PREPARATION AND PURIFICATION OF COENZYME A CONCENTRATES

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No pure substance with coenzyme A (CoA) activity has yet been isolated despite the great biochemical importance of CoA and the expenditure of much time and effort in several laboratories (1-4). Previous work in this laboratory (3) has, therefore, been continued. It was considered essential to develop a cheap, practical, large scale procedure for preparation of preliminary concentrates, which would serve both as a source of CoA for biochemical studies and as a starting point for further purification.

A promising lead in this direction was uncovered by the discovery that an intermediate fraction from the preparation of triphosphopyridine nucleotide (TPN) according to the procedure of LePage and Mueller (5) contained CoA in relatively high concentration. This fraction was the crude nucleotide mixture precipitated by acetone from charcoal eluates derived from aqueous extracts of fresh hog liver (5). Use of this procedure, with minor modifications, has made possible the preparation of some 260 gm. of a stable, dry, light colored powder containing approximately 1 per cent of pantothenic acid (PA). This product will be designated Fraction A. None of the PA in this fraction was free, and essen-

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‡ Traveling Fellow of the Rockefeller Foundation, on leave from Oregon State College, 1949-50.
COENZYME A CONCENTRATES

Technically all of it was present in a CoA-active form as judged by its ability to promote acetylation of aromatic amines.

This material has been subjected to a variety of further fractionation procedures, most of which led either to large losses of activity or to only small increases in purity. Of the procedures tried, solvent distribution gave the greatest purification.

EXPERIMENTAL

Analytical Methods—CoA activity was determined both by sulfanilamide acetylation (6) and by the modified acetylation method of Handschumacher et al. (7). Recently a transacetylation test suggested by Ochoa has also been used. This assay was based on the CoA-dependent destruction of acetyl phosphate by arsenoanalysis in the presence of bacterial transacetylase (8–10), the disappearance of the acetyl phosphate being followed by the hydroxamic acid method of Lipmann and Tuttle (11). Nearly all the fractions have also been assayed for free and total PA with Lactobacillus arabinosus, as previously described (3, 12). Except where otherwise indicated, PA values refer to bound, CoA-active PA. Purity of the various fractions is expressed as the percentage of PA present on the dry basis or as Lipmann CoA units per mg. of dry weight. 1 Lipmann unit is considered equivalent to 0.65 γ of PA (13). Consequently, the percentage of PA is equal to Lipmann units per mg. divided by 15. The CoA purity should be about 4 times the PA purity based on a molecular weight of 800 ± 50 for CoA (14).

Preparation of Fraction A

The procedure of LePage and Mueller (5) was followed exactly through the precipitation of the charcoal eluate with acetone. The precipitate was collected by centrifugation, rubbed up with acetone, and centrifuged several times until it became granular, and was finally dried in vacuo at room temperature. A typical flow sheet for one of the more successful preparations (Preparation 2, Table I) is given. This procedure has been applied to approximately 3000 pounds of hog liver, in batches ranging from about 10 to 400 pounds each.2 A summary of most of the runs made is presented in Table I.

Although somewhat variable, the yields and potency of Fraction A were reasonably satisfactory except for three runs. The very low yields in these cases were traced to the use of a particular lot of Nuchar C-190,

1 S. Ochoa, New York University, private communication.
2 Most of the large scale preparations were carried through the charcoal adsorption stage at The Wilson Laboratories, Chicago. This assistance, and particularly that of Dr. Stanley Hier, is gratefully acknowledged.
which held the activity too tightly to permit elution. This lot was of a finer mesh size than batches previously used, but was thought to be otherwise comparable.

Preparation of CoA Concentrate from Hog Liver

Fresh, trimmed livers, 86 lbs.

1. Divided into 2 portions for grinding, heating, and cooling (5)
2. Filtered through cheese-cloth and sparkler filter

Combined filtrate, 100 liters (1365 mg. PA, 4% free, 1120 mg. CoA-active PA (7))

1. Divided into 2 portions
2. Each portion treated with 260 gm. Nuchar C-190 carbon

Combined charcoal

1. Elute with 5200 ml. 10% pyridine in water
2. Extract eluate with 2 × 1.5 liters chloroform

Chloroform (discard)

Aqueous eluate (328 mg. PA, none free, 365 mg. CoA-active PA)

1. HNO₃ to pH 2
2. 5 volumes acetone, overnight at 5°C

Supernatant (113 mg. PA, 16% free, 36.5 mg. CoA-active PA)

1. Washed with acetone
2. Dried in vacuo

Pale buff powder, 12.46 gm. (200 mg. PA, none free, 200 mg. CoA-active PA)

In a preliminary study of the charcoal adsorption step, it was found that adsorption was equally good from the liver filtrate at any pH between 2.0 and 6.5, but that the latter value permitted better subsequent elution and precipitation with acetone. In one trial, preliminary deproteinization of the filtrate with trichloroacetic acid resulted in poorer adsorption. CoA
adsorbed on charcoal was found to be stable at least for several days at room temperature. Water saturated with phenol at room temperature was as good an eluant as 10 per cent pyridine. Study of the adsorption and elution of CoA on various carbons is being continued.

Attempts were made to substitute other adsorbents for carbon in the preparation of Fraction A. It was found that silicic acid,3 Silene-EF (a hydrated calcium hydrogen silicate),4 Hi-sil,4 and Florex-XXX (an activated Florida fullers' earth)6 adsorbed 60 to 90 per cent of the CoA activity from boiled aqueous liver extracts, but that negligible amounts were removed by elution with 4 per cent aqueous ammonia. Florisil6 adsorbed less than half of the activity from extracts at pH 6.5. Adsorption on Florisil was better at pH 2, but elution in each case was poor both with aqueous ammonia and aqueous pyridine. Since the results with these silicate type adsorbents did not appear promising, no further efforts were made to use them for the preliminary purification of CoA.

**Composition of Fraction A**—The suitability of concentrates such as Fraction A as a source of CoA for other biochemical investigations will in

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### Table I

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>Liver used</th>
<th>Acetone ppt., Fraction A</th>
<th>Purity*</th>
<th>Yield per 100 lbs. liver†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lbs.</td>
<td>gm.</td>
<td>per cent</td>
<td>units per mg.</td>
</tr>
<tr>
<td>1</td>
<td>31</td>
<td>4.8</td>
<td>1.6</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td>12.5</td>
<td>1.6</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>7.9</td>
<td>2.0</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>172</td>
<td>19.8</td>
<td>1.5</td>
<td>22.5</td>
</tr>
<tr>
<td>5</td>
<td>86</td>
<td>9.3</td>
<td>0.9</td>
<td>13.5</td>
</tr>
<tr>
<td>6</td>
<td>86</td>
<td>11.6</td>
<td>0.55</td>
<td>8.2</td>
</tr>
<tr>
<td>7</td>
<td>86</td>
<td>19.4</td>
<td>0.25</td>
<td>3.7</td>
</tr>
<tr>
<td>8</td>
<td>72</td>
<td>9.9</td>
<td>1.2</td>
<td>18</td>
</tr>
<tr>
<td>9</td>
<td>400</td>
<td>40.9</td>
<td>1.4</td>
<td>21</td>
</tr>
<tr>
<td>10</td>
<td>400</td>
<td>47.0</td>
<td>0.9</td>
<td>13.5</td>
</tr>
<tr>
<td>11</td>
<td>486</td>
<td>50.0</td>
<td>0.25</td>
<td>3.7</td>
</tr>
<tr>
<td>12†</td>
<td>963</td>
<td>30.3</td>
<td>0.95</td>
<td>14.3</td>
</tr>
</tbody>
</table>

* Expressed as per cent of total PA on the dry basis or as Lipmann units per mg. of dry weight. Substantially all of the PA present appeared to be in the CoA-active form.

† Expressed as mg. of total PA.

‡ Two runs combined.

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3 Merck, reagent grade.

4 Pittsburgh Plate Glass Company, Columbia Chemicals Division, Barberton, Ohio.

general depend to an important degree on the presence or absence of various B vitamins and coenzymes in the preparations. In order to obtain some information along this line, typical samples were analyzed for the components listed in Table II. Only the pyridine nucleotides, of those determined, were present in appreciable amounts.

**Further Purification of Fraction A**

**Barium Salt Fractionation**—A sample of Fraction A (230 mg. containing 3.68 mg. of PA; purity 1.6 per cent) was suspended in 5 ml. of water,

**Table II**

<table>
<thead>
<tr>
<th>Preparation No.*</th>
<th>Component</th>
<th>Amount found</th>
<th>Bibliographic reference to analytical method used</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Total pyridine nucleotides</td>
<td>10.6</td>
<td>(15)</td>
</tr>
<tr>
<td>9</td>
<td>&quot; &quot; &quot; &quot;</td>
<td>6.9</td>
<td>(15)</td>
</tr>
<tr>
<td>4</td>
<td>TPN</td>
<td>7.8</td>
<td>(5)</td>
</tr>
<tr>
<td>9</td>
<td>&quot; &quot;</td>
<td>4.9</td>
<td>(5)</td>
</tr>
<tr>
<td>4</td>
<td>Flavin-adenine dinucleotide</td>
<td>0.36</td>
<td>(16)</td>
</tr>
<tr>
<td>9</td>
<td>&quot; &quot;</td>
<td>0.44</td>
<td>(16)</td>
</tr>
<tr>
<td>4</td>
<td>Cocarboxylase†</td>
<td>&lt;1.5</td>
<td>per gm.</td>
</tr>
<tr>
<td>4</td>
<td>Biotin</td>
<td>&lt;0.5</td>
<td>(17)</td>
</tr>
<tr>
<td>4</td>
<td>Vitamin B₁₂</td>
<td>30</td>
<td>(18)</td>
</tr>
<tr>
<td>4</td>
<td>&quot; &quot; B₁₂</td>
<td>0.9</td>
<td>(19)</td>
</tr>
</tbody>
</table>

* The numbers refer to preparations listed in Table I.
† Determined by I. C. Gunsalus.

about 50 mg. of insoluble, inactive material were centrifuged, and the supernatant was treated with 1 ml. of 25 per cent aqueous barium acetate solution. The mixture was adjusted to pH 6, the water-insoluble barium salts removed, and the filtrate diluted with 3 to 4 volumes of ethanol. The precipitate was twice reprecipitated with ethanol from water solution and was finally obtained as a white powder weighing 72 mg. and containing 2.17 mg. of bound PA (yield 59 per cent; purity 3.0 per cent).

**Ion Exchange**—It has been observed repeatedly that the CoA activity of Fraction A and similar concentrates is not held to an important degree by cation exchange resins, but is bound by anion exchangers. A typical run with each type of exchanger is summarized in Table III. Recoveries from the anion columns were fairly good, although never complete, but the purity on the dry basis was not increased. This was at least partly
due to salts introduced during elution. Recovery from cation columns was good, and purity values were increased about 1.5- to 2-fold. Similar results were obtained with Amberlites IR-4B and IR-100, respectively.

**Florisil Columns**—Florisil appeared to be a more useful adsorbent for partially purified CoA preparations than for crude liver extracts. Adsorption was essentially complete when an aqueous solution of Fraction A adjusted to pH 2 was passed through a 60 to 100 mesh Florisil column previously washed with water acidulated to pH 2. Elution with 3 per cent pyridine in water was quantitative. However, the adsorbent was dissolved to an appreciable extent under the acidic conditions used.

### TABLE III

**Behavior of CoA Concentrates toward Ion Exchange Resins**

<table>
<thead>
<tr>
<th>Type of exchanger</th>
<th>Anion; Dowex-1 (Cl⁻ form)</th>
<th>Cation; Dowex-50 (H⁺ form), 200-400 mesh</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bed dimensions, mm.</td>
<td>7 × 20</td>
<td>10 × 15</td>
</tr>
<tr>
<td>Percolation rate, ml. per min.</td>
<td>1.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Sample volume and pH</td>
<td>50 ml., pH 7.4</td>
<td>80 ml., pH 6.6</td>
</tr>
<tr>
<td>&quot; PA content, γ.&quot;</td>
<td>1460</td>
<td>2300</td>
</tr>
<tr>
<td>&quot; PA in original filtrate, γ.</td>
<td>23</td>
<td>2010*</td>
</tr>
<tr>
<td>&quot; &quot; washings, γ†</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot; eluate, γ‡</td>
<td>980</td>
<td></td>
</tr>
<tr>
<td>Total recovery, %</td>
<td>79</td>
<td>87.5</td>
</tr>
</tbody>
</table>

* The purity increased from 1.4 to 2.7 per cent.
† Column washed with 50 ml. of 0.01 N HCl, followed by 50 ml. of a solution 0.01 N in HCl and 0.02 N in NaCl.
‡ Elution accomplished with 50 ml. of a solution containing 0.01 equivalent of HCl and 0.2 equivalent of NaCl per liter.

Florisil columns adsorbed practically no CoA from neutral solutions of Fraction A, but in some cases did retain considerable amounts of impurities. Thus, when 0.9 gm. of Preparation 10 (Table I) dissolved in 180 ml. of water was adjusted to pH 7, passed through a 20 × 100 mm. column of 30 to 60 mesh Florisil, and the column washed with 40 ml. of water, 97 per cent of the PA in the sample was recovered in the effluent and washings, and the purity was increased from 0.9 to about 1.6 per cent. Subsequent elution of the column with 10 per cent aqueous pyridine removed considerable amounts of fluorescent material, probably flavin derivatives. Use of such columns would probably offer a convenient means of separating CoA from FAD.

**Readsorption on Charcoal**—The charcoal column procedure of LePage and Mueller (5) gave poor over-all recoveries of activity (30 to 40 per cent) and resulted in only about a 2-fold increase in purity when applied to
several samples of Fraction A. The chief difficulty appeared to lie in the elution step. On the other hand it was possible to obtain a somewhat better concentration by using charcoal for the selective adsorption of the impurities. For example when a solution of 200 mg. of Preparation 8 (Table I) in 120 ml. of water was adjusted to pH 5.5 and stirred with 200 mg. of Nuchar C-190, 62 per cent of the CoA activity remained in the solution after filtration, and the purity was increased from 1.2 to 2.7 per cent.

**Solvent Distribution**—The amyl alcohol-octadecylamine system of Plaut, Kuby, and Lardy (20), for distribution of organic phosphate esters, proved applicable to the concentration of CoA. The separation from other nucleotides was sufficiently sharp so that only two or three extractions were needed to effect a 3- to 4-fold increase in PA content. In a typical experiment 1 gm. of Fraction A (containing 1.6 per cent PA) was dissolved in 40 ml. of water and freed by centrifugation from the small amount of inactive insoluble matter which it contained. The supernatant was adjusted to pH 4.85 and mixed with 10 ml. of 0.1 M citrate buffer, which had previously been equilibrated at pH 4.85 with a 5 per cent solution of octadecylamine in n-amyl alcohol. To this aqueous mixture was added 50 ml. of the amine-alcohol solution (saturated with buffer). After vigorous shaking for 1 to 2 minutes, the system was centrifuged at low speed and the amine layer was withdrawn and reextracted twice with 30 ml. aliquots of fresh buffer. The CoA was then released into an aqueous phase by extracting the amine with 20 ml. of 1.1 per cent NH₄OH. The amine was discarded, and the aqueous extract was neutralized with glacial acetic acid and extracted twice with ether to remove the bulk of the dissolved amine and amyl alcohol.

After adjusting the pH to 7.4 and adding 10 ml. of 0.1 M ammonium acetate buffer saturated with the alcohol-amine system (at pH 7.4), the solvent extraction was repeated. After the CoA-amine salt had been decomposed with NH₄OH, the final aqueous extract was treated with 5 volumes of cold acetone and centrifuged. The centrifugate was thus essentially freed from traces of amine and the bulk of the inorganic salts, which remained in solution. When the centrifugate was dissolved in 10 ml. of water and dried from the frozen state, 48 mg. of a product were obtained containing 3.4 mg. of bound PA (yield 21 per cent; purity 7.1 per cent).

**DISCUSSION**

*Preparation of Fraction A*—It was necessary to select as the starting material only firm, small livers, and to avoid those from old sows, which
are likely to be large, fatty, light colored, and friable. The latter were found to be low in PA and yielded very cloudy filtrates, unsuitable for charcoal adsorption. The livers were trimmed of excess fat, but were otherwise kept intact and were chilled on crushed ice from the time of collection at the slaughter-house until they could be extracted with hot water (5). Autolytic destruction of CoA is not extensive during a period of 1 to 2 hours under these conditions.

In order to simplify the filtration process it was found desirable to grind the livers through rather large holes, approximately $\frac{1}{4}$ to $\frac{3}{8}$ inch in diameter. Ice was added to the hot extraction mixture as recommended (5), but this is probably unnecessary unless TPN is also desired, since CoA is stable at 90–100° in approximately neutral solutions. After the bulk of the solids was removed (5), the extract was passed through a small filter press6 precoated with a diatomaceous filter aid.7 The more active filtrates had a yellowish brown cast and were only faintly cloudy (transparent through at least 5 cm.). Tap water was used for the extraction.

Separation of the charcoal adsorbate was greatly facilitated by using a rather coarse charcoal8 and allowing the mixture to settle for 1 to 2 hours. The bulk of the fluid could then be siphoned or decanted with negligible losses, and the carbon collected on large Büchner funnels.

The great variation in the yields of Fraction A (Table I) emphasizes the necessity of using a charcoal which will permit satisfactory elution. The present results show that such charcoals exist, but indicate that the suitability of each new lot must be separately determined for this specific purpose. Although the over-all yield has never exceeded 10 to 15 per cent, the method described nevertheless offers a convenient and practical way of obtaining a highly potent CoA concentrate in relatively large amounts and at a total expenditure of not over 2 days time. The low over-all yield is attributable in large part to incomplete adsorption on and elution from the charcoal (see scheme). A certain amount of free PA and bound PA not in a CoA-active form also is present in various side fractions. The latter is especially abundant in the final acetone supernatant (see scheme). These are probably to be regarded mainly as degradation products of CoA, since nearly all of the PA in strictly fresh liver is present in a CoA-active form (3, 12, 13).

Further Purification of Fraction A—A wide variety of other fractionation procedures based on fundamentally different principles has been applied to Fraction A and to other concentrates prepared previously (3). With very few exceptions, no procedure has yielded a single fraction con-

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6 A sparkler filter, model 8-6 (Sparkler Manufacturing Company, Mundelein, Illinois) was used.
7 Celite 503, Johns-Manville, Chicago.
8 Nuchar C-190, "unground," approximately 20 to 60 mesh.
taining all, or nearly all, of the CoA activity, but instead the activity as a rule has divided itself among several fractions. This behavior suggests that the CoA activity of the original liver may well be due not to a single chemical substance but to several substances, each measured as CoA by the analytical methods currently in use.

Several other lines of evidence also point to the multiple nature of CoA. Solvent distribution studies indicating the existence of at least two forms have already been reported (3). Similarly, in the present work distribution with the amine solvent system (20) has split the activity of many of the Fraction A samples tested into relatively amine-soluble and amine-insoluble fractions. *Lactobacillus bulgaricus* factor (LBF) concentrates from natural sources, when subjected to paper chromatography, give rise to as many as seven zones showing LBF activity (21-23). It is likely that at least some of these represent mixed disulfides produced by combination of the reduced, —SH form of LBF with other biologically common —SH compounds such as cysteine and reduced glutathione (24). Since LBF (25) has been demonstrated to be a part of the CoA molecule (26, 27), similar considerations may also be applicable to the coenzyme. Still another possible form of CoA is the acetyl derivative, which Lynen and Reichert claim to have extracted in an impure state from actively respiring yeast (28). The fundamentally different forms of CoA are probably the reduced —SH form, the oxidized S—S form (of which a series of mixed types may exist), and the acetyl derivative.

Indications of more than one form would also result from any CoA fragments capable of showing activity in the acetylation assays (6, 7) because of resynthesis to the intact molecule during the test. LBF, for example, has been shown to produce a limited amount of CoA activity under favorable conditions (27). Such resynthesis would not be expected in the transacetylase assay, since no ATP is involved. It has in fact been found that LBF is completely inactive and that various CoA concentrates are somewhat less active for this test than for acetylation of aromatic amines.

For all these reasons it seems most probable that liver extracts, and perhaps other natural materials, contain not one but a group of pantothenic acid derivatives which possess CoA activity to varying degrees.

**SUMMARY**

Coenzyme A (CoA) concentrates have been prepared from hog liver on a relatively large scale by a charcoal adsorption process. The yield

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9 No acetyl derivative has been detected in the CoA concentrates obtained during the present work, since all samples tested without adenosine triphosphate (ATP) failed to show activity in the acetylation test (7).
of the product, which contained about 1 per cent of bound pantothenic acid (probably corresponding to about 4 per cent of CoA), depended greatly on the particular charcoal used. A wide variety of further fractionation procedures was applied to such concentrates, but relatively little additional purification resulted. The possible existence of a group of CoA-active substances is discussed.

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