THE PURIFICATION OF COENZYME A BY ION EXCHANGE CHROMATOGRAPHY

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With the increasing demand for coenzyme A (CoA) to be used as a substrate in enzyme studies, a need has developed for additional methods of isolating this substance from natural sources. Two methods have been published whereby CoA of reasonably high purity can be prepared (1, 2). Both of these procedures are lengthy and the over-all yields are relatively poor (10 to 30 per cent). One of these methods is relatively expensive, since large amounts of glutathione are required (2).

In the present communication a method is described for the preparation of CoA of 50 to 65 per cent purity from crude yeast extract in fair yield (50 to 55 per cent) by a relatively simple two-step chromatographic procedure. In this procedure the CoA is adsorbed on charcoal and is concentrated by selective elution with ammoniacal acetone. The CoA concentrate thus obtained is adsorbed on a Dowex 1 resin and is isolated as a distinct fraction by elution with strong buffer. The final product contains 200 to 270 units (1) of CoA per mg.

Materials and Methods[1]

Preparation of Charcoal—Acid-treated, degassed charcoal is prepared by suspending about 400 gm. of unground Nuchar C charcoal in 4 liters of 6 N HCl. The suspension, in two 4 liter flasks, is kept under a vacuum overnight during which time the charcoal settles, leaving a clear supernatant solution. Sufficient charcoal is transferred to a large glass column (10 cm. in diameter) to give a bed height of 29 cm. The glass column is fitted at the bottom with a stainless steel screen (80 mesh) which is packed around the edges with glass wool. The charcoal is washed with distilled water until the pH of the effluent is 3.5 to 4.0.

Preparation of Resin—Dowex 1 × 2 (2 per cent cross-linked resin)[2]

[1] All operations are carried out at room temperature unless otherwise indicated.

[2] Dowex 1 × 10 (10 per cent cross-linked resin) was previously tested by us and found unsatisfactory for CoA isolation. We are indebted to Dr. Waldo Cohn, who called our attention to the availability of Dowex resins with low cross-linkage (low divinylbenzene content).

(200 to 400 mesh) is washed by suspending 5 pounds in 30 liters of 3 N HCl. The suspension is stirred mechanically for 8 hours and then allowed to settle for 2 days and the supernatant solution is decanted. This step is repeated three to four times until the ultraviolet absorption of the supernatant solution at 260 mμ is 0.080 or less in a 1 cm. cell. The resin is washed with 30 liter batches of water (two to three times) until the test for chloride ion in the wash water is faint. The resin is transferred to a glass column fitted with a porous sintered glass base and is washed with 3 M sodium formate until the chloride ion test on the effluent is faint. Finally, it is washed with water until the effluent is practically salt-free (20 parts per million on Barnstead purity meter, referred to NaCl).

Other anion exchange resins tested, including Duolite A-3 chloride, Dowex 2 × 10 formate, and Dowex 1 × 10 chloride and formate proved unsatisfactory.

CoA Assay—All purification steps are followed by direct CoA analysis by means of the phosphotransacetylase assay system (3) with a slight modification to permit more rapid determinations. The method is as follows: Water to give a final volume of 1.0 ml.; tris(hydroxymethyl)aminomethane hydrochloride buffer (1 M, pH 8.0), 0.1 ml.; dilithium acetyl phosphate, 6 μM; cysteine hydrochloride, 10 μM; an aliquot of the test solution containing 0.5 to 3.0 units of CoA; and phosphotransacetylase (4), 8 units, are added in the indicated order. The reaction mixture is incubated at 28° for 5 minutes and then 0.1 ml. of potassium arsenate (0.5 M, pH 8.0) is added. After 10 minutes, the residual acetyl phosphate is estimated by the hydroxamic acid method (5). Under these conditions the amount of acetyl phosphate decomposed is proportional to the amount of CoA present in the reaction mixture. Up to twelve samples can be conveniently examined in a single assay. For reference, a standard tube containing 2 to 2.5 units of CoA and a control sample containing no CoA are included in each assay. All CoA samples must be adjusted to pH 7.5 to 8.0 prior to testing.

The reference standard used in this study was an acetyl CoA sample prepared by the enzymatic acetylation of CoA and isolated by paper chromatography (6). The acetyl-adenine ratio of this preparation was 0.97 and it was assumed to contain 316 units of CoA per μM (1).

Preparation of Yeast Extract—3 kilos of dried yeast (Anheuser-Busch, strain G) are dropped into 15 liters of boiling water. The suspension is stirred and boiled vigorously for 5 minutes. 25 pounds of cracked ice are added and the cold suspension is centrifuged at about 2000 × *g* for 30 minutes. The slightly turbid supernatant solution (19 liters) should contain 100,000 ± 30,000 units of CoA.

Charcoal Chromatography of Yeast Extract[3]—19 liters of yeast extract are adjusted to pH 3.0 with 6 N HCl (200 to 250 ml.). The extract (which becomes turbid upon acidification) is passed through a charcoal column (10 cm. × 29 cm.) at a rate of 1 liter per 3 minutes. The milky white effluent is discarded. The charcoal is washed with 10 to 15 liters of distilled water, 2 to 4 liters of 40 per cent aqueous acetone, and then with 40 per cent aqueous acetone containing 1.0 ml. of concentrated ammonium hydroxide solution (28 per cent) per liter. The eluate is collected in 2 liter fractions. The pH of successive fractions increases gradually from 3.3 to 5.0 and then increases sharply to pH 7.5 to 9.0. In most runs the CoA is concentrated in the eluates having a pH of 3.8 to 7.0; occasionally, however, the fraction on either side of these limits will contain some CoA

TABLE I

Charcoal Chromatography of CoA in Crude Yeast Extract

Fraction No. (2 liters)	Color	pH	CoA, per cent of initial
1	Straw, turbid	3.4	0
2	"	3.6	0
3	" clear	3.9	7
4	" "	4.0	26
5	Yellow, "	4.2	28
6	Deep amber	5.0	26
7	" "	8.5	7
8	Amber	9.4	0
Total			94

also. Data from a typical experiment showing the distribution of CoA in the eluates from a charcoal column are given in Table I.

The fractions containing CoA are pooled, the pH is adjusted to 1.7 with 6 N HCl (any precipitate which forms is removed by filtration through a fluted Schleicher and Schüll No. 588 filter paper), and the CoA is precipitated by the addition of 5.5 volumes of cold (2°) acetone. After 1 to 2 hours the precipitate is recovered by filtration through a Büchner funnel (10 cm. in diameter). The precipitate is washed with acetone and with ether and dried in a vacuum desiccator over P_2O_5. 5 to 6.5 gm. of dry acetone powder are obtained containing 10 to 18 units of CoA per mg. The over-all recovery based on the boiled yeast extract is about 80 to 95 per cent.

Chromatography on Dowex 1 Resin—15 gm. of acetone powder are dis-

[3] This charcoal step is a modification of a method developed by M. Soodak and F. Lipmann (private communication).

solved in water and the pH is adjusted to 8.1 with 2 N KOH and the volume to 200 ml. Any material which does not dissolve is removed by centrifugation.

The crude CoA solution is placed on a Dowex 1 × 2 formate column (13 cm. × 15 cm. square). Invariably the colored front progresses unevenly, due presumably to severe shrinkage of the resin caused by the high

TABLE II

Ion Exchange Chromatography of CoA

Fraction No. (500 ml.)	Adenine*	CoA†	$\frac{\mu M \text{ CoA}}{\mu M \text{ adenine}}$
	μM	μM	
0‡	165	0	0
1	9,900	0	0
2	8,500	0	0
3	1,500	0	0
4	660	6	0.009
5	435	5	0.011
6	520	0	0
7	490	0	0
8	275	0	0
9	151	25	0.16
10	113	75	0.66
11	123	86	0.70
12	117	89	0.76
13	107	75	0.70
14	85	49	0.58
15	69	19	0.28
Total recovered.....	23,210	429	
" initial.....	25,900	450	

* Calculated from optical density at 260 mμ on the basis of a molecular extinction coefficient of 15.9×10^6 sq. cm. per mole.

† Calculated on a value of 316 units of CoA per μM (1).

‡ Filtrate collected during the adsorption and washing.

salt concentration of the CoA solution. Therefore, when the farthest edge of the front has progressed approximately 1 cm., the upper 1 cm. layer of resin is gently stirred up with the overlying solution. Following this treatment the progress of the front appears uniform. After the CoA solution has been applied, the column is washed with two 100 ml. portions of water and is eluted with a solution containing 25.8 ml. of formic acid (88 per cent) per liter (0.6 M) and 18.9 gm. of ammonium formate per liter (0.3 M). The rate of flow (attained with a hydrostatic pressure of 2 to 5 feet) is about 450 ml. per hour. 500 ml. fractions are collected and assayed for CoA and for adenine (optical density at 260 mμ).

Results showing the development of a typical chromatogram are given in Table II. Elution of the CoA begins when 20 to 23 resin bed volumes (eight or nine fractions) of eluate have been collected. The CoA is completely eluted when an additional 10 to 12 resin bed volumes (Fractions 9 to 15) are collected. In the experiment described, the CoA-adenine ratio of Fractions 9 to 15 varied from 0.16 to 0.76. In other experiments ratios as high as 1.0 have been obtained. The over-all recovery of CoA from the chromatograms is generally 80 to 95 per cent; however, in some instances recoveries as low as 70 per cent have been observed.

Concentration of Eluate—Since the CoA from the Dowex 1 column is distributed in a large volume (2500 to 3000 ml.) of strong formate buffer, it is desirable to concentrate it and remove the excess salt before attempting to recover the CoA by acetone precipitation. This is accomplished by readsorbing the CoA on a small charcoal column and eluting with ammoniacal acetone. To obtain CoA of highest purity, the eluates from the ion exchange chromatogram in which the CoA-adenine ratio is 0.6 or greater are pooled; the pH is adjusted to 2.0 with 6 N HCl (about 100 ml. per 2000 ml. of eluate) and the solution is passed through a small charcoal column (2.5 cm. in diameter × 12 cm.) at a rate of 1.5 liters per hour. The optical density of the effluent at 260 mμ should not exceed 0.020. The column is washed with 300 ml. of water and with 300 ml. of 40 per cent acetone. The CoA is finally eluted with 40 per cent acetone containing 1 ml. of concentrated ammonium hydroxide per liter. The flow rate is adjusted to about 3.0 ml. per minute. 50 ml. fractions are collected. The alkaline fractions are neutralized with 4 N nitric acid. The CoA is generally all eluted when the pH of the effluent is alkaline.

The CoA in these fractions is sufficiently concentrated to permit detection by the nitroprusside test. Drops of the various fractions are placed on a strip of No. 3 Whatman filter paper and dried, and the paper is dipped first into Reagent I and then into Reagent II of Toennies and Kolb (7). The appearance of a red spot, which is greatly intensified by shaking the paper strip in ethyl ether, indicates the presence of CoA. The fractions containing CoA (usually Fractions 3 to 6) are pooled (final volume, 150 to 200 ml.). The pooled sample is acidified to pH 1.7 with 4 N nitric acid and the CoA is precipitated by the addition of 7 volumes of cold acetone. The precipitate is allowed to settle overnight, the supernatant solution is decanted, and the CoA is transferred to a centrifuge tube. The precipitate is washed two times with acetone (room temperature), once with ether, and dried in a vacuum desiccator over P_2O_5.

From 15 gm. of CoA concentrate (10 units per mg.) 300 to 400 mg. of purified material are obtained containing 200 to 270 units of CoA per mg. The over-all yield is generally 50 to 55 per cent. Occasionally, yields as

low as 40 per cent and as high as 70 per cent have been obtained. In addition to this CoA of relatively high purity, CoA preparations containing 150 to 200 units per mg. can be obtained as a by-product by working up the eluates from the Dowex 1 column having a CoA-adenine ratio of less than 0.6.

Composition—The compositions of some purified preparations are given in Table III. For comparison, analytical data on a sample of Pabst CoA are also presented. The contents of phosphorus, adenine, and enzymatically active CoA are 65 to 67 per cent of theory. The rather good stoichiometry among these three components precludes the presence of other adenine nucleotides as major contaminants.

TABLE III

Analysis of CoA Preparations

Sample No.	Total P*	Adenine†	CoA‡	$\frac{\mu M\ P}{\mu M\ CoA}$	$\frac{\mu M\ adenine}{\mu M\ CoA}$
	μM per mg.	*μM per mg.*	*μM per mg.*		
VIIa	2.56	0.82	0.77	3.32	1.06
XIII	2.55	0.91	0.83	3.07	1.10
Pabst (Lot 401-27)	2.66	0.87	0.85	3.13	1.02
Pure CoA§	3.9	1.3	1.3	3.00	1.00

* Determined by the method of Fiske and Subbarow (8).

† Calculated from optical density at 260 mμ on the basis of an assumed molecular extinction coefficient of 15.9×10^6 sq. cm. per mole.

‡ Determined by arsenolysis of acetyl P with transacetylase.

§ Theoretical values (1).

It should be pointed out that CoA samples of lesser purity prepared from the fractions just preceding the peak fractions from the Dowex 1 column (*i.e.* the material with a CoA-adenine ratio of less than 0.6) do contain significant amounts of ATP.

Since no reductive steps are employed in the above chromatographic procedure, the CoA isolated should be present mainly as the mixed disulfide derivative of other sulfhydryl compounds (1, 9, 10). Paper chromatography of the isolated material in an 80 per cent phenol-20 per cent water solvent shows that the CoA moves to a spot identical with a ninhydrin-reactive material (R_F = 0.35). Following reduction of the CoA with hydrogen sulfide it moves with an R_F of 0.51, while the amino compound has an R_F of 0.46, which is identical with that found for glutathione. These observations suggest that the CoA isolated may be present largely as the mixed disulfide derivative of glutathione.

Chromatography on Cation Resin—A further purification of the CoA may be achieved by reduction with hydrogen sulfide followed by chromatog-

raphy on Dowex 50 (H^+) to remove the amino compounds as previously described (2). In a single experiment, 100 mg. of CoA (263 units per mg.) were neutralized with KOH (400 μeq.), and hydrogen sulfide was passed through the solution (25 ml.) for 3.5 hours. The solution was adjusted to pH 2.5 and gassed with helium to remove the hydrogen sulfide. The acidified solution (3.0 ml.) was passed through a Dowex 50 (H^+) column (3 cm. $\times$ 1 cm. square) and the CoA in the percolate (6.0 ml.) was precipitated by the addition of 70 ml. of acetone. 45 mg. of precipitate were recovered containing 328 units of CoA per mg., corresponding to a purity of 78 per cent. Since all of the adenine-absorbing material initially present was recovered in the percolate from the Dowex 50 column, the low recovery of CoA (55 per cent) is due either to incomplete acetone precipitation of the reduced CoA or to decomposition of the CoA on the acid resin.

Comments

The ion exchange chromatography of CoA concentrates has also been used on a scale one-third, one-half, and 2 times that described. In these instances the size of the resin column was varied proportionally by varying the cross-sectional area of the column while maintaining the height of the resin bed constant. The rate of elution and volumes of the fractions collected were varied correspondingly. The elution pattern, yields of CoA, and purity of the product obtained were similar, except that a somewhat poorer over-all yield (40 per cent) was obtained in a single experiment at 2 times the scale described.

Usually 14 to 16 hours are required to elute the CoA from the Dowex 1 column. In the absence of an automatic fraction collector which permits uninterrupted elution overnight, the elution can be discontinued and finished on the following day.

In addition to the charcoal-treated, yeast acetone powder concentrates a number of other CoA concentrates have been tested as sources of CoA for ion exchange chromatography. Of those tested, an Armour CoA concentrate of hog liver (13 units per mg.) and a Sigma triphosphopyridine nucleotide concentrate (10 per cent triphosphopyridine nucleotide, 6.5 units of CoA per mg.) gave products of lower purity (about 150 units per mg.) and lower yields (30 to 35 per cent), while chromatography of a CoA concentrate (6.0 units per mg.) obtained from *Streptomyces fradiae* gave poor results. On the other hand, a crude Pabst CoA concentrate from yeast containing only 1.3 units of CoA per mg. gave results similar to those obtained with the more purified starting material prepared by us. However, the capacity of the resin column in terms of CoA was only about one-seventh as great as when the more purified material was used. A preliminary charcoal purification of the Pabst CoA concentrate gave a CoA

preparation (10 units per mg.) which for resin chromatography is equally as good as the material prepared by us from crude yeast extracts. Thus, charcoal treatment of other commercially available crude CoA concentrates may provide suitable starting materials for the ion exchange chromatography.

SUMMARY

A method has been developed for the purification of coenzyme A by charcoal and anion exchange chromatography. When the procedure was applied to hot water extracts of dried yeast, preparations containing 200 to 270 units of coenzyme A per mg. (50 to 65 per cent pure) were obtained in yields of 50 to 55 per cent.

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