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# THE OCCURRENCE OF COENZYME A IN PLANTS[1]

ELI SEIFTER[2,3]

BOTANICAL LABORATORIES, UNIVERSITY OF PENNSYLVANIA, PHILADELPHIA, PENNSYLVANIA

Coenzyme A is of interest as it is a necessary cofactor in the Krebs tricarboyxlic acid cycle, and by implication one would expect it to be present in higher plants. However, there is little direct information on the distribution and the amounts of Coenzyme A (Co A) in higher plants. This paper establishes the widespread occurrence of this coenzyme in vascular plants.

## MATERIALS AND METHODS

The plant materials were chosen as representative of the various families of flowering plants. More detailed work was done on spinach, mung bean, pea, wheat germ, and corn since these plants are being widely used in current studies of plant metabolism.

DIRECT ASSAYS: Seeds, dried plant parts, or materials high in Co A may be directly assayed for Co A by the methods listed below: Weighed samples were homogenized with an equal weight of water at 80° C. After two minutes in the Waring blendor, the material was transferred to a beaker and boiled for five minutes. Occasionally more water had to be added at this point. The material was filtered through cheesecloth and clarified by centrifuging at $4600 \times g$. The sediment was re-extracted in a similar manner and the filtrates combined. It was found that a third extract did not contain a measurable amount of Co A. The combined filtrates were directly assayed for Co A.

CONCENTRATION OF Co A: Since the extracts must contain five units of Co A per ml for the methods used here, the extracts of many plants cannot be directly assayed. In such cases extracts prepared as above were concentrated at reduced pressure below the freezing point to one-fourth of their original volume. The concentrated solutions were used for Co A assay.

In some experiments the plant tissues were dried and defatted with cold acetone. After the acetone treatment the tissue was dried in vacuo over $P_2O_5$; and these powders were then extracted and assayed by the direct method. The acetone powders were useful for the preparation of enzymes active with Co A. Attempts to make acetone powders containing Co A by acetone precipitation of plant extracts failed.

A coenzyme A standard was obtained from Dr. Lawrence L. Lachat of Armour and Company which, according to analysis in that laboratory contained 10 units of Co A per mg. In addition two standard coenzyme preparations were made following the method described by Kaplan and Lipmann (4) and Lipmann, Kaplan, et al (5). The first of these prepared as an acetone powder from a boiled extract of rabbit liver was found by Mr. H. S. Moyed to contain approximately 0.3 unit per mg. The second standard was obtained as a barium salt from a yeast coenzyme concentrate. Comparison with the other two standards showed a content of 5.3 units of Co A per mg.

ASSAY PROCEDURE: The Co A was assayed by the method of Kaplan and Lipmann (4). When extracts of acetone powders of pigeon livers are aged, the Co A is destroyed and the ability to acetylate sulfanilamide is lost; but is regained upon the addition of Co A. Since the acetylated sulfanilamide fails to react in the Bratton and Marshall (2) method for the determination of free sulfanilamide, the quantity of Co A can be determined by the disappearance of sulfanilamide. The colored compound resulting from the reaction of sulfanilamide with nitrous acid and 1-naphthyl ethylenediamine is read in a photocell colorimeter with a filter giving a peak transmission at 530 to 540 $m\mu$.

Another method of assay for Co A used was based on the procedure of Stadtman et al (8). The method depends upon the enzyme transacetylase (Stadtman, 7) which catalyzes the reversible reaction

(1) acetyl phosphate + Co A $\longrightarrow$ acetyl Co A + phosphate

The enzyme is also capable of catalyzing an irreversible reaction

(2) acetyl Co A + arsenate $\longrightarrow$ acetate + Co A

In a system supplied with acetyl phosphate, Co A, and arsenate the transacetylase quantitatively converts acetyl phosphate into acetate and phosphate. Stadtman has shown that this arsenolysis is proportional, within very wide ranges, to Co A concentration when the system is supplied with ample transacetylase enzyme and arsenate. Since the disappearance of acetyl phosphate is a function of Co A concentration the reaction may be followed quantita-

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[2] Supported by a grant of the National Cancer Institute of the U. S. Department of Health, Education and Welfare.

[3] Present address: Central Research Department, Monsanto Chemical Co., Dayton, Ohio.

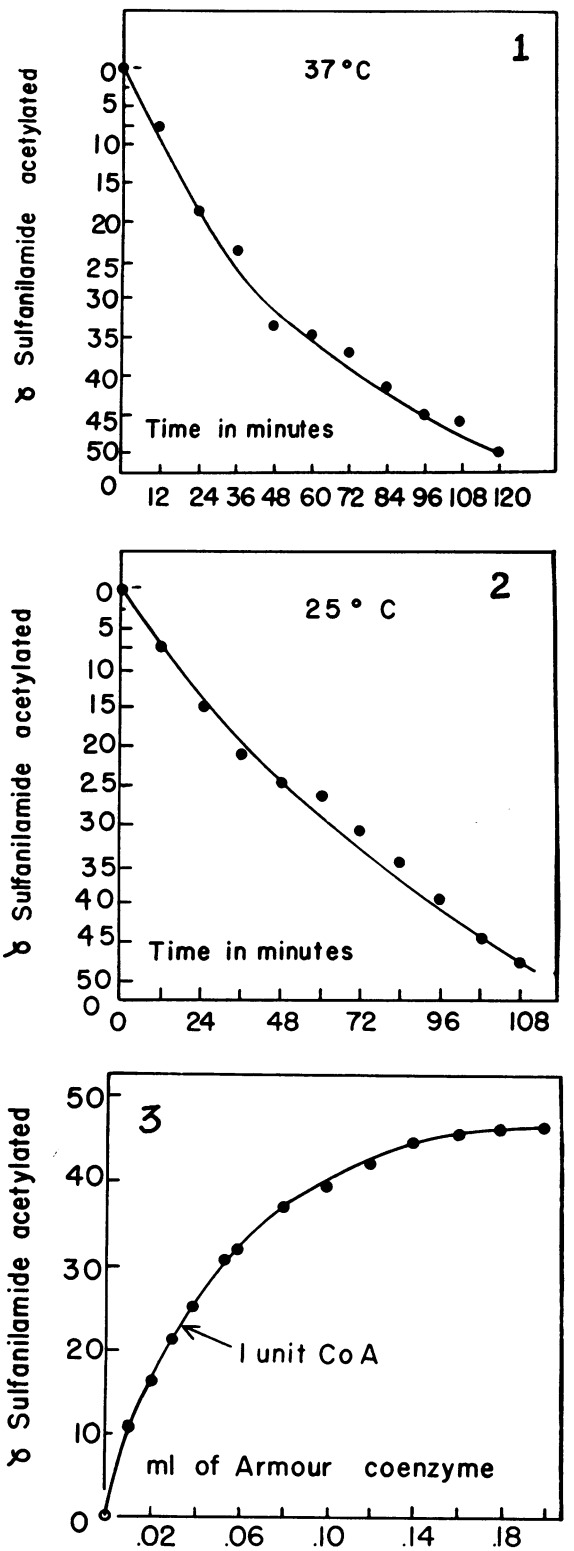


FIG. 1. Acetylation of sulfanilamide by Co A with pigeon liver enzyme; total volume 1.0 ml, pH 7.1.

FIG. 2. Similar experiment to that shown in figure 1, except the temperature was 25° C instead of 37° C.

tively by the method of Lipmann and Tuttle (6) for the determination of acyl phosphates. The latter workers have adapted a well known organic reaction in which an acid anhydride of an organic acid is reacted in acidic solution with hydroxylamine. The reaction product is the hydroxamic acid. The latter compound reacts with ferric chloride to form a complex possessing a characteristic color.

As a standard for the determination of acetyl phosphate the monohydroxamic acid of succinic acid was used. This was prepared by the method of Fieser (3) from succinic acid and acetic anhydride. Succinic anhydride thus prepared melted at 119° C and was free from acetic anhydride, acetic or succinic acids.

Using the purest acetyl phosphate he was able to obtain, Lipmann determined that 1 mole of the hydroxamic acid formed from succinic acid produces 80 % of the color produced by 1 mole acetohydroxamic acid, when both react with an excess of ferric ions.

The enzyme preparation, transacetylase, was prepared from *Streptococcus hemolyticus* kindly supplied by Lederle Laboratories. It has been assayed by Mr. Harris Moyed and found to contain 75 units of transacetylase per ml of enzyme solution. One unit is that amount of enzyme which catalyzes, when supplied with five units of Co A, the arsenolysis of 1 $\mu$M of acetyl phosphate in 15 minutes, at 25° C and pH = 7.5.

The standard enzyme system defined by Lipmann is one in which 0.25 ml of the enzyme solution, when saturated with Co A (5 units) and supplied with adequate amounts of acetate, adenosine triphosphate (ATP), sulfanilamide and proper activators, and reacting in a volume of approximately 1 ml, is able to acetylate approximately 50 $\mu$g of sulfanilamide in 2 hours at 37° C.

The enzyme prepared from pigeon liver was assayed and found to be acceptable for use in analyzing materials for Co A. This is illustrated in figure 1 in which activity is plotted against time.

The amount of coenzyme which will produce half maximal activity in the standard system has been defined as 1 Lipmann unit. By microbiological assay it has been found to represent 0.7 $\mu$g of bound pantothenic acid. Under conditions of this assay the Armour sample contained 11 units of Co A per mg.

It has been shown by Kaplan and Lipmann (4) that the unit is a constant and is independent of the absolute amount of acetylation taking place in a particular reaction. Therefore the course of the reaction was followed under two different temperature conditions. The time course at 37° C has already been shown in figure 1, while figure 2 shows the time course at 25° C. It will be seen that at 25° C less product is formed, but the shape of the curve is es-

FIG. 3. The relation between Co A and amount of sulfanilamide remaining. One ml of Co A solution contained 2.5 mg of Armour Co A; or 11 units per mg. The pH was 7.1, 37° C; time = 125 min.

sentially the same as that obtained at 37° C. Incubation for 90 minutes yields more than 90 % of the product that is formed in a 2 hour period. Accordingly the conditions of the experiment were changed so that the tubes were incubated for 90 minutes at 25° C.

For all assay experiments a standard sulfanilamide reaction mixture with the following composition was prepared:

| | |
|---|---|
| 10.0 ml | 0.004 M sulfanilamide |
| 2.5 ml | M magnesium acetate |
| 8.0 ml | 0.05 M potassium adenosine triphosphate (ATP) |
| 10.0 ml | 0.2 M potassium citrate |

The mixture was kept in 5 ml vials in a frozen state. It was not kept for more than one week before use. For the assay of Co A the following were used:

| | |
|---|---|
| 0.3 ml | standard sulfanilamide reaction mixture |
| 0.1–0.3 ml | solution being analyzed |
| 0.1 ml | 0.15 M neutralized cysteine solution |
| 0.25 ml | pigeon liver enzyme preparation |
| | water to make volume 1 ml |

The pH of the reaction mixture was maintained slightly basic to brom thymol blue.

After incubation the reaction was stopped by the addition of 4 ml of 5 % trichloroacetic acid (TCA). The reaction mixture was then centrifuged and 1 ml aliquots were taken for analysis of residual sulfanilamide.

Having standardized the conditions it was then possible to determine the response of the standard acetylating mixture to varying amounts of Co A. Figure 3 shows the effect of concentration of the coenzyme upon the acetylating ability of the standard enzyme system.

For this experiment a stock solution containing 25 mg of the coenzyme concentrate (Armour) was dissolved in 10 ml. Aliquots ranging from 0.05 to 0.3 ml were added to tubes containing the standard amounts of enzyme and reaction mixture. Amounts corresponding to the addition of 0.01 to 0.06 ml of the coenzyme solution were made by diluting 1 ml of the stock solution five-fold. Figure 3 shows that 45 μg of sulfanilamide were acetylated in 2 hours, and that 0.03 to 0.04 ml were required to produce half maximal activity.

TABLE I

COMPARISON OF TWO METHODS OF ASSAY FOR COENZYME A

| SOURCE | PREPARATION | UNITS PER GM DRY WT | |
|---|---|---|---|
| | | ACETYLATION | ARSENOLYSIS |
| Armour Coenzyme Concentrate | Acetone powder | 11,000 | 10,500 |
| Brewers yeast | Ba salt of coenzyme conc. | 5,300 | 6,000 |
| Rabbit liver | Acetone powder | 300 | 300 |
| Wheat germ | Direct * | 41 | 40 |
| Corn germ | Direct | 38 | 39 |
| Corn meal | Direct | 22 | 21 |
| Pea meal | Direct | 44 | 43 |
| Tomato fruit | Conc.** | 11.2 | 12 |

* Direct = hot water extract.
** Conc. = hot water extract, concentrated.

TABLE II

COENZYME A CONTENT OF SAMPLES OF REPRESENTATIVE PLANTS AND PLANT TISSUES

| SOURCE | TISSUE | PREPARATION * | UNITS PER GM DRY WT | TEST USED ** |
|---|---|---|---|---|
| Apple | Fruit | VC | 3 | Ac |
| Brussels sprouts | Bud | D | 20.6 | Ac |
| *Bryophyllum calycinum* | Leaf | VC | 6.2 | Ac |
| *B. crenatum* | Leaf | VC | 5.1 | Ac |
| Buckwheat | Seed | D | 30 | Ac |
| Cabbage | Leaf | VC | 10.4 | Ac |
| Cabbage | Bud | VC | 10.4 | Ac |
| Canada balsam | Pollen | D | 38 | Ac |
| Carrot | Xylem | VC | 16.7 | Ar |
| Carrot | Phloem | VC | 21.6 | Ar |
| Cattail | Pollen | D | 27.3 | Ac |
| Corn | Pollen | D | 42.2 | Ac |
| *Cryptostegia grandiflora* | Seed | D | 32.7 | Ac |
| *C. madagascariensis* | Seed | D | 30 | Ac |
| *Cryptostegia* hybrid (largely sterile) | Seed | D | 13 | Ac |
| *Hevea* | Seed | D | 41 | Ac |
| Kok Saghyz | Seed | D | 28.5 | Ac |
| Kok Saghyz | Root | D | 19 | Ac |
| Kok Saghyz | Leaves | D | 9.3 | Ac |
| Lettuce | Leaf | VC | 14.2 | Ac |
| Mustard | Seed | D | 39.7 | Ar |
| Onion | Bulb | AP | 18.1 | Ac |
| Orange | Seed | D | 14.2 | Ac |
| Orange | Rind | VC | 2.1 | Ar |
| Orange | Pulp | VC | 36 | Ar |
| Peanut | Seed | D and AP | 47 | Ac and Ar |
| Pine | Seed | D | 34 | Ar |
| *Polypodium* sp. | Leaf | VC | 4.1 | Ar |
| *Polypodium* sp. | Spores | D | 23.5 | Ar |
| Potato | Buds | D | 17.6 | Ar |
| Potato | Tuber | AP | 5.3 | Ar |
| Spinach | Leaf | VC | 8.2 | Ac |
| *Valeriana* sp. | Root | D | 24 | Ac |
| *Valeriana* sp. | Leaves | VC | 13.2 | Ac |
| Yam | Root | AP | 13 | Ac |

* AP = Acetone powder; D = Direct; VC = Vacuum concentrated.
** Ac = Acetylation; Ar = Arsenolysis.

## EXPERIMENTAL RESULTS

Table I presents assay data obtained under the modified conditions with extracts from specified

sources. The data obtained by using the arsenolysis with primary and secondary Co A standards are also presented in this table. The excellent agreement between the results obtained by both methods is more apparent than real. This is due to the fact that the standard Co A used in the arsenolysis experiments was itself standardized by the acetylation test.

Table II lists the results of assaying a large number of plants and plant parts for Co A. The wide distribution of Co A in plants is apparent.

The distribution of Co A in the various parts of 5 day old mung bean seedlings are shown in table III. Except for the low content in seed coats no marked inequality in the distribution in the parts was found.

An attempt was made to further concentrate Co A from plant material. The method of preparation consisted of a modification of that used by Beinert et al (1).

Two kg of wheat germ were boiled for ten minutes with five liters of water and after ten minutes

TABLE III

COENZYME A CONTENT OF TISSUES OF MUNG BEAN (*Phaseolus aureus*) SEEDLINGS *

| TISSUE | UNITS PER GM DRY WT |
|---|---|
| Plumule and first leaves ..... | 22.8 |
| Cotyledons ..... | 15.1 |
| Hypocotyl ..... | 14.7 |
| Radicle ..... | 24 |
| Seed coat ..... | 2.4 |
| Whole seed ** ..... | 42 |

* Five day old seedlings; acetone powders were prepared and twice extracted with water at 85° C.
** Ungerminated.

at 100° C the preparation was centrifuged. The protein was precipitated by the addition of 10 % TCA and removed by centrifugation. The supernate was neutralized to pH 7 with 0.1 M NaOH and a second precipitate formed. This was removed by centrifugation and the volume was made up to 5,000 ml. The pH was adjusted with 1 *N* HCl to 5.5. Fifty grams of Nu-Char charcoal was added and the mixture was put in the cold for 4 hours. The charcoal containing absorbed Co A was recovered by centrifugation and filtration, and then was extracted with 500 ml of water-saturated phenol, at room temperature for three hours. After filtration, the cold filtrate was extracted with two 100 ml portions of chloroform to remove the phenol. The water solution of the coenzyme was made 0.5 *N* with respect to $H_2SO_4$. The coenzyme was precipitated from this acid solution by the cautious addition of crystalline CuCl prepared according to the method of Fieser (3). The cuprous salt of the coenzyme was collected on a filter and then suspended in 20 ml of water. It was decomposed by treatment with excess $H_2S$ for several hours. The precipitated copper sulfide was then removed by filtration and excess $H_2S$ by a stream of hydrogen. After this aeration the solution was treated with 10 volumes of acetone and kept in the deep freeze for 24 hours, during which time a precipitate formed, this was dried over $P_2O_5$. Upon analysis by the acetylation procedure it was found to contain 49 units of Co A per mg. Microscopic observation indicated that a good portion of the residue consisted of cellulose fibers. The preparation of this sample represents approximately one thousand fold increase in the activity of Co A on a dry weight basis. However, such a preparation is still only 10 % pure.

## SUMMARY

The study of the distribution of Co A in plants made use of the assay procedures described as acetylation and arsenolysis. Both of these were adequate from the standpoint of sensitivity. Aqueous extracts of plant materials generally required concentration before they could be assayed for Co A content. Co A was found to be present in a large number of flowering plants, and non-flowering vascular plants.

In general, the coenzyme is present in highest concentration in the seed and rather low concentration in leaf tissue, even when these are considered on a dry weight basis. The lowest value detected was 2.1 units per gm dry weight in the orange rind, the highest was found in the peanut seed—47 units per gm dry weight.

The coenzyme was isolated from wheat germ in an impure state by a chemical technique based upon its properties as a mercaptan and as a nucleotide.

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