STUDIES ON BIOSYNTHESIS AND METABOLIC FUNCTION OF COENZYME A

by

RUSSELL LAGRANGE JOLLEY, JR.

A THESIS

submitted to

OREGON STATE COLLEGE

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1954

APPROVED:

[redacted]

Professor of Chemistry

In Charge of Major

[redacted]

Chairman of Department of Chemistry

[redacted]

Chairman of School Graduate Committee

[redacted]

Dean of Graduate School

Date thesis is presented 4-1-'54

Typed by Mary Willits

ACKNOWLEDGMENT

The author wants to express his thanks to Dr. Vernon H. Cheldelin for his guidance, help and encouragement during the course of this work, and to Dr. T. E. King for many helpful suggestions and criticisms.

TABLE OF CONTENTS

STUDIES ON BIOSYNTHESIS AND METABOLIC FUNCTION OF COENZYME A

Part One

STUDIES ON BIOSYNTHESIS OF COENZYME A

Introduction

In the past five years, the number of metabolic reactions in which coenzyme A has been proved to play a part has increased manyfold, and there is reason to expect that future investigations will yield discoveries of yet more such reactions. As a result, coenzyme A has been a common component in enzyme studies. It is, of course, desirable that the components of enzyme studies be of the highest possible purity. Until recently, however, the only coenzyme A available was in the form of crude concentrates from liver, yeast, or other sources, and even though a high-purity (ca. 80%) preparation is now available, the cost is still quite high. This high cost has been due, not so much to the low concentration in which CoA[1] is generally found in animal tissues and microorganisms, as to the difficulties in isolation and purification. Recent improvements in the methods

---

[1] The following abbreviations will be used: CoA, coenzyme A; acetyl CoA or S-acetyl CoA, acetyl coenzyme A; LBF, Lactobacillus bulgaricus factor = pantetheine; LBF-γ-PO_4, pantetheine-4'-phosphate; ATP, adenosine-triphosphate; AMP, adenosine-5'-phosphate (muscle adenylic acid); cysHCl, cysteine hydrochloride; Tris, tris(hydroxymethyl)aminomethane; µg., microgram (10^{-6} gm.); µM, micromole (10^{-6} mole).

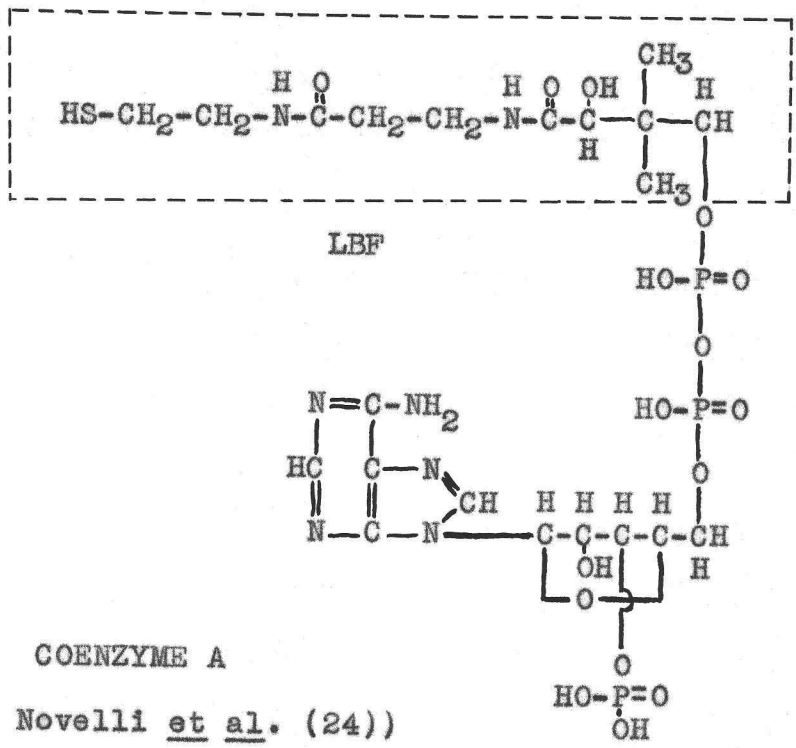
for purification of CoA from yeast have been developed by Beinert *et al.* (4) and by Stadtman and Kornberg (29), and it is likely that the high-purity product will be made more easily available than it has been in the past.

Coenzyme A has not yet, as of this date, been prepared by organic synthesis, although LBF (pantetheine), the β-mercaptoethylamine derivative of pantothenic acid, has been synthesized (27) (13). Recently, LBF-γ-PO_4 has also been synthesized (2). But even if CoA is synthesized in the near future, it is doubtful that strict organic synthesis would provide a cheap source of the compound.

In 1951, King and Strong (12) reported that LBF concentrates were able to partially replace CoA in the CoA-catalyzed acetylation of sulfanilamide by pigeon liver extracts. Partial purification of the acetylating enzyme system by ammonium sulfate fractionation led to the conclusion that LBF itself was not able to catalyze acetylation but that another enzyme system in the crude pigeon liver extract was able to synthesize CoA from LBF. On the basis of a microbiological assay for total pantothenic acid equivalents in the LBF concentrate, 15-20% of the LBF was found to have been converted to CoA. Later, Govier and Gibbons (10), using synthetic LBF, found that up to 12% of the LBF was converted to CoA by the crude pigeon liver system.

Novelli and Levintow (23), in studies on the mechanism of CoA synthesis, were able to obtain considerable

purification of the CoA-synthesizing enzyme system of pigeon liver and partial separation of the individual enzymes. In 1952, they reported that the synthesis occurred in three successive steps: (1) phosphorylation of LBF in the gamma (4') position; (2) coupling of this product to an ATP molecule to form the di-nucleotide; and (3) phosphorylation at the three position of the ribose moiety to form CoA. Each step was found to require the expenditure of one molecule of ATP. Magnesium ion was also required as a cofactor.



COENZYME A

(cf. Novelli et al. (24))

When LBF had been synthesized by a method which promised to be adaptable to relatively large scale production, it was thought that the combination of organic synthesis of LBF with enzymatic biosynthesis of CoA from synthetic LBF

might be economically feasible. In order for such a method to rival CoA production from yeast or from Streptomyces fradiae, which synthesizes large amounts of CoA during fermentation (9), the yields in the enzymatic conversion of LBF to CoA would have to be high. Moreover, the source of the crude enzyme would have to be a tissue cheaper and more conveniently used than pigeon liver.

In approaching this problem, it was decided to study in detail the quantitative aspects of the conversion of LBF to CoA by pigeon liver and then to make a survey of the synthetic abilities of a number of other animal tissues. The outcome of these studies is given in parts I and II of Results and Discussion.

Since CoA is the only pantothenic acid derivative thus far known to be metabolically active as such[2], it is assumed that any organism which demonstrates a nutritional requirement for (or the ability to utilize) pantothenic acid or a pantothenic acid derivative other than CoA must be able to synthesize CoA from the intermediate utilized.

For certain of the lactobacilli, LBF is a more potent growth factor than pantothenic acid (8). Thus it is evident that these LBF-utilizing bacteria must synthesize CoA from LBF. It was considered likely that resting cells, deprived

---

[2]There are indications that dephosphoCoA, the product of step (2) in the synthesis of CoA from LBF, can replace CoA in certain enzyme systems (32).

of nitrogen needed for growth, but supplied with an energy source, might synthesize considerable amounts of CoA. The outcome of studies on the conversion of LBF to CoA by resting Lactobacillus acidophilus cells is given under Results, part III.

METHODS AND MATERIALS

Preparation of tissue extracts - The preparation of acetone powders of tissues and of the bicarbonate buffer extracts of these powders followed the procedure described by Kaplan & Lipmann (11).

Dowex treatment of tissue extracts - The treatment with Dowex-1 (anion exchange resin) to remove endogenous CoA was carried out by the method of Novelli & Schmetz (22).

CoA assay by sulfanilamide acetylation - This assay method was the one developed by Kaplan & Lipmann (11). The sulfanilamide determination was carried out according to the procedure of Bratton & Marshall (5). A standard curve was prepared relating the number of µg. of sulfanilamide acetylated to the number of units of CoA present (Table 1a).

Preparation of cell-free extracts of Clostridium kluyveri - Dried Cl. kluyveri cells received in February 1951 from Dr. E. R. Stadtman were used in the preparation of a potent cell-free extract by the method outlined by Stadtman & Barker (28). Dowex-1 treatment of the cell-free extract was that used by Stadtman, Novelli, and Lipmann (30).

CoA assay by the phosphotransacetylase system - This method, employing the Dowex-treated cell-free extract of Cl. kluyveri, is based on the CoA-catalyzed arsenolysis of acetyl phosphate. The assay method was that used by Stadtman and Kornberg (29). The determination of acetyl

phosphate followed the procedure of Lipmann & Tuttle (16). From Table 1b it can be seen that the arsenolysis of acetyl phosphate is strictly proportional to the CoA concentration up to the level at which about 90% of the acetyl phosphate present is arsenolyzed.

Culture medium for Lactobacilli - The batches of cells required in the experiments described in part II were grown on a broth containing 0.5% each of glucose and Difco peptonized milk, 1% Difco yeast extract, and 0.2% Wilson's Liver; made up in distilled water. The pH was adjusted to 6.8-7.0.

Medium for microbiological assays - The assay medium for use with L. acidophilus was that used by Craig & Snell for Group C microorganisms (8).

Microbiological assays - The LBF standard used was "Basamin Busch" (obtained from Anheuser-Busch, Inc.). The unknowns were assayed at graded levels and only those samples having optical density values (at 550 mμ) falling on the straight line ("log phase") portion of the standard curve were taken as significant.

Preparation of cell-free extract of L. acidophilus (UT)[3] - 20 liters of the above-described culture medium were sterilized, cooled and inoculated with a one-liter inoculum of L. acidophilus (UT) cells. After 36 hours at 37°C, the

[3](UT) - University of Texas strain.

cells were harvested in a Sharples supercentrifuge, washed twice with distilled water by ordinary centrifugation, and lyophilized. The yield was 4.2 gm. of lyophilized cells. 1.8 gm. of the lyophilized cells were ground with alumina; ruptured cells and alumina were suspended in 22 ml. of cold phosphate buffer, pH 7, and centrifuged in the cold, yielding about 17 ml. of a clear reddish brown supernate. Both the cell-free extract and the cell debris, which layered out above the alumina during centrifugation, were kept frozen for later use.

Coenzyme A standard - Pabst CoA was used as a standard in all experiments. It was stated by the manufacturer to be at least 75% pure. This was borne out by the fact that 3.06 µg. gave approximately one half-maximum acetylation of sulfanilamide in the pigeon liver system of Kaplan & Lipmann. The amount required for half-maximum acetylation, defined as one unit, contains 0.7 µg. pantothenic acid equivalent (= 2.45 µg. pure CoA). The purity of the CoA standard was, therefore, 2.45/3.06, or 80%.

LBF (Pantetheine) - The LBF used in these experiments was a synthetic product prepared in this laboratory by Mr. C. J. Stewart, and kindly donated by him for use in these experiments. It was assayed with L. acidophilus (UT) against two different standards, one the Anheuser-Busch standard yeast, "Basamin Busch", the other a Parke-Davis LBF solution. By the first standard the purity of the

synthetic LBF was about 40%; assay against the second standard indicated a purity of about 70%. An arbitrary value of 50% purity was chosen as a basis for future calculations. One of the impurities was water, as the preparation was very hygroscopic; other possible impurities being pantothenic acid, β-alanyl-β-mercaptoethylamide (β-alatheine), and traces of methanol.

LBF-γ-PO_4 and Pantothenylcysteine - The LBF-γ-PO_4 used was furnished by Baddiley and Thain. The pantothenylcysteine was supplied as the disodium salt by J. F. Cavalla, Parke, Davis & Co., London.

RESULTS AND DISCUSSION

I

Coenzyme A Synthesis by Pigeon Liver Extracts

An attempt was made to measure the conversion of LBF to CoA by use of the sulfanilamide acetylation system. It was found that this system could be used to indicate the presence and probably concentration of the synthetic enzyme system in a preparation. At rather low levels of LBF (15 µg./tube) the yields appeared to be as high as 7.5% (Table 2), but never as high as the yields reported by King and Strong and by Govier and Gibbons. At higher levels of LBF (over 50 µg./tube) the amount of acetylation by LBF (and thus the apparent yield of CoA) fell off (Table 3), a maximum total acetylation being obtained at a level of about 50 µg. per tube. The apparent yield of CoA at this level was about 2.5 units, while at 500 µg. per tube the apparent yield was 0.40 units. This led to the conjecture that LBF was actually inhibiting the acetylation system, possibly by competition with CoA in the formation of the enzyme-CoA complex.

This was borne out by the results of an experiment in which known amounts of CoA were incubated with various levels of LBF in the sulfanilamide acetylation system. Acetylation was found to fall off markedly with increasing LBF concentration (Table 4). Some intermediate in the

formation of CoA, such as LBF-γ-PO_4, may well have been the inhibitor directly involved. The possible inhibitory action of high levels of LBF-γ-PO_4 could be tested similarly if sufficient quantities of it become available, although no inhibitory effect was noted at a level of 40 µg. per tube. The possibility must not be discounted, however, that the actual inhibitory substance was not LBF itself, but an impurity present (perhaps only in trace amounts) in the synthetic LBF preparation.

It was also found that high levels of ATP were inhibitory in the sulfanilamide acetylation system (Table 5).

Time studies indicated that acetylation using LBF reached a half-maximum value in about 60 minutes and reached a maximum at about 120 minutes (Table 2).

The fact that LBF, particularly at high levels, was found to inhibit the acetylation of sulfanilamide meant that this system was unsuitable as a measure of the amount of CoA formed, except at very low levels of LBF where inhibition of acetylation could be neglected. This led to consideration of other possible assay methods for CoA which would be independent of the amount of LBF present. Two possibilities presented themselves, one an assay method based upon adsorption of CoA (but not of LBF) on a Dowex 1x2 (2% cross-linked) resin column and subsequent elution and assay by sulfanilamide acetylation. This procedure, based upon work of Stadtman and Kornberg (29), would have

effectively separated LBF and CoA but might not have separated CoA and LBF-γ-PO_4. A further obvious disadvantage lay in the fact that the high concentration of ammonium formate required for elution of CoA from the Dowex column was strongly inhibitory to the pigeon liver sulfanilamide-acetylation system. Recovery from the eluate and assay of the CoA would have required additional steps which would have made the method tedious and of doubtful reliability.

A second possibility was the use of the CoA-catalyzed arsenolysis of acetyl phosphate in the phosphotransacetylase system developed by Stadtman (28). This assay system had been used by Govier and Gibbons (10). The phosphotransacetylase system was found to be only slightly inhibited by low levels of LBF, and, at very high levels, the inhibition amounted only to about 10-15% (Table 6). This relatively small inhibition was neglected in all subsequent experiments in which this assay system was used.

Two facts were brought out in the subsequent work with pigeon liver preparations. The first was that the assayed yields of CoA were of about the same order as had been concluded from sulfanilamide acetylation assays (Table 7, 8). It is difficult to account for the discrepancy between these values and the values obtained by other groups, particularly King and Strong (12), who reported yields over twice as great as those obtained in the experiments reported here. These workers, however, had used LBF concentrates from

natural materials instead of synthetic preparations. Thus the presence of possible inhibitory impurities in the synthetic LBF preparation might have been responsible for lower yields of CoA than those obtained with LBF concentrates. Furthermore, the LBF assay method used by previous workers was not a direct microbiological assay for LBF but was based on enzymatic digestion to break the LBF down to pantothenic acid, followed by microbiological assay for pantothenic acid. Such a procedure, if the enzymatic breakdown were not quantitative, would have given falsely low values for the LBF content of the concentrates and the values for the percentage of LBF converted to CoA would have been too high. Finally, there is the possibility that the pigeon liver preparations used in these experiments were abnormally low in ability to synthesize CoA. This does not seem too likely, however, since, in the presence of five units of CoA, all of the five preparations used were able to acetylate 80-90% of the sulfanilamide in the Kaplan & Lipmann assay system. It seems unlikely that the preparations would be of normal potency in CoA-synthesizing ability while of lower than normal potency in acetylation.

The second fact brought out in this work was that there appeared to be a CoA-destroying enzyme active in the pigeon liver preparation after the 4 hour aging period prescribed by Kaplan & Lipmann (11), and even after an additional 4 hours aging (Table 8). CoA incubated with the aged pigeon

liver preparation (in the absence of ATP) showed a marked loss in activity for the phosphotransacetylase system when compared with CoA incubated for the same time in the absence of pigeon liver enzyme (Table 9). The presence of fluoride was not found to have any appreciable inhibitory effect on the CoA-destroying activity. However, it was found that inclusion of ATP in the incubation mixture to a large extent prevents the loss of CoA (Table 10). This could be due to an ATP-requiring resynthesis of CoA after cleavage by the CoA-destroying enzyme. This idea, however, conflicts with the finding of Novelli that the CoA-destroying enzyme of pigeon liver cleaves the molecule at the peptide linkage between the pantothenic acid and β-mercaptoethylamine moieties rather than at the phosphate linkage (21) (24). He further states that pigeon liver does not contain the necessary enzymes for resynthesis of the peptide bond. Reference to Table 8 shows that the CoA-synthesizing enzyme system in pigeon liver is quite active and that considerable final yields of CoA are obtained in spite of an apparent destruction of around one-half of the CoA which was incubated along with the enzyme preparation.

The total yields of CoA increased proportionately with increasing levels of LBF in the incubation mixture up to 250 µg. per tube (Table 7). The increase in LBF level to 500 µg. per tube did not bring a proportional increase in CoA yield, but the apparent leveling-off effect is actually

less marked when one recalls that there is about a 10% inhibition of the assay system at this high level of LBF. Nevertheless, a glance at Table 11 shows quite clearly that the system is practically saturated with LBF at 500 µg. per tube. Here also, as in the sulfanilamide acetylation system, an optimum ATP concentration was found (ca. 10 µM/ tube), and the yields of CoA appeared to fall off when the amount of ATP was increased above this level.

It was thought that perhaps some means of trapping the CoA as it is formed might allow accumulation of CoA with no concurrent loss due to the CoA-destroying enzyme(s). In a single experiment acetate was included in the reaction mixture with the possibility in view that acetyl-CoA might be formed and accumulated, but direct assay for "active acetate" by the hydroxamic acid method of Lipmann and Tuttle showed that inclusion of acetate had no significant effect (Table 12). Inclusion of sulfanilamide with acetate in the reaction mixture also had no effect.

The use of acetone powder extracts in these experiments was based on the assumption that any denaturation of the CoA synthesizing enzymes by acetone precipitation would be negligible. To determine whether or not this assumption was justified, 9 gms. of freshly excised pigeon liver were homogenized in 10 ml. of cold 0.02 M $NaHCO_3$ solution, centrifuged, and the supernate tested for ability to synthesize CoA. Comparison of the results shown in Table 13 with those

for an acetone powder extract, as in Table 8, certainly point to some loss of activity during the acetone treatment. In other work with the buffer extract (not tabulated), it was found that the freshly prepared enzyme gave twice the conversion obtained with 4 hour-aged enzyme. Apparently the synthesizing enzymes lose activity faster with aging than do the splitting enzymes. This is also clear from Table 8 in which 4 hour and 8 hour agings are compared.

II

Synthesis of CoA by Tissues Other Than Pigeon Liver

While the sulfanilamide acetylation system was perhaps an adequate means for measurement of the CoA synthetic ability of pigeon liver extracts at low levels of LBF, it was obviously not satisfactory for testing of other tissues, since a number of tissues, including guinea pig liver and hog kidney, did not appear to acetylate sulfanilamide at all. It was, of course, not feasible to incubate the extracts with LBF, and then to assay these in the pigeon liver system, since the unconverted LBF would be a substrate for CoA synthesis by the pigeon liver, and the "assay" would be meaningless. Consequently, the phosphotransacetylase system was used to assay for the CoA synthesized by the various tissue extracts.

On the basis of yield of CoA per unit volume of extract used, none of the tissues tested excelled pigeon liver in ability to synthesize CoA. However, on basis of yield per milligram of dry enzyme solids, some of the tissues, such as rabbit and hog liver, appear to have synthetic abilities approaching that of pigeon liver. Furthermore, the tissues differed markedly in their CoA-destroying properties, so that the final figure for the amount of CoA synthesized represented the resultant of the two opposing activities, synthesis and cleavage. The enzyme systems involved in the

two activities, however, are, reportedly, partly or entirely different (21) (24). Thus there is no question here of an equilibrium catalyzed by a single enzyme.

Not all the tissue extracts were tested under identical conditions. In addition to differing amounts of solids per ml., some extracts were Dowex-treated, others not. The amounts of LBF, as well as of cysteine and Mg^{++}, were not the same in all the tests.

In spite of the above differences in the testing conditions, the percent of LBF converted to CoA was of such an order that a variation of as much as 50% in the values did not alter the general picture, and thus a comparison is still possible. Considerable variations were occasionally obtained, inexplicably, even with the same extract under apparently identical testing conditions.

Typical values obtained in the comparison tests are summarized in Table 14.

Dowex-treated extracts - Dowex-treated pigeon liver preparations had been found to require added Mg^{++} (Table 15). Similarly, Dowex-treated guinea pig liver preparations showed a Mg^{++} requirement (Table 16), the optimum amount being between 4 and 10 micromoles per tube for the conditions specified. Using the same guinea pig liver preparation, the response to added ATP was obtained, showing a maximum at about 5 micromoles (Table 17).

Time-reaction studies which were made using a Dowex-treated guinea pig liver preparation indicate that maximum yield is obtained in about 4 hours, half-maximum in about 45 minutes (Table 18). The time required to reach maximum was not influenced by the amount of enzyme present. As can be seen from Table 18, the amounts of CoA produced were roughly proportional to the enzyme concentration at the two levels used.

III

CoA Synthesis by L. acidophilus

Incubation of LBF with a heavy suspension of resting L. acidophilus cells in 1% KCl, together with ATP as an energy source, followed by heat-rupture of the cells by a five-minute immersion in a boiling water bath, showed negligible synthesis of CoA (Table 19). This was apparently due to failure of ATP to be utilized by the cell, possible because of inability of ATP to cross the cell membrane. When, however, ATP was replaced in the medium by glucose, inorganic phosphate, and adenylic acid (adenine was almost equally effective), the yields were greatly improved, indicating that the resting cells could manufacture the required ATP from the simpler substances (Table 20). Incubation for 12 hours gave a total increase in yield of about 40% over the yield at 2 hours (Table 21), but at a level of 50 µg. LBF/tube, the yield was still small; not more than 5% of the LBF was converted. In the use of animal tissue extracts, the conversion of LBF to CoA had been found to be roughly proportional to the amount of crude enzyme added. Similarly, CoA synthesis by resting cells was roughly proportional to the amount of cells present (Table 22). The proportionality did not hold at high cell concentrations, possibly because accumulation of the cells at the bottom of the tube prevented adequate access of the

cells to the substrates. At an LBF concentration of 5 µg. per tube the yields were about 2/3 as great as were obtained at levels of 50 µg. per tube (Table 23); at 5 µg. per tube over 10% of the LBF was converted to CoA. Thus the concentration of cells was a limiting factor. It was thought possible that the permeability of the cell membrane for the various reactants might also be limiting.

To determine whether or not permeability was a limiting factor, a cell-free extract of L. acidophilus cells was prepared (cf. Methods). The results are shown in Table 24. In Part A one notes the high degree of conversion when ATP is supplied, particularly in the presence of added magnesium ion, known to be a required cofactor. In one experiment (not tabulated) in which twice as much enzyme was used as in Part A (thus 11.7 mg.), 53% of the LBF was converted. It seems clear, therefore, that the extremely low yields obtained with resting cells plus ATP (Table 19) were due to the impermeability of the cell membrane to ATP. On the other hand, CoA synthesis by the cell-free extract, using glucose, inorganic phosphate, and AMP in place of ATP (Part B), was rather low and was not significantly improved by addition of cell debris to the reaction mixture. This low yield is obviously not due to absence of the CoA synthesizing enzyme system, but may be connected with loss in activity of some of the enzymes involved in glycolysis, resulting in a deficiency in ATP. This effective loss of

activity could conceivably be due, in part, to the breakdown of cellular organization as well as to actual destruction of the enzymes themselves.

Although synthesis of CoA by resting cells, using pantothenate as a substrate, had not been tested in this series of experiments, it was known from Snell's work that pantothenate is a far less potent growth factor for L. acidophilus than LBF, and it seemed of interest to ascertain whether the same ratio of activities would be found in CoA synthesis by the cell-free extract. Part C of Table 24 shows that pantothenate is, indeed, less active than LBF on the basis of equimolar quantities. The addition of cysteine as a precursor to the β-mercaptoethylamine moiety appeared to increase the CoA yield somewhat by supplementing the endogenous sources of cysteine, although part of the effect of cysteine may have been due to its action in maintaining CoA in the metabolically active, reduced form.

Comparison of CoA-synthesizing abilities of several strains of L. acidophilus and of L. bulgaricus indicated some variation among the former (Table 25), but CoA synthesis by resting L. bulgaricus cells proved to be poor.

In a test of the loss of activity by CoA incubated for four hours at 37°C, it was found that while known amounts of CoA, when incubated with water alone, lost about 1/2 of the original activity, equal amounts, when incubated with resting L. acidophilus cells, lost only about 10% of the

original activity (Table 26). One factor causing this result may have been the reducing atmosphere provided by the resting cells.

Two English workers, Pierpoint and Hughes (25), studying the synthesis of CoA by L. arabinosus from pantothenate and cysteine, recently reported that with suspensions of washed L. arabinosus cells, addition of Mg^{++} increased the CoA yield around 70%. This was readily explained on the basis of Novelli & Levintow's work which had shown Mg^{++} to be a cofactor required in the phosporylation steps leading from LBF to CoA. Pierpoint & Hughes also found that addition of adenine to the medium did not improve the yields, the required adenine apparently being obtained entirely from endogenous sources.

Table 27 shows that Mg^{++} did not improve yields of CoA from LBF with L. acidophilus. Nor did cysteine, although probably the only role for cysteine here would be to maintain the CoA and LBF in the reduced form. Table 27 also shows that somewhat better yields were obtained with adenylic acid in the incubation mixture than without it. The failure of added Mg^{++} to improve yields of CoA from LBF might be attributed to a high endogenous Mg^{++} content, or, since the cells were washed (by centrifugation) only once, to the presence of traces of Mg^{++} remaining over from the culture broth.

In general, the yields obtained with L. acidophilus, using LBF as a substrate, were in the range of 300-400 units of CoA/mg. cells (dry wt.). This compares with about 500 units/mg. obtained by Pierpoint and Hughes, working with L. arabinosus, using LBF as substrate. Working with L. arabinosus, but with pantothenate and cysteine as substrates, these workers obtained a yield of 600-800 units/mg.

IV

Application of Microbiological Assay Methods

Although the work of Novelli and Levintow had shown the synthesis of CoA from LBF to be a three-stage process, it seemed worthwhile to see if the amount of CoA synthesized could be measured indirectly by microbiological assays of the LBF content before and after incubation of LBF with the crude pigeon liver enzyme, or, which is practically the same thing, to measure the difference in LBF content between two incubated systems, one containing the enzyme, and the other not containing the enzyme.

If the synthesis of CoA from LBF were a simple one-step process, it might be expected that synthesis of CoA would be paralleled by a decrease in LBF concentration and a consequent decrease in growth-stimulatory activity for L. acidophilus, since CoA itself is not a growth factor for that organism except in relatively high concentrations. But, in fact, the LBF incubated with the pigeon liver extract showed a greater growth-stimulating activity than did the LBF incubated without enzyme (Table 28). That the growth-stimulating factor must have been an enzymatic product was shown by the fact that neither enzyme without LBF nor boiled enzyme plus LBF gave comparable activity. This interesting result, then, on the basis of Novelli's three-stage synthesis, must mean that at least one of the intermediates

between LBF and CoA is a more potent growth factor for L. acidophilus than LBF itself. Pantothenylcysteine, though more likely a precursor of LBF than an intermediate between LBF and CoA, was checked because of its close relationship to LBF, but its activity proved to be less than that of LBF (Table 29). A sample of LBF-γ-PO_4, obtained from Baddiley, also showed somewhat less activity than LBF.

SUMMARY

1. The values obtained for conversion of LBF to CoA by extracts of pigeon liver acetone powder were found to lie in the range from 3.5 to 5.5%, while those for extracts of pigeon liver homogenized directly in buffer solution ranged from 6 to 10%.

2. "Aging" of the pigeon liver extracts was found to result in decreased CoA synthesis.

3. Extracts of pigeon liver, hog kidney, and, to a lesser degree, most of the other tissues tested, were found to contain a powerful CoA-splitting enzyme. The strength of this enzyme was not appreciably decreased by 8 hours "aging" at room temperature.

4. Under the conditions used, the yield of CoA was proportional to the amount of LBF present, up to a level of about 300 μg. The system was nearly saturated with LBF at a level of about 500 μg.

5. On a basis of CoA yield per mg. dry enzyme solids, rabbit and hog liver extracts were found to have a synthetic ability comparable to, but not exceeding, that of pigeon liver extracts.

6. Resting cells of L. acidophilus incubated with LBF, glucose, inorganic phosphate, and adenylic acid were found to synthesize 300-400 units of CoA per mg. cells (dry wt.).

7. LBF incubated with pigeon liver extract and ATP was found to have greater growth-promoting activity for L. acidophilus than LBF alone.

TABLE 1a

Catalysis of Sulfanilamide Acetylation by CoA
(Pigeon Liver System)

CoA, Units*	Sulfanilamide Acetylated, μg.
0.00	0
0.33	5
0.83	12
1.70	16.5
2.50	19
3.30	23
5.00	26

* 1 unit = 2.45 μg. CoA (= 0.7 μg. PA equivalent)
Experimental conditions: cf. Kaplan & Lipmann (11).

TABLE 1b

Catalysis of Acetyl Phosphate Arsenolysis by CoA
(Phosphotransacetylase system)

CoA, Units	Acetyl Phosphate Arsenolyzed, %
0.00	0.0
0.83	32.3
1.67	65.7
2.50	91.9
3.33	98.3
4.17	98.2
5.00	98.4

Experimental conditions: cf. Stadtman & Kornberg (29).

TABLE 2

Time of Incubation vs. Synthesis of CoA from LBF
(Determined from Sulfanilamide Acetylation)

Time, Minutes	Net CoA Synthesized, Units	LBF Converted, Percent
0	0.00	0.00
15	0.30	0.80
30	0.65	1.72
45	1.00	2.65
60	1.40	3.71
90	2.15	5.70
120	2.80	7.42
180	2.85	7.56

Other components of the system as follows: Potassium citrate, 20 µM; di-sodium ATP, 4 µM; sodium acetate, 25 µM; sulfanilamide, 0.4 µM; sodium bicarbonate, 100 µM; cysteine hydrochloride, 20 µM; LBF, 33 µg.; crude pigeon liver extract, ca. 22 mg. dry wt.; water to 1.0 ml. final volume. Incubated at 37°C.

TABLE 3

Variation of CoA Yield with LBF Concentration
(Determined from Sulfanilamide Acetylation)

LBF, µg.	Apparent CoA Synthesized, Units	LBF Converted, Percent
0	0.00	-
17	0.70	3.60
33	1.35	3.57
50	2.55	4.50
100	2.00	1.75
150	1.50	0.88
200	0.85	0.37
250	1.30	0.45
500	0.40	0.07

Other components of the system same as in Table 2, except: di-potassium ATP, 2.5 µM; LBF, as tabulated. Incubation time, 2 hours at 37°C.

TABLE 4

Inhibition of Sulfanilamide Acetylation by Increasing Levels of LBF (and LBF-γ-PO_4)

LBF-γ-PO_4, µg.	LBF, µg.	Sulfanilamide Acetylated, µg.
0	0	39
0	16	39
0	50	33
0	100	28
0	500	20
0	1000	15
20	0	39

Other components and conditions of the system same as in Table 2, except: CoA, 5 units; LBF, as tabulated; water to 1.3 ml. final volume. Incubation time, 2 hours at 37°C.

TABLE 5

Effect of ATP Concentration on CoA Synthesis by Pigeon Liver Extracts (Determined from Sulfanilamide Acetylation)

Additional ATP (di-potassium salt), µM	CoA Synthesized, Units
0	2.00
2.5	2.50
5	1.70
10	1.15
15	0.80

Other components of the system same as in Table 2, except: LBF, 50 µg.; additional ATP, as tabulated; water to 1.3 ml. final volume. Incubation, 2 hours at 37°C.

TABLE 6

Inhibition of the Phosphotransacetylase System by LBF

LBF, µg.	Assayed Amount of CoA, Units	Inhibition of Assay System, %
50	1.70	0
500	1.50	12.0
1000	1.42	16.4

Components of solution: CoA, 1.70 units, LBF as tabulated. Solution assayed by use of phosphotransacetylase system.

TABLE 7

Variation of CoA Yield with LBF Concentration
(By Phosphotransacetylase Assay)

LBF, µg.	CoA Synthesized, Units	LBF Converted, Percent
0	0	-
25	0.51	1.69
50	1.82	3.18
150	5.57	3.24
250	10.07	3.55
500	14.17	2.47

Components of system: Potassium bicarbonate, 100 µM; cysteine hydrochloride, 5 µM; di-potassium ATP (pH 6.5), 10 µM; magnesium sulfate, 1 µM; 4 hour-aged Dowex-treated pigeon liver extract, ca. 22 mg. (dry wt.); LBF, as tabulated; water to 1.2 ml. final volume; incubation, 2 hours at 37°C.

TABLE 8

Comparison of CoA Synthesis by Crude Enzymes "Aged" For Four and Eight Hours, Respectively

"Aging" Time, Hrs.	Added Reactants	CoA at End of Incub., Units	CoA Synthesized, Units/gm. Dry Wt.	LBF Converted Percent
4	LBF, 150 µg.	9.5	905	5.5
4	CoA, 5 units	2.9	-	-
8	LBF, 150 µg.	6.3	600	3.7
8	CoA, 5 units	2.8	-	-

Components of the system: Potassium bicarbonate, 100 µm.; cysteine hydrochloride, 5 µM; di-potassium ATP (pH 6.5), 10 µM; $MgSO_4$, 1 µM; Dowex-treated pigeon liver extract, 10.5 mg. dry wt.; LBF and CoA as tabulated; water to 1.1 ml. final volume. Incubation, 2 hours at 37°C.

TABLE 9

Loss of CoA Activity During Incubation of Added CoA with "Aged" Tissue Extracts

Crude Enzyme	CysHCl μM	Amt. Added CoA Remaining After Incubation, Units	CoA Lost, Percent
-	-	4.44	11.2
-	10	4.48	10.4
Pigeon Liver	-	0.12	97.5
Pigeon Liver	10	0.14	97.2
Hog Kidney	-	0.08	98.4

Components of the system: Potassium bicarbonate, 100 μM; CoA, 5 units; others as tabulated; water to 1.0 ml. final volume. Extracts Dowex-treated and "aged" four hours at room temperature. Incubation, 2 hours at 37°C.

TABLE 10

Effect of ATP on Retention of Activity by CoA Incubated with Crude Pigeon Liver Extract (Assayed by Phosphotransacetylase System)

di-potassium ATP (pH 6.5), μM	CoA Activity After Incubation, Units
0	1.32
10	2.80

Components of the system as follows: Potassium bicarbonate, 100 μM; cysteine hydrochloride, 10 μM; CoA, 5 units; aged pigeon liver extract, ca. 22 mg. dry wt.; di-potassium ATP (pH 6.5), as tabulated; water to 1.0 ml. final volume. Mixture incubated 2 hours at 37° C.

TABLE 11

Variation of CoA Yield with LBF Concentration
(Assayed by Phosphotransacetylase System)

LBF, µg.	CoA Synthesized, Units	LBF Converted, Percent
0	0	-
50	0.43	0.75
150	1.38	0.80
500	1.78	0.30

Components of the system as follows: Potassium bicarbonate, 100 µM; di-potassium ATP (pH 6.5), 5 µM; potassium citrate, 20 µM; cysteine hydrochloride, 20 µM; pigeon liver extract, ca. 22 mg. dry wt.; LBF, as tabulated; water to 1.0 ml. final volume. Incubation, 2 hours at 37°C.

TABLE 12

Test of Possible Formation of Acetyl CoA by Inclusion of Acetate in the Incubation Mixture

Potassium Acetate, µM	Equivalent Acetyl Phosphate, µM
0	0.05
30	0.06

Components of the system: Di-potassium ATP (pH 6.5), 20 µM; potassium bicarbonate, 100 µM; potassium citrate, 20 µM; cysteine hydrochloride, 10 µM; LBF, 500 µg.; aged pigeon liver extract, ca. 22 mg. dry wt.; water to 1.0 ml. final volume. Incubation, 2 hours at 37°C.

TABLE 13

CoA Synthesis by a Buffer Extract of Pigeon Liver

Added LBF, µg.	CoA Synthesized, Units	Units per mg. d. w.	LBF Conv., %
150	16.8	1480	9.8

Conditions same as in Table 8, except that the enzyme used was Dowex-treated buffer extract, aged 4 hours, ca. 11.4 mg. dry wt.

TABLE 14

Typical Values for the Synthesis of CoA from LBF by Various Tissue Extracts

Tissue Extr.	Dowex Treatment	Dry Wt. Solids mg./3ml.	Cys-HCl µM	$MgSO_4$ µM	LBF µg.	CoA Syn. Units	Units CoA Per mg. d. w.	LBF Converted, Percent
Rabbit Kidney	-	?	-	-	500	0.74	-	0.13
Rabbit Liver	-	9.0	-	-	500	0.94	0.10	0.16
Guinea Pig Liver	-	8.5	-	-	500	1.44	0.17	0.25
Hog Liver	-	10.0	-	-	500	0.88	0.09	0.14
Pigeon Liver	-	22.0	-	-	500	4.38	0.20	0.77
Beef Liver	+	12.0	-	10	500	1.04	0.09	0.18
Beef Kidney	+	8.0	-	10	500	1.58	0.20	0.28
Rat Liver	+	14.0	-	10	500	1.44	0.10	0.25
Rat Kidney	+	8.5	-	10	500	0.68	0.08	0.12
Rabbit Kidney	+	?	5	-	150	1.00	-	0.58
Rabbit Liver	+	9.0	5	-	150	1.36	0.15	0.79
Rabbit Liver	+	9.0	5	1	150	2.10	0.23	1.22
Hog Liver	+	10.0	5	1	150	2.44	0.24	1.42
Hog Kidney	+	5.0	5	1	150	0.21	0.04	0.12
Pigeon Liver	+	22.0	5	1	150	5.57	0.25	3.25

TABLE 15

Effect of Added Mg^{++} on CoA Synthesis by Dowex-treated Pigeon Liver Extract

Magnesium Sulfate, μM	CoA Synthesized, Units
0	3.95
1	5.57

Components of the system as follows: Potassium bicarbonate, 100 μM; cysteine hydrochloride, 5 μM; di-potassium ATP (pH 6.5), 10 μM; LBF, 150 μg.; Dowex-treated pigeon liver extract, ca. 22 mg. dry wt.; water to 1.2 ml. final volume; magnesium sulfate, as tabulated. Incubation, 2 hours at 37°C.

TABLE 16

Effect of Added Mg^{++} on CoA Synthesis by Dowex-treated Guinea Pig Liver Extract

Magnesium Sulfate, μM	CoA Synthesized, Units
0	1.00
1	1.50
2	1.60
4	1.58
10	1.66
20	1.28

Components of the system as follows: Potassium bicarbonate, 100 μM; di-potassium ATP (pH 6.5), 10 μM; LBF, 500 μg.; $MgSO_4$ as tabulated; Dowex-treated guinea pig liver extract, ca. 8.5 mg. dry wt.; water to 1.0 ml. final volume. Incubation time, 2 hours at 37°C.

TABLE 17

Effect of Added ATP on CoA Synthesis by Dowex-treated Guinea Pig Liver Extract

Di-potassium ATP (pH 6.5), μM	CoA Synthesized, Units
0	1.04
5	2.10
10	1.60
20	0.98

All conditions same as in Table 16, except: Magnesium sulfate, 1 μM; di-potassium ATP, as tabulated.

TABLE 18

Time Study of CoA Synthesis by Dowex-treated Guinea Pig Liver Extract

	CoA Synthesized, Units	
Time, Hours	Crude Enzyme, 8.5 mg. (Dry Weight)	Crude Enzyme, 17 mg. (Dry Weight)
0	0.68	0.92
1/2	1.08	1.90
1	1.16	3.01
2	2.12	4.00
3	2.50	4.50

Components as follows: Potassium bicarbonate, 100 μM; LBF, 500 μg.; di-potassium ATP (pH 6.5), 10 μM; magnesium sulfate, 1 μM; water to 1.0 ml. final volume; enzyme preparation, as tabulated. Incubation, 2 hours at 37°C.

TABLE 19

Effect of Added ATP on CoA Synthesis by Resting L. acidophilus (UT)* Cells

LBF, μg.	Di-potassium ATP (pH 6.5), μM	CoA Per Tube (Total), Units	CoA Synthesized, Units	LBF Converted, %
0	0	0.54	0	-
500	20	0.72	0.18	0.04

* (UT) - University of Texas Strain.
Dry weight of cells (suspended in 1% KCl), unknown (approx. 4-5 mg.); water to 1.0 ml. final volume. Incub., 3 hrs.,37°C.

TABLE 20

CoA Synthesis by Resting L. acidophilus (UT) Cells with Added Glucose, Inorganic Phosphate, and Adenylic Acid (AMP)

LBF, µg.	CoA Synthesized, Units	CoA Synthesized per gm. Dry Cells	LBF Converted, Percent
50	1.52	355	3.00
500	2.24	523	0.45

Components as follows: Glucose, 50 µM; resting cells, 4.28 mg. (dry wt.); water to 1.0 ml. final volume; K_2HPO_4, 30 µM; AMP, 10 µM. Incubation time, 4 hours at 37°C.

TABLE 21

Effect of Incubation Time on Yield of CoA Synthesized from LBF by Resting L. acidophilus (UT) Cells

Time of Incubation, Hours	Total CoA Per Tube, Units
2	1.90
4	2.36
8	2.38
12	2.72

Conditions same as in Table 18, except: LBF, 500 µg.; time of incubation, as tabulated.

TABLE 22

Effect of Increasing Cell Concentrations on Conversion of LBF to CoA by L. acidophilus (UT)

Wt. of Cells, mg. dry wt.	CoA Synthesized, Units	CoA Per gm. Cells
7.00	0.76	95
3.50	0.78	195
1.75	0.54	270
0.70	0.18	225

Components of the system as follows: K_2HPO_4, 30 µM; AMP, 10 µM; glucose, 50 µM; LBF, 5 µg.; water to 1.0 ml. final volume; cells, suspended in 1% KCl, as tabulated. Incubation, 4 hours at 37°C.

TABLE 23

CoA Synthesis by Resting L. acidophilus (UT) Cells at Low Concentrations of LBF

LBF, µg.	CoA Synthesized, Units	CoA Per gm. Cells	LBF Converted, Percent
0	0	0	-
2.5	0.64	120	22.4
5.0	0.58	110	10.2
50.0	0.96	182	1.7

Components of the system as follows: K_2HPO_4, 30 µM; AMP, 10 µM; glucose, 50 µM; LBF, as tabulated; water to 1.0 ml. final volume; cells (in 1% KCl), 5.5 mg. dry wt. Incubation, 4 hours at 37°C.

TABLE 24

CoA Synthesis by Cell-free Extracts of L. acidophilus (UT)

Part A: Effect of Mg^{++} ion on Synthesis from LBF and ATP

$MgSO_4$, uM	CoA Synthesized, Units*	CoA Synthesized Per gm. d. w., Units	LBF converted, %
1	36.6	6260	21.4
0	11.6	1980	6.8

Components of the system: Potassium bicarbonate, 100 µM; cysteine hydrochloride, 5 µM; di-potassium ATP (pH 6.5), 10 µM; cell-free extract, 5.9 mg. dry wt.; LBF, 150 µg.; $MgSO_4$, as tabulated; water to 1.1 ml. final volume. Incubation, 2 hours at 37°C.

Part B: Effect of Replacing ATP by a Mixture of Glucose, Inorganic Phosphate, and Adenylic Acid; Effect of Adding Back Debris from Ground Cells

Suspended Cell Debris	CoA Synthesized, Units*	CoA Synthesized Per gm. d. w., Units	LBF Converted, %
-	4.3	735	2.5
+	5.0	852	2.9

Components of the system: Potassium bicarbonate, 100 µM; cysteine hydrochloride, 5 µm; cell-free extract, 5.9 mg. dry wt.; $MgSO_4$, 1 µM; glucose, 50 µM; K_2HPO_4, 30 µM; AMP, 10 µM; LBF, 150 µg.; water to 1.1 ml. final volume. Incubation, 2 hours at 37°C.

Part C: Synthesis from Pantothenate and ATP with and without Added Cysteine

CysHCl, µm.	CoA Synthesized, Units*	CoA Synthesized Per gm. d. w., Units	LBF Converted, %
5	6.3	1080	3.7
0	3.6	615	2.1

Components of the system: Same as in Part A, except: $MgSO_4$, 1 µM; LBF, none; calcium pantothenate, 130 µg.; cysteine hydrochloride as tabulated.

* Average of two assay determinations.

TABLE 25

Comparison of the Abilities of Several Microorganisms to Synthesize CoA from LBF

Organism	D. Wt. of Cells, mg.	CoA Synthesized, Units	CoA Per gm., Units
L. acidophilus (ATCC 832)	11.2	2.54	227
L. acidophilus (Iowa State)	11.2	3.56	320
L. acidophilus (OSC)	9.2	3.70	402
L. acidophilus (U. of Texas)	6.9	2.76	403
L. bulgaricus (ATCC 8001)	5.4	0.64	120

Components as follows: Glucose, 50 µM; adenylic acid, 10 µM; K_2HPO_4, 30 µM; LBF, 50 µg.; cells (suspended in 1% KCl) as tabulated; water to 1.0 ml. final volume. Incubation, 2 hours at 37°C.

TABLE 26

Loss of Activity by CoA Incubated 4 Hours at 37°C

Added CoA, Units	mg. d. wt. Cells	CoA, After Incubation, Units	Percent Original Activity Lost
5	0	2.34	53.2
5	4.3	4.52	11.6
0	4.3	0.10	-

Conditions as follows: L. acidophilus (UT) cells, as tabulated; CoA, as tabulated below; final volume, 1.0 ml.; incubation, 4 hours at 37°C. CoA activity by phosphotransacetylase assay.

TABLE 27

Effect of Added Adenylic Acid, Mg^{++}, and Cysteine on CoA Synthesis by L. acidophilus (UT)

Added Components, µM	CoA Synthesized, Units	CoA Per gm. Cells	LBF Converted, Percent
-	1.56	142	2.73
AMP, 10	2.40	218	4.20
AMP, 10; $MgSO_4$, 1.	1.86	169	3.26
AMP, 10; CysHCl, 10.	1.50	136	2.63
AMP, 10; CysHCl, 10; $MgSO_4$, 1.	1.14	104	2.00

Components as follows: Glucose, 50 µM; K_2HPO_4, 30 µM; water to 1.0 ml. final volume; cells (in 1% KCl), 11 mg. dry wt; LBF, 50 µg.; other components as tabulated. Incubation, 4 hours at 37°C.

TABLE 28

Growth-stimulatory Activity (for L. acidophilus (UT)) of LBF Incubated with Crude Pigeon Liver Extract

	Pigeon Liver Extract Alone	LBF Alone, 0.03 µg.	LBF, 0.03 µg. Boiled P. L. Extr.	LBF, 0.03 µg. P. L. Extr.
Run #1	-	0.025	-	0.080
Run #2	0.007	0.039	-	0.097
Run #3	-	0.040	0.039	0.064
Avg. of 3 runs	0.007	0.035	0.039	0.080

Components of incubated systems: LBF, 50 µg.; di-sodium ATP, 10 µM; sodium bicarbonate, 100 µM; cysteine hydrochloride, 20 µM; water to 1.0 ml. final volume. Incubation, 2 hours at 37°C. Values given in the table are activities in terms of LBF, given in µg. (as read from the standard curve).

TABLE 29

Growth-promoting Activities (for L. acidophilus (UT)) of CoA, LBF-γ-PO_4, and Pantothenylcysteine (PA-cysteine), Compared to that of LBF

	LBF Molar-equivalent*, µg.	Activity in Terms of LBF, µg.	Activity Compared to LBF (100%)
CoA	0.10	0.006	6%
LBF-γ-PO_4	0.041	0.024	59%
PA-cysteine	0.011	0.005	46%

* An LBF molar-equivalent as here expressed means that the quantity of CoA, LBF-γ-PO_4, or PA-cys used was equivalent in molarity to the indicated weight of LBF.

Part Two

STUDIES ON THE METABOLIC FUNCTION OF COENZYME A

Introduction

In the original work by Lipmann, a dialyzable cofactor, which he named coenzyme A, was found to be necessary, together with other factors, for the enzymatic acetylation of sulfanilamide (17). Shortly thereafter, coenzyme A was found to be catalytic in the acetylation of choline (18), the acetylcholine formed in the latter reaction being of importance in the transmission of nerve impulses.

In 1949, Stern & Ochoa reported that the condensation of "active acetate" with oxaloacetate to form citrate, a key reaction in the metabolism of all higher organisms and many microorganisms, required CoA as a cofactor (31). Then, in 1951, Lynen, working with yeast, was able to isolate the "active acetate" and identify it as S-acetyl CoA (19). This compound belongs to the group known as "high-energy" compounds, among which are ATP, phosphocreatine, phosphoenolpyruvate, and others, all having values of ΔF^{o} (of hydrolysis) ranging from -10,000 to -16,000 calories per mole. This same compound was also shown to be the acetyl donor in the formation of citrate, acetylsulfanilamide, and acetylcholine (7).

It should be emphasized that, while the acetyl group is always attached through the carboxyl group to the sulfur

atom of CoA, the activation of the acetyl group in the acetyl CoA linkage is not necessarily restricted to the carboxyl end. In the condensation of "active acetate" and oxaloacetate, the acetate is "tail-activated" and the linkage with oxaloacetate takes place through the methyl carbon. The apparent differences in the position of the reactive center must be attributed to characteristic differences in the enzyme systems concerned.

The function of CoA was found, however, not to be limited to activation of acetate. The "active succinate" involved in the only substrate level oxidative phosphorylation in the Krebs cycle was found to be succinyl CoA (26).

The true proportions of the role of CoA in intermediary metabolism were not realized until the now generally accepted scheme for the synthesis and degradation of fatty acids was being worked out. This development has taken place only in the last few years. It now appears that coenzyme A is the "vehicle" for the acid group in every step of the so-called fatty acid "cycle". In one turn of the cycle, for example, two molecules of acetyl CoA condense to form acetoacetyl CoA, which accepts two hydrogens to form β-hydroxybutyryl CoA, which loses water to form crotonyl CoA, which, in turn, accepts two hydrogen atoms to form butyryl CoA (20). This compound can be hydrolyzed to the free acid or condense with another acetyl CoA to continue the buildup of the fatty acid chain. Fatty acid degradation,

with a two-carbon unit being split off with each turn of the cycle, is simply the reverse of the buildup process. Every substrate in the scheme is in the form of an acyl CoA, and it appears that, before a fatty acid can enter the cycle, it must first be converted to the acyl CoA with the expenditure of one ATP molecule.

It was reported in 1952, by Kornberg & Pricer (15), that CoA is also required in phospholipid synthesis. Here again, the substrate appears to be an acyl CoA (e.g., stearyl CoA).

The part played by CoA in the acetylation of amines, such as sulfanilamide, glucosamine, and others, has been recognized for some time, but there are indications that it may play a more general role in synthesis of peptide linkages. In 1951, Chantrenne, working with rat liver homogenates, showed CoA to be an essential cofactor in the well-known detoxication mechanism whereby ingested benzoic acid is conjugated with glycine to form the non-toxic benzoylglycine (hippuric acid) (6). In the living animal, the hippuric acid is subsequently eliminated as such in the urine. This work was particularly significant in that it was an example clearly showing the involvement of CoA in the synthesis of a simple peptide bond.

In a similar detoxication process, perfused rabbit livers have been shown to possess the ability to conjugate furoic acid (furan-2 carboxylic acid) with glycine to form

furoylglycine (1) (14). *In vivo* experiments with rabbits have shown that furoylglycine is eliminated in the urine, and the quantitative estimation of the furoylglycine excreted following ingestion of furoic acid has been used as an *in vivo* test of liver function. Since the detoxication of furoic acid appears to follow a path similar to that for benzoic acid, it was thought that here, too, a CoA dependency might be found. This has proved to be true, as shown by the experiments reported here.

Methods

Preparation of tissue extracts - Acetone powders of various animal tissues were prepared and the powders re-suspended in buffer solution. The procedure followed here was identical to that used by Kaplan and Lipmann for the preparation of pigeon liver extracts (11).

Dowex-treatment of extracts - The treatment of tissue extracts with Dowex-1 (anion exchange resin) for removal of endogenous CoA followed the procedure outlined by Novelli and Schmetz (22), except that the extracts were treated only once with the resin instead of twice.

Incubation of reactants - Prior to use, the ATP and furoic acid solutions were brought to a pH of approximately 6.5 with KOH. Final volumes ranged from 1.4 to 1.6 ml. in different experiments. All incubations were for three hours at 37°C. At the end of the incubation period the volumes were brought to 2.0 ml. with distilled water.

Preparation of synthetic furoylglycine - The synthetic product was prepared from furoyl-2 chloride and glycine (both Eastman "white label") using the procedure of Baum (3). The product melted sharply at 166°C (value reported by Baum, 165°C).

Estimation of furoylglycine - Furoylglycine was estimated colorimetrically essentially according to the method of Kitamura (14), and a standard curve relating optical

density to the amount of furoylglycine present was prepared using synthetic furoylglycine (Table 1). In the preparation of the standard curve, it was found that reproducible values were obtained when a stream of air saturated with bromine was bubbled vigorously through the furoylglycine solution (2 ml. volume) for three minutes, followed immediately by a stream of air alone for fifteen minutes to remove excess bromine. To prevent solutions containing protein from foaming over, a small amount of Dow-Corning anti-foam agent was placed midway up the inner wall of the testtube. After aeration, eight ml. of 20% ammonium carbonate was added, but the additional five ml. of water prescribed by Kitamura was omitted. The solution was centrifuged, and after thirteen minutes had elapsed since the addition of the ammonium carbonate solution, the optical density was read on a Bausch & Lomb colorimeter, using a 550 mμ. filter. This procedure was followed for all furoylglycine determinations. The arbitrary thirteen minute period of waiting before reading the optical density was important since it was observed that the violet color faded somewhat with time.

Results and Discussion

It was found that rat liver extract and both liver and kidney extracts from the rabbit, guinea pig, cattle, and hog were able to synthesize some furoylglycine when incubated with furoic acid, glycine, and ATP, and that the amount synthesized was increased by the addition of cysteine. However, when these extracts were treated with Dowex-1, which removes most of the endogenous CoA and a large proportion of the metallic ions such as Mg^{++}, only a negligible amount of furoylglycine was synthesized (Table 2).

When CoA was then added in graded levels to a reaction mixture containing Dowex-treated enzyme, Mg^{++}, ATP, furoic acid, glycine, cysteine, and Tris buffer, a response was obtained which showed that CoA is required in the synthesis of furoylglycine (Table 3; Figure 1). Using a fixed amount of Dowex-treated enzyme preparation, response curves were obtained for each of the other components of the reaction mixture (Tables 4-9). A time-reaction study was also run (Table 10).

Because of greater convenience in preparation, beef liver extracts were used in these tests in preference to extracts of other tissues, such as rabbit kidney and guinea pig liver, although these latter appeared to be the most potent of the tissues tested. It is interesting to note that the one other tissue extract tested, that of pigeon liver,

showed only a negligible amount of synthesis, at most not over one-fourth as much as any of the others. This may be due, in part, to the presence in pigeon liver of a very active CoA-destroying enzyme (11) (21), but it is likely that pigeon liver simply does not contain appreciable amounts of the furoylglycine synthesizing system, since hog kidney, which also contains a high level of the CoA-destroying enzyme, is able to synthesize approximately ten times as much furoylglycine.

These in vitro studies indicate that kidney, as well as liver, contains the requisite enzyme systems for conjugation of furoic acid, as Akiwa had previously found from perfusion studies (1). Thus there may be a question as to whether in vivo studies based on the excretion of furoylglycine into the urine are completely valid as a test of liver function alone.

Summary

Liver and kidney extracts of various animals, when incubated with furoic acid, glycine, and ATP, are able to synthesize furoylglycine. Treatment of the extracts with Dowex-1 inactivates the system, but the inactivated system is reactivated by the addition of coenzyme A.

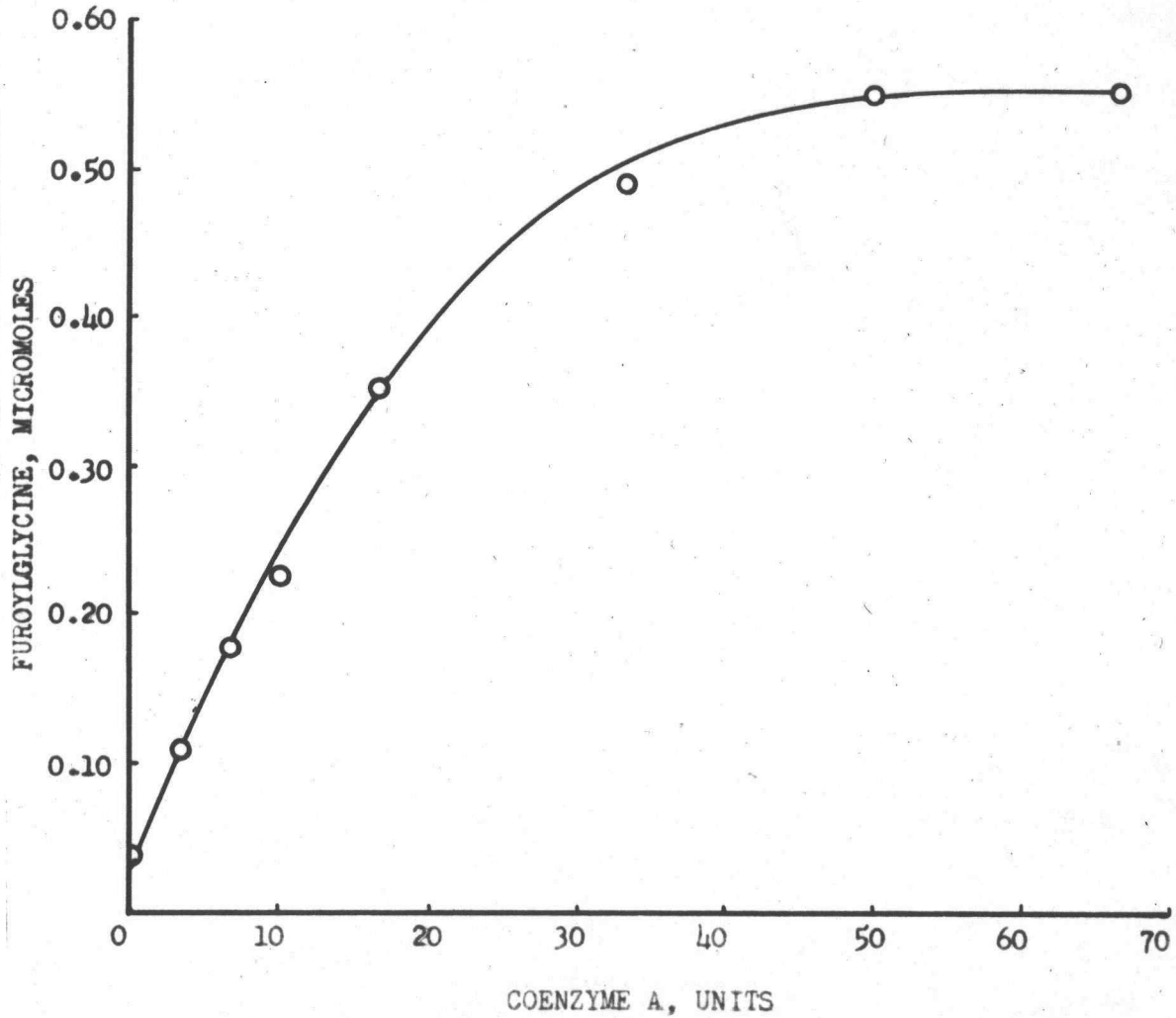


FIGURE 1. EFFECT OF ADDED COENZYME A ON FUROYLGLYCINE SYNTHESIS

TABLE 1

Standard Curve for Furoylglycine Determination

Furoyl-glycine, µg.	Furoyl-glycine, µM	Optical density (550 mµ)
0	0	0
10	0.059	0.030
20	0.118	0.060
30	0.178	0.097
40	0.236	0.124
50	0.296	0.150
60	0.355	0.179
70	0.414	0.201
80	0.473	0.220
100	0.592	0.261
150	0.890	0.359
200	1.181	0.441

Conditions of assay: Volume of furoylglycine solution, 2 ml.; time of bromination, 3 minutes; time of aeration, 15 minutes; 20% ammonium carbonate solution, 8 ml.; time before reading optical density, 13 minutes; optical density read with 550 mµ filter on Bausch & Lomb colorimeter.

TABLE 2

Effect of Dowex-treatment on Furoylglycine Synthesis by Beef Liver Extract

Dowex-1 Treatment	Added CoA, Units	Furoylglycine Formed, µM
-	0	0.175
+	0	0.050
+	10	1.277

Components of the system as follows: Tris buffer, 200 µM; glycine, 25 µM; furoic acid, 10 µM; di-potassium ATP, 10 µM; cysteine hydrochloride, 20 µM; $MgSO_4$, 1 µM; beef liver extract, 20 mg., dry wt.; final volume, 1.4 ml.

TABLE 3

Effect of Added CoA on Furoylglycine Synthesis by Dowex-treated Beef Liver Extract

CoA, Units	Furoylglycine, μM
0.0	0.037
3.3	0.108
6.7	0.176
10.0	0.225
16.7	0.352
33.3	0.490
50.0	0.550
66.7	0.551

Components of the system as follows: Tris, 200 μm; glycine, 10 μM; furoic acid, 10 μM; cysteine hydrochloride, 20 μM; $MgSO_4$, 1 μM; di-potassium ATP, 10 μM; Dowex-treated enzyme, ca. 20 mg. (dry weight); water to 1.5 ml. final volume. Incubation, 3 hours at 37°C.

TABLE 4

Effect of Increasing Concentrations of Furoic Acid on Furoylglycine Synthesis by Dowex-treated Beef Liver Extract

Furoic Acid, μM	Furoylglycine, μM
0	0.018
0.5	0.096
1.0	0.133
2.0	0.181
3.0	0.244
5.0	0.268
10.0	0.275

Components of the system as follows: Tris buffer, 200 μM; glycine, 25 μM; cysteine hydrochloride, 20 μM; $MgSO_4$, 1 μM; di-potassium ATP, 10 μM; coenzyme A, 10 units; Dowex-treated enzyme, ca. 20 mg.; water to 1.6 ml. final volume. Incubation, 3 hours at 37°C.

TABLE 5

Effect of Increasing Concentrations of Glycine on Furoylglycine Synthesis by Dowex-treated Beef Liver Extract

Glycine, μM	Furoylglycine, μM
0	0.108
5	0.213
10	0.250
20	0.276
30	0.267
50	0.295

Components of the system: Tris buffer, 200 μM; cysteine hydrochloride, 20 μM; di-potassium ATP, 10 μM; furoic acid, 10 μM; coenzyme A, 10 units; $MgSO_4$, 1 μM; Dowex-treated enzyme, ca. 20 mg.; water to 1.6 ml. final volume. Incubation, 3 hours at 37°C.

TABLE 6

Effect of Increasing Concentrations of ATP on Furoylglycine Synthesis by Dowex-treated Beef Liver Extract

Di-potassium ATP, μM	Furoylglycine, μM
0	0.053
5	0.190
10	0.295
15	0.198
20	0.130
30	0.097

Components of the system: Tris buffer, 200 μM; cysteine hydrochloride, 20 μM; $MgSO_4$, 1 μM; glycine, 50 μM; furoic acid, 30 μM; coenzyme A, 10 units; Dowex-treated enzyme, ca. 20 mg.; water, 1.6 ml. final volume. Incubation, 3 hours at 37°C.

TABLE 7

Effect of Increasing Concentrations of Mg^{++} ion on Furoylglycine Synthesis by Dowex-treated Beef Liver Extract

$MgSO_4$, µM	Furoylglycine, µM
0	0.127
0.5	0.230
1.0	0.244
2.0	0.212
3.0	0.195
5.0	0.150

Components of the system: Tris buffer, 200 µM; cysteine hydrochloride, 20 µM; di-potassium ATP, 10 µM; glycine, 10 µM; furoic acid, 10 µM; coenzyme A, 10 units; Dowex-treated enzyme, ca. 20 mg.; water to 1.6 ml. final volume. Incubation, 3 hours at 37°C.

TABLE 8

Effect of Increasing Concentration of Cysteine on Furoylglycine Synthesis by Dowex-treated beef Liver Extract

CysHCl, µM	Furoylglycine, µM
0	0.033
10	0.121
20	0.280
30	0.292
40	0.245

Components of the system: Tris buffer, 200 µM; $MgSO_4$, 1 µM; di-potassium ATP, 10 µM; glycine, 25 µM; furoic acid, 10 µM; coenzyme A, 10 units; Dowex-treated enzyme, ca. 20 mg. dry wt.; water to 1.5 ml. final volume. Incubation, 3 hours at 37°C.

TABLE 9

Effect of Tris Buffer Concentration on Furoylglycine Synthesis by Dowex-treated Beef Liver Extract

Tris, μM	Furoylglycine, μM
0	0.082
100	0.222
200	0.280
300	0.202
400	0.080

Components of the system: $MgSO_4$, 1 μM; di-potassium ATP, 10 μM; cysteine hydrochloride, 20 μM; glycine, 25 μM; furoic acid, 10 μM; coenzyme A, 10 units; Dowex-treated enzyme, ca. 20 mg. dry wt.; water to 1.5 ml. final volume. Incubation, 3 hours at 37°C.

TABLE 10

Synthesis of Furoylglycine (Time-reaction Study)

Time, Hours	Furoylglycine, μM
0	0.052
1/2	0.088
1	0.130
2	0.208
3	0.242

Components of the system: Tris, 200 μM; $MgSO_4$, 1 μM; cysteine hydrochloride, 20 μM; glycine, 50 μM; furoic acid, 30 μM; coenzyme A, 10 units; di-potassium ATP, 15 μM; Dowex-treated enzyme, ca. 20 mg. dry wt.; water to 1.5 ml. final volume. Incubation at 37°C.

BIBLIOGRAPHY

1. Akiwa, Jiro. Furoylglycine method as a liver function test. Hukuoka acta medica 44:290-295. 1953. (Abstracted in Chemical abstracts 47:11304h. 1953.)

2. Baddiley, J., E. M. Thain, G. D. Novelli and F. Lipmann. Structure of coenzyme A. Nature 171:76. 1953.

3. Baum, Erich. Brenzschleimsäurechlorid als Acylirungsmittel. Synthese der Pyromycursäure. Berichte der deutschen chemischen Gesellschaft 37:2949-2961. 1904.

4. Beinert, Helmuth, et al. A method for the purification of coenzyme A from yeast, Journal of biological chemistry 200:385-400. 1953.

5. Bratton, A. Calvin and E. K. Marshall Jr. A new coupling component for sulfanilamide determination. Journal of biological chemistry 128:537-550. 1939.

6. Chantrenne, Hubert. The requirement for coenzyme A in the enzymatic synthesis of hippuric acid. Journal of biological chemistry 189:227-233. 1951.

7. Chou, T. C. and Fritz Lipmann. Separation of acetyl transfer enzymes in pigeon liver extracts. Journal of biological chemistry 196:89-103. 1952.

8. Craig, Jean A. and Esmond E. Snell. The comparative activities of pantethine, pantothenic acid, and coenzyme A for various microorganisms. Journal of bacteriology 61:283-291. 1951.

9. DeVries, W. H., et al. Purification of coenzyme A from fermentation sources and its further identification. Journal of the American Chemical Society 72:4838. 1950.

10. Govier, Wm. M. and Anna J. Gibbons. Activity of LBF (pantethine) in acetylation of sulfanilamide by pigeon liver extract. Archives of biochemistry and biophysics 32:347-348. 1951.

11. Kaplan, Nathan O. and Fritz Lipmann. The assay and distribution of coenzyme A. Journal of biological chemistry 174:37-44. 1948.

12. King, Tsoo E. and F. M. Strong. Some properties of coenzyme A. Journal of biological chemistry 189:325-333. 1951.

13. King, Tsoo E., Charles J. Stewart and Vernon H. Cheldelin. β-Aletheine and pantetheine. Journal of the American Chemical Society 75:1290-1292. 1953.

14. Kitamura, Hiroshi. A new method of estimating furoylglycine. Furoic acid test as an indicator of liver function. Kyushu memoirs of medical science 1:7-14. 1950. (Abstracted in Chemical abstracts 46:2668g. 1952.)

15. Kornberg, Arthur and W. E. Pricer Jr. Enzymatic synthesis of phosphorus-containing lipides. Journal of the American Chemical Society 74:1617. 1952.

16. Lipmann, Fritz and L. Constance Tuttle. A specific micromethod for the determination of acyl phosphates. Journal of biological chemistry 159: 21-28. 1945.

17. Lipmann, Fritz. Acetylation of sulfanilamide by liver homogenates and extracts. Journal of biological chemistry 160:173-190. 1945.

18. Lipmann, Fritz and Nathan O. Kaplan. A common factor in the enzymatic acetylation of sulfanilamide and choline. Journal of biological chemistry 162:743-744. 1946.

19. Lynen, Feodor, Ernestine Reichert and Luistraud Rueff. Zum biologischen Abbau der Essigsäure VI. "Aktivierte Essigsäure", ihre Isolierung aus Hefe und ihre chemische Natur. Justus Liebigs Annalen der Chemie 574:1-32. 1951.

20. Lynen, Feodor. Functional group of coenzyme A and its metabolic relations in the fatty acid cycle. Federation proceedings 12:683-691. 1953.

21. Novelli, G. David. Enzymatic synthesis and structure of coenzyme A. Federation proceedings 12:675-680. 1953.

22. Novelli, G. David and Frank J. Schmetz Jr. An improved method for the determination of pantothenic acid in tissues. Journal of biological chemistry 192:181-185. 1951.

23. Novelli, G. David and L. Levintow. Enzymatic synthesis of coenzyme A. Abstracts of 122nd meeting of the American Chemical Society. Sept. 1952. Abstract 33c.

24. Novelli, G. D., Frank J. Schmetz Jr., and Nathan O. Kaplan. Enzymatic degradation and resynthesis of coenzyme A. Journal of biological chemistry 206:533-545. 1954.

25. Pierpoint, W. S. and D. E. Hughes. The synthesis of coenzyme A by L. arabinosus 17-5. The biochemical journal 56:130-135. 1954.

26. Sanadi, D. R. and J. W. Littlefield. Studies on α-ketoglutaric oxidase. I Formation of "active succinate". Journal of biological chemistry 193:683-689. 1951.

27. Snell, Esmond E., et al. Chemical nature and synthesis of the Lactobacillus bulgaricus factor. Journal of the American Chemical Society 72:5349-5350. 1950.

28. Stadtman, E. R. and H. A. Barker. Fatty acid synthesis by enzyme preparations of Clostridium kluyveri. I Preparation of cell-free extracts that catalyze the conversion of ethanol and acetate to butyrate and caproate. Journal of biological chemistry 180:1085-1093. 1949.

29. Stadtman, E. R. and A. Kornberg. The purification of coenzyme A by ion exchange chromatography. Journal of biological chemistry 203:47-54. 1953.

30. Stadtman, E. R., G. D. Novelli and Fritz Lipmann. Coenzyme A function in and acetyl transfer by the phosphotransacetylase system. Journal of biological chemistry 191:365-376. 1951.

31. Stern, Joseph R. and Severo Ochoa. Enzymatic synthesis of citric acid by condensation of acetate and oxalacetate. Journal of biological chemistry 179:491-492. 1949.

32. Wang, T. P. and Nathan O. Kaplan. Kinases for the synthesis of coenzyme A and triphosphopyridine nucleotides. Journal of biological chemistry 206:311-325. 1954.