2	0.4	1:25	0.063	133	8.4	21.0
3	0.5	1:25	0.088	133	11.7	23.3
4	0.7	1:50	0.057	265	15.3	21.8
5	0.9	1:50	0.072	265	19.1	21.3
6	Blank	1:25	0.010	133	1.3	

* Corrected for blank.

† *Free* pantothenic acid 0.7 γ per ml. of liver extract (determined by direct microbiological assay with 0.1 ml. of liver sample, undiluted).

or slightly alkaline reaction. The readings were made, after 16 to 20 hours incubation, with a Klett colorimeter. The pantothenic acid values were read from a standard curve run with each assay.

Free pantothenic acid is determined by direct microbiological assay on the sample at conveinent levels.

Comparison of Coenzyme Function and Pantothenic Acid Content in Tissues

The main purpose of the experiments reported in this paragraph was to determine how large a part of tissue pantothenic acid was bound in coenzyme A. Boiled tissue extracts were prepared as described previously (5), and particular care was taken to avoid autolysis before boiling the tissue. The procedure of enzymatic liberation was then used on such tissue extracts for pantothenic acid determination.

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The experiment of Table V, which was presented as an example for the procedure, was carried out with samples of boiled liver extract and likewise belongs with this series. It showed that, in the fresh tissue, only 3.3 per cent of the total pantothenic acid was present in free form. The fact that so large a quantity of pantothenic acid was liberated with the method developed for pantothenic acid determination in coenzyme A indicated that pantothenic acid in liver was present mostly, if not entirely, bound in coenzyme A. An even more convincing test was possible by comparison of the pantothenic acid values by microbiological assay with coenzyme activity expressed in our units. It was reported previously (1) that coenzyme A preparations contained, independent of purity, 0.65 γ of pantothenic acid per unit.

TABLE VI

Relation of Coenzyme A Activity and Pantothenic Acid Content in Organs of Rabbit

All values are given per gm. of wet weight of tissue.

	Coenzyme A	Pantothenic acid			
		Free	Total	Bound	
				Found	Calculated*
	units	γ	γ	γ	γ
Liver	112	1.2	75	74	73
Heart	26.4	3.3	20.7	17	17
Kidney	49.5	2.7	45	42	32
Brain (cortex)	40.5	3.0	18	15	26
Testes	25.6	6.0	20.4	14	17
Muscle (skeletal)	6	5.1	9.9	5	4

* Calculated by multiplication of the unit value by 0.65, the average pantothenic acid content in micrograms of a unit of coenzyme A.

Experiments were carried out, therefore, to compare coenzyme activity and pantothenic acid content in various tissues. The data obtained are reproduced in Table VI. Again free pantothenic acid generally is only a small fraction of the total pantothenic acid. Only muscle appears to contain a larger quantity, about 50 per cent, of free pantothenic acid. In the two last columns of Table VI bound pantothenic acid is compared with coenzyme units. The pantothenic acid equivalent of the unit values is obtained by multiplying by 0.65, the average pantothenic acid content in micrograms of a unit of coenzyme A. The correspondence between pantothenic acid bound as calculated and determined is reasonably good. Only brain shows a lower value of bound pantothenic acid than would be expected from unit determination. This may be due to a considerable tur-

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bidity blank in the case of the brain, even with high dilutions, in the microbiological assay.

The same correspondence of liberated pantothenic acid and coenzyme A content is found in bacteria. In Table VII such data are similarly arranged; again the calculated and determined values for pantothenic acid corresponded very closely. Some of such data had been mentioned in an earlier publication (9).

A further proof for a binding of most or all of the pantothenic acid in coenzyme A is given in the experiment represented in Table VIII. Here

TABLE VII

Pantothenic Acid-Coenzyme A Relationship in Some Representative Bacteria

	Coenzyme A	Pantothenic acid			
		Free	Total	Bound	
				Found	Calculated*
	units per gm.	γ	γ	γ	γ
Lactobacillus arabinosus (deficient)	54	7	40	33	35
Propionic acid bacteria	410	52	330	268	268

* From coenzyme A unit value, as indicated in Table VI.

TABLE VIII

Changes of Coenzyme A Activity and Free Pantothenic Acid during Liver Autolysis

The values are given per gm. of fresh tissue.

	Coenzyme A	Free pantothenic acid	Liberated pantothenic acid / Coenzyme A disappearing
	units	γ	
Fresh	45	0	
After autolysis	12	22.5	
	−33	+22.5	22.5/33 = 0.68

free pantothenic acid and coenzyme A activities were compared in fresh and autolyzed liver. A liberation of pantothenic acid by liver autolysis was very early recognized by Williams (10). In our experiment the liver was removed as fast as possible after the animals were killed, and a sample was taken at once and immediately boiled. The rest of the liver was then incubated in a moist chamber for 3 hours at 37°, and samples were taken after autolysis. Determination of the coenzyme activity before and after autolysis shows that 73 per cent of the coenzyme was destroyed. The disappearance of coenzyme was accompanied by an appearance of considerable

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amounts of free pantothenic acid. A closer comparison of coenzyme A which disappeared and liberated pantothenic acid gives an indication how much of the latter had been derived from coenzyme A. It is shown in the last column of Table VIII that 0.68 γ of pantothenic acid was liberated for every unit of coenzyme A disappearing, while 0.65 γ of pantothenic acid had been found per unit of isolated coenzyme A.

DISCUSSION

In a recent review of the microbiological assay of pantothenic acid, Jukes (11) summarizes the previous efforts to liberate the vitamin in natural materials and to make it available to the microorganism. Particularly with yeast, liver, and similar sources there remained the impression that the problem was never solved satisfactorily. The microbiological return of pantothenic acid never reached the levels obtained in the chick test whatever enzyme or enzyme mixture was used for its liberation. It was suggested that the results may indicate a linked form of pantothenic acid which was not available in the microbiological assay. During our recent work with coenzyme A, we became very well acquainted with a compound which contained pantothenic acid bound in just such a manner. The pantothenic acid bound in intact coenzyme A appeared practically unavailable to living microorganisms (1). In the chick test, however, the coenzyme returned most or all its pantothenic acid (2). In the attempts to liberate, for microbiological assay, pantothenic acid from coenzyme A, we inadvertently obtained, at the same time, a reliable method for microbiological determination of pantothenic acid. This is indicated in the results reported here, but it appears still more clearly in a comparative study by Neilands and Strong (12) on a great variety of food materials, comparing the yield of pantothenic acid by the earlier mylase method (older treatment) and by our present enzyme mixture. Although, against untreated samples, mylase treatment increased the yields, with the mixture of liver enzyme and intestinal phosphatase up to 4 times more pantothenic acid appeared, particularly from animal materials. These results are in accord with our experience on the treatment of coenzyme A with mylase or clarase. We find that very little, if any, pantothenic acid was liberated from intact coenzyme. If coenzyme A, however, had already undergone preliminary autolysis, clarase and mylase tended to split out more pantothenic acid. As will be reported in more detail elsewhere, fragments of coenzyme A may be attacked by mylase and clarase, while the intact coenzyme is stable with these enzymes.

These observations already indicated that a large fraction of tissue pantothenic acid was bound in coenzyme A. It is well known that some vitamins appear in a variety of coenzymes, while others seem to be present in

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only one metabolically active form. It appeared, therefore, important to explore how far pantothenic acid could be identified quantitatively with coenzyme A. For this purpose values for the coenzyme unit were compared with those for the pantothenic acid by use of the previously reported factor of 0.65 γ of pantothenic acid per unit of coenzyme A. The practical identity of calculated and found values of bound pantothenic acid in liver and the general close correspondence indicate that pantothenic acid appears in living cells largely, maybe only, in one metabolically active form.

SUMMARY

1. The enzymatic inactivation of coenzyme A is compared with the liberation of pantothenic acid and inorganic phosphate; with intestinal phosphatase only about one-third of the pantothenic acid is liberated with complete dephosphorylation and inactivation. With pigeon liver extract complete inactivation is parallel with partial liberation of pantothenic acid and phosphate; phosphate, as well as pantothenic acid, liberation may be suppressed by fluoride without effect on functional inactivation of the coenzyme.

2. A mixture of intestinal phosphatase and fresh pigeon liver extract liberates practically all of the pantothenic acid from coenzyme A, and makes it available for microbiological assay. A procedure is described for microbiological determination of pantothenic acid in coenzyme A-containing material with this enzyme mixture.

3. A close correspondence is found between the determined pantothenic acid content and the pantothenic acid equivalent of coenzyme A activity for animal tissues and other living cells. It is concluded that most, possibly all, cellular pantothenic acid is bound in coenzyme A.

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