[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WASHINGTON]

Chemical Synthesis and Properties of Butyryl Adenylate[1]

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Butyryl adenylate has been prepared in good yield by the direct condensation of butyric acid and adenylic acid, promoted by dicyclohexylcarbodiimide. After purification by column and paper chromatography, the material behaved as a single, homogeneous substance upon paper chromatographic examination, and gave the correct analytical data for various functional groups. Fatty acid activating enzyme was used to study the participation of butyryl adenylate in the enzymatic synthesis of butyryl coenzyme A.

The enzymatic synthesis of "active acetate," or acetyl CoA,[2] from acetate, CoA, and ATP is catalyzed by the acetate-activating enzyme present in a variety of tissues, including brain,[3] yeast[4] and heart.[5,6] The over-all reaction for this process follows the stoichiometry of equation 1

$$\text{acetate} + \text{ATP} + \text{CoA} \longrightarrow \text{acetyl CoA} + \text{AMP} + \text{PP} \quad (1)$$

Several stepwise mechanisms and intermediates[5,7] have been suggested to account for the over-all reaction. Recently, Berg[8] has proposed the sequence shown in equations 2 and 3

$$\text{acetate} + \text{ATP} \longrightarrow \text{acetyl AMP} + \text{PP} \quad (2)$$

$$\text{acetyl AMP} + \text{CoA} \longrightarrow \text{acetyl CoA} + \text{AMP} \quad (3)$$

to explain the net reaction (1). The intermediate, acetyl AMP,[9] which Berg synthesized by treating disilver AMP with acetyl chloride, was enzymatically active when tested with the acetate-activating system from yeast. In the presence of PP and the enzyme, acetyl AMP was converted to ATP, while the combination of CoA and acetyl AMP with the enzyme resulted in the synthesis of acetyl CoA. These findings fulfill the requirements of equations 2 and 3.

Several other reactions of biological importance, notably the activation of other fatty acids or α-amino acids, have an over-all stoichiometry analogous to that of equation 1. In these cases, too, it may be envisioned that the acyl group becomes "activated" by ATP, followed by the reaction of the acyl adenylate with an acceptor molecule (*e.g.*, CoA or an α-amino group).

In view of the widespread occurrence and importance of these reactions, it seemed desirable to investigate methods for the chemical synthesis of acyl adenylates, and to study the properties of these compounds. Butyryl adenylate has been prepared in good yield by the condensation of butyric acid and AMP using dicyclohexylcarbodiimide as the dehydrating agent. Carbodiimides have been used widely to promote synthetic reactions involving dehydrations.[10-13]

The chemical properties of BuAMP have been studied, as well as its participation as an intermediate in the enzymatic formation of butyryl CoA, by reactions analogous to equations 1–3 wherein butyric acid replaces acetic acid.[14]

$$\text{butyrate} + \text{ATP} + \text{CoA} \longrightarrow \text{butyryl CoA} + \text{AMP} + \text{PP} \quad (1')$$

$$\text{butyrate} + \text{ATP} \longrightarrow \text{BuAMP} + \text{PP} \quad (2')$$

$$\text{BuAMP} + \text{CoA} \longrightarrow \text{butyryl CoA} + \text{AMP} \quad (3')$$

Experimental

Materials.—AMP, ATP and CoA were obtained from the Sigma Chemical Company and Pabst Laboratories, Inc. Dicyclohexylcarbodiimide was purchased from Dajac Laboratories, or was generously made available as a gift from the California Foundation for Biochemical Research. Hexokinase and glucose-6-phosphate dehydrogenase were obtained from the Sigma Chemical Company.

FAAE was isolated from beef liver by the method of Mahler, *et al.*,[15] except that the initial acetone powder was prepared from an homogenate rather than from the mitochondrial fraction of beef liver.

Methods.—The enzymatic formation of butyryl CoA according to equations 1′ or 3′ was followed by the nitroprusside method of Mahler, *et al.*[15] BuAMP was estimated by the hydroxylamine–$FeCl_3$ method.[16] The formation of ATP by the reversal of equation 2′ was carried out by linking the reaction with the hexokinase-glucose-6-phosphate dehydrogenase[17] system, and following the appearance of TPNH at 340 mμ.

Ascending paper chromatography for analytical purposes was carried out on 28 × 42 cm. sheets of Whatman No. 1 paper using: (A) isobutyric acid:NH_3:water (66:1:33) or (B) isopropyl alcohol:water (70:30) as the solvent systems. Adenine-containing compounds were located on paper chromatograms as "quenching spots" when viewed under a Mineralight ultraviolet lamp. Phosphate esters were located by means of the molybdate spray reagent,[18] anhydrides by the hydroxylamine–$FeCl_3$ spray reagent,[19] and

(1) Aided by grants from the Office of the Surgeon General, U. S. Army and Eli Lilly and Company.

(2) The following abbreviations will be used: CoA, coenzyme A; AMP, adenosine 5′-phosphate; ATP, adenosine triphosphate; BuAMP, butyryl adenylate; PP, pyrophosphate; FAAE, fatty acid activating enzyme; DCC, dicyclohexylcarbodiimide; TPNH, reduced triphosphopyridine nucleotide.

(3) D. Nachmansohn and A. L. Machado, *J. Neurophysiol.*, **6**, 397 (1943).

(4) F. Lipmann, M. E. Jones, S. Black and R. M. Flynn, THIS JOURNAL, **74**, 2384 (1952).

(5) H. Beinert, D. E. Green, P. Hele, H. Hift, R. W. von Korff and C. V. Ramakrishnan, *J. Biol. Chem.*, **203**, 35 (1953).

(6) P. Hele, *ibid.*, **206**, 671 (1954).

(7) M. E. Jones, F. Lipmann, H. Hilz and F. Lynen, THIS JOURNAL, **75**, 3285 (1953).

(8) P. Berg, *ibid.*, **77**, 3163 (1955).

(9) In a mixed anhydride of this type, either the name "adenyl acetate," as proposed originally by Berg,[8] or "acetyl adenylate" as used here, would be equally correct.

(10) H. G. Khorana, THIS JOURNAL, **76**, 3517 (1954); **76**, 5056 (1954).

(11) G. W. Kenner, A. R. Todd and R. F. Webb, *J. Chem. Soc.*, 2843 (1954).

(12) E. P. Kennedy and S. B. Weiss, THIS JOURNAL, **77**, 250 (1955).

(13) F. M. Huennekens and G. L. Kilgour, *ibid.*, **77**, 6716 (1955).

(14) While this manuscript was in preparation, we were kindly informed by Prof. D. E. Green that Dr. C. H. Lee Peng had carried out a similar investigation on the chemical synthesis of BuAMP. We are indebted to these investigators for informing us of their results prior to publication.

(15) H. R. Mahler, S. J. Wakil and R. M. Bock, *J. Biol. Chem.*, **204**, 453 (1953).

(16) F. Lipmann and L. C. Tuttle, *ibid.*, **159**, 21 (1945).

(17) A. Kornberg, *ibid.*, **182**, 779 (1950).

(18) R. S. Bandurski and B. Axelrod, *ibid.*, **193**, 405 (1951).

(19) E. R. Stadtman and H. A. Barker, *ibid.*, **184**, 769 (1950).

vicinal hydroxyl groups by the periodate spray reagent.[20]

The following methods were employed for quantitative assay of functional groups: total phosphate by the Fisk–SubbaRow method,[21,22] free *vicinal* hydroxyl groups by the periodate method,[23] anhydride linkages by the hydroxylamine–$FeCl_3$ method,[16] and free purine amino groups by deamination with HNO_2.[24]

Synthesis of BuAMP.—Two and four tenths ml. (2.6×10^{-2} mole) of butyric acid, 300 mg. (8.6×10^{-4} mole) of AMP, 2.4 ml. (1.33×10^{-1} mole) of water and 8 ml. (9.9×10^{-2} mole) of pyridine were mixed rapidly by mechanical stirring in a round-bottom flask at 0° for several minutes, after which 3 g. (1.4×10^{-2} mole) of DCC dissolved in 7 ml. (8.7×10^{-2} mole) of pyridine at 0° was added. The resulting mixture was then stirred for 40 minutes at 0°, whereupon the reaction was stopped by the addition of 25 ml. of ice-cold water. The mixture was then stirred for an additional 5 minutes, followed by rapid filtration through a sintered glass funnel in the cold to remove the insoluble dicyclohexylurea The aqueous pyridine filtrate, containing the BuAMP and unreacted AMP, was then extracted several times with 2 volumes of cold ether to remove unreacted butyric acid. The aqueous solution was reduced to a small volume at 0–5° on a Rinco rotating evaporator,[25] followed by several extractions with 5 volumes of acid-free chloroform at 0° to remove residual pyridine.

BuAMP was separated from AMP by adsorption chromatography on a cellulose column using isopropyl alcohol:water (70:30) as the solvent system. Whatman No. 1 paper powder (coarse grade) was suspended by vigorous stirring in isopropyl alcohol:water and poured into a 7×75 cm. glass column with a stopcock at the lower end. Under gentle air pressure, the column of adsorbent was packed evenly and without channels to a height of 40 cm. In all subsequent operations the liquid was allowed to flow through under hydrostatic pressure of the liquid reservoir at the top. The column was washed with solvent for 3–4 days until effluent aliquots had an optical density at 260 mμ of less than 0.050 when measured in 1 cm. Quartz cells with the Beckman spectrophotometer, Model DU.

The sample containing about 300 mg. of solids in 10–15 ml. of water was added to the top of the column which had been taken to incipient dryness. The aqueous solution was allowed to percolate into the column, followed by 30 ml. of isopropyl alcohol. At this point the column was transferred to a cold room, the isopropyl alcohol:water mixture was passed through the column, and the effluent was collected with a Technicon time-flow fraction collector set for 300 drops (*ca.* 8.0 ml.) per tube. The emergence of material was determined from optical density measurements (at 260 mμ) of every fifth or tenth tube. After approximately 12 hours, tubes 0–90, having an optical density of essentially zero, had been collected.[26] BuAMP then appeared in the effluent tubes 100–130. AMP followed BuAMP in the effluent (tubes 130–150), and in some columns there was a slight overlapping of the two components. The tubes containing BuAMP were pooled and reduced in volume at 0–5° using a Rinco evaporator. By evaporation of this solution to dryness, or by lyophilization, BuAMP in solid form could be obtained, but, owing to the lability of the material, it was usually stored frozen as the concentrated solution. The yield of BuAMP, including the isolation procedure, varied between 50–75% for all preparations.

An alternate method of separating BuAMP from AMP involved the use of large-scale paper chromatography. An aqueous concentrate of BuAMP (*ca.* 25 mg. in 0.2 ml.) was applied as a streak to the starting line of a 28×42 cm. sheet of Whatman No. 1 paper, and the chromatogram was developed (ascending) with a mixture of isobutyric acid:NH_3:H_2O (66:1:33). BuAMP ($R_f = 0.68$) was located by inspection under a Mineralight, and subsequently eluted with water. This method was more rapid than the columns and was employed routinely for smaller preparations (*ca.* 100 mg.).

Attempts were made to separate BuAMP and AMP on Dowex-1 anion-exchange columns using the batch method of Carter and Cohn,[27] or the gradient elution method of Hurlbert, *et al.*[28] In both instances BuAMP preceded AMP, as expected, but the lability of the former in prolonged contact with the acidic eluent mixtures, limited the use of this technique.

Results and Discussion

The use of a carbodiimide to effect a condensation between the free acid and AMP would appear to be, at present, the most satisfactory method for the preparation of this class of mixed anhydrides. In preliminary experiments directed toward the synthesis of acetyl AMP, acetyl chloride, acetic anhydride or isopropenyl acetate were somewhat unsatisfactory owing to the attack of the acylating agent upon other positions of the AMP. Even with the carbodiimide method, it was found that syntheses run at room temperature, or above, gave rise to polyacetylated products of AMP which were characterized by an absence or reactivity toward periodate, showing that the 2′–3′ positions were blocked.

Stability tests indicated that BuAMP was decomposed slowly even when stored in the frozen state. The stability of BuAMP at *p*H 7.0 as a function of temperature is shown in Fig. 1. At room temperature, BuAMP has its maximum stability in the *p*H region of 4–6.

The homogeneity of BuAMP was demonstrated by means of paper chromatography. BuAMP moved as a single, homogeneous spot in solvent systems A and B (R_f values of 0.68 and 0.30, respectively) and was readily separable from AMP (R_f values of 0.40 and 0.20). BuAMP was located on these paper chromatograms by its "quenching" spot when viewed under a Mineralight. It also reacted positively to the molybdate, hydroxylamine–$FeCl_3$ and periodate spray reagents, and in each case there was only a single colored area which coincided with the "quenching" spot.

The authenticity and purity of the BuAMP was established further by means of quantitative assay for the various functional groups. Ultraviolet absorption spectrum of the material at *p*H 7 revealed no absorption other than a symmetrical peak at 259 mμ.[29] It is assumed that BuAMP has the same extinction coefficient as AMP under these conditions (*i.e.*, $\epsilon = 15.4 \times 10^3$). Aliquots of this solution were analyzed for the following functional groups: (a) adenine; (b) total phosphate; (c) anhydride linkage; (d) *vicinal* hydroxyl groups; and (e) free amino group on the 6-position of the purine. The ratio of these assays a/b/c/d/e was 1.00/1.00/0.90/0.98/1.07. The material appears to be reasonably pure and of the correct structure (*i.e.*, a single acyl group in an anhydride linkage with the phosphate group of AMP). The slightly

(20) J. G. Buchanan, C. A. Dekker and A. G. Long, *J. Chem. Soc.*, 3162 (1950).

(21) Phosphate analyses were kindly performed by Mrs. Ruth Caffrey.

(22) P. B. Hawk, B. L. Oser and W. H. Summerson, "Practical Physiological Chemistry," 12th ed., Blakiston Co., Philadelphia, 1949, p. 579.

(23) J. S. Dixon and D. Lipkin, *Anal. Chem.*, **26**, 1092 (1954).

(24) G. L. Kilgour, Ph.D. thesis, University of Washington, 1955.

(25) Obtained from A. S. Aloe Co., 1920 Terry Avenue, Seattle, Washington.

(26) At this point an unknown material emerged and was collected in tubes 90–100.

(27) W. Cohn and C. E. Carter, THIS JOURNAL, **72**, 4273 (1950).

(28) R. B. Hurlbert, H. Schmitz, A. F. Brumm and V. R. Potter, *J. Biol. Chem.*, **209**, 23 (1954).

(29) This observation affords further proof that the product is devoid of acylation on the 6-amino group, since model compounds, such as 6-acetamidopurine, have their absorption band shifted to 280 mμ. (P. T. Talbert and F. M. Huennekens, unpublished observation.)

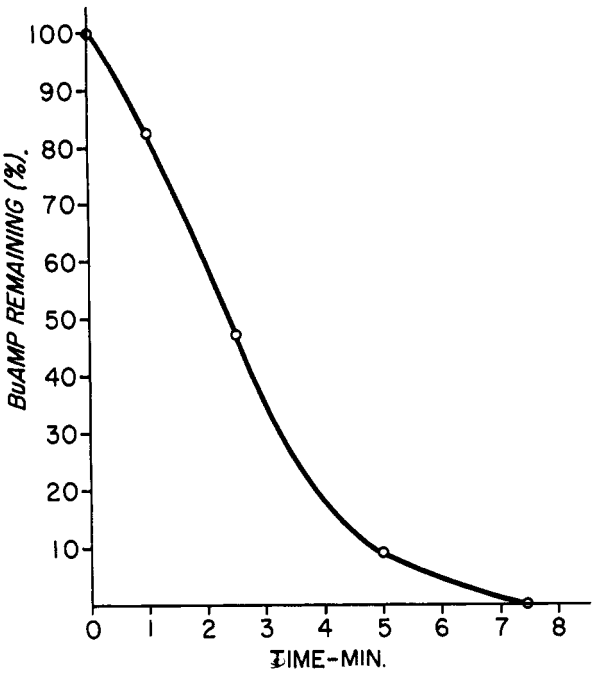

Fig. 1.—Heat stability of BuAMP. Each tube contained 3.45 μmoles of BuAMP, 50 μmoles of phosphate buffer, *p*H 7.0 and H_2O to make 1.0 ml. The tubes were immersed in a water-bath at 100° for the indicated time, chilled in an ice-bath, and immediately assayed for residual BuAMP by the hydroxamic acid method.

low value for the anhydride linkage is due probably to the lability of the compound.

BuAMP has been assayed enzymatically with FAAE. From an inspection of equation 2′ it is evident that BuAMP should replace butyrate and ATP in the enzymatic synthesis of butyryl CoA. The results of a typical experiment[30] are shown in Table I. The lower over-all conversion of BuAMP, relative to butyrate plus ATP, could be due to: (a) contamination of BuAMP by traces of AMP, which is a potent inhibitor ($K_I = 2.4 \times 10^{-4}$ *M*) of the FAAE system[15]; (b) hydrolysis of BuAMP; or (c) the possibility that BuAMP is not an intermediate on the main pathway of butyryl CoA formation (see below).

TABLE I

COMPONENT STUDY OF BUTYRYL CoA FORMATION

The complete system contained 1 μmole of CoA, 2 μmoles of ATP, 5 μ moles of butyrate, 2.5 μmoles of $MgCl_2$, 1 μmole of KBH_4, 25 μmoles of glycyl glycine buffer, *p*H 8.0, 0.1 ml. of FAAE and water to make 0.4 ml. After incubation for 30 minutes at 38°, the residual CoA was assayed by the nitroprusside method.

	CoA disappearance, μmoles
Complete system	0.290
No butyrate	.000
No ATP	.029
No FAAE	.000
BuAMP replacing butyrate and ATP[a]	.097

[a] 0.48 μmoles of BuAMP replaced butyrate and ATP.

(30) Different preparations of FAAE have always shown an absolute requirement for butyrate, but a variable, and partial, requirement for ATP.

The effect of BuAMP concentration upon butyryl CoA synthesis is illustrated in Fig. 2. The Michaelis constant (K_m) for BuAMP, calculated by the Lineweaver–Burk method,[31] is 2.5×10^{-4} *M*. This K_m value for BuAMP is somewhat lower (and, therefore, a reflection of the higher affinity between enzyme and substrate) than the reported constants[15] for ATP ($K_m = 5 \times 10^{-4} M$) and butyrate ($K_m = 1.59 \times 10^{-3} M$) with FAAE.

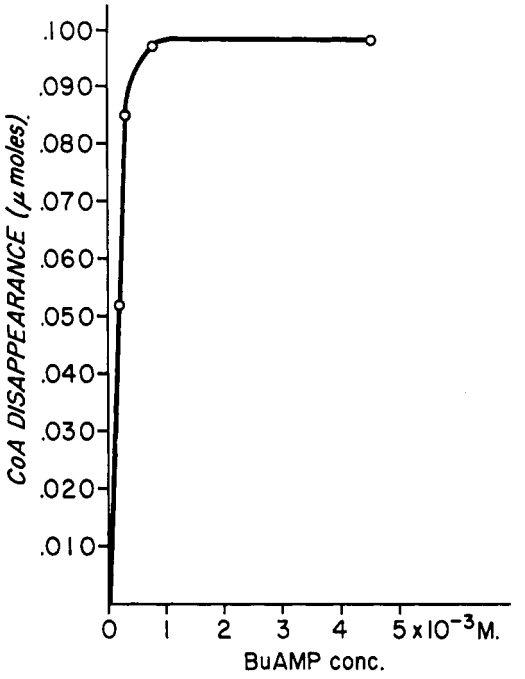

Fig. 2.—Dependence of CoA disappearance on BuAMP concentration. Experimental conditions as in Table I with variable amounts of BuAMP added. Velocity is expressed as μmoles of CoA disappearing in 30 min.

The required stoichiometry for reaction (3′) was obtained also from the data of Fig. 2. For the lowest amount of BuAMP employed, 5.5×10^{-2} μmoles, the reaction proceeded essentially to completion during the time of the assay and caused the disappearance of 5.3×10^{-2} μmole of CoA. With larger initial amounts of BuAMP, the amount of butyryl CoA formed was less than the BuAMP disappearing. This may be attributed to the enzymatic hydrolysis of BuAMP, wherein water competes with CoA for the BuAMP. The magnitude of the hydrolytic effect is illustrated in Fig. 3. It should be noted that the amount of enzyme has been increased threefold, compared to the amount normally used (*cf.* Table I), in order to emphasize the hydrolysis reaction. Since water and CoA may compete as acceptors in equation 3′, it is difficult to utilize the data from Fig. 3 to ascertain the amount of hydrolysis occurring in the *presence* of CoA, *i.e.*, during the enzymatic synthesis of butyryl CoA. Furthermore, until completely pure preparations of FAAE are available, it is not possible to decide whether hydrolysis is an inherent reaction of the FAAE system, or whether there is contamination by the hydrolase.

(31) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

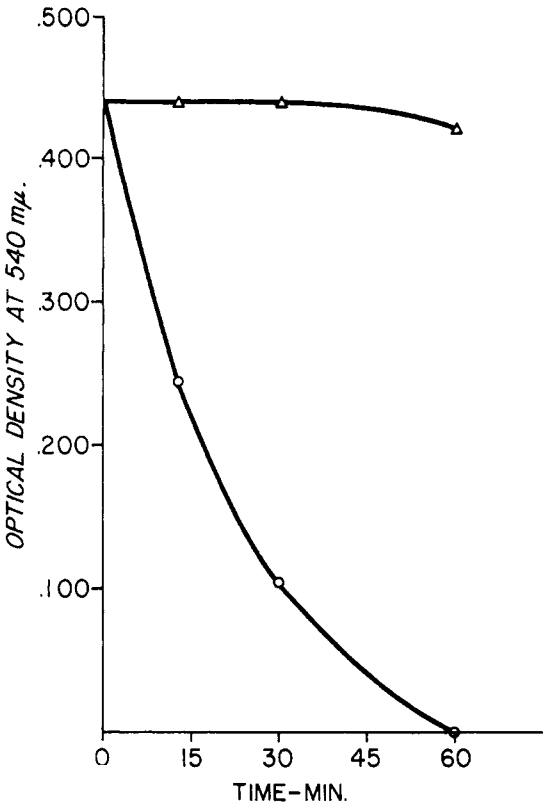

Fig. 3.—Enzymatic hydrolysis of BuAMP. The system contained 4 μmoles of BuAMP, 50 μmoles of phosphate buffer, *p*H 7.0, 0.3 ml. of FAAE and water to make 1.0 ml. The tubes were incubated at 38° for the indicated periods and analyzed immediately for residual BuAMP by the hydroxylamine method. The results are expressed in terms of optical density at 540 mμ O, enzymatic reaction; Δ, enzyme omitted.

BuAMP may be assayed also in a system based upon equation 2′ in reverse, which traps the ATP by further reaction with the hexokinase-glucose-6-phosphate dehydrogenase systems. As shown in Table II, there was an absolute requirement for BuAMP and FAAE in order to form ATP (as measured by TPNH formation), but a variable, partial requirement for the other substrate, PP. The rate of TPNH formation increased with increasing BuAMP concentration, but the individual rate curves were erratic, indicating that some component of the FAAE system interfered with the ATP utilizing system. In no case was more than 10–15% of the initial BuAMP converted to ATP.[32]

TABLE II

The complete system contained in a 1 cm. Corex cuvette 1 mg. of TPN, 2 mg. of hexokinase, 0.8 mg. of glucose 6-phosphate dehydrogenase, 20 μmoles of glucose, 50 μmoles of glycyl glycine buffer, *p*H 7.5, 20 μmoles of PP, 10 μmoles of Mg^{++}, 2.4 μmoles of BuAMP, 0.3 ml. of FAAE and water to make 3.0 ml. TPN was omitted in the blank. The reaction was started by the addition of FAAE and the change in optical density measured over a 30-min. period.

Conditions	TPNH formed, μmoles
Complete system	0.10
Less PP	.05
Less BuAMP	0
Less FAAE	0

The above findings that BuAMP serves as a substrate for equation 2′ in the reverse direction, and for equation 3′ in the forward direction, and that in the latter case the correct stoichiometry is observed and the K_m value is lower than that of either butyrate or ATP, may be taken as supporting evidence that equations 2′ and 3′ represent the correct mechanism for reaction (1′). We have been unable to demonstrate, however, the direct enzymatic formation of BuAMP from butyrate and ATP, as required by equation 2′, whether the reactions were carried out in the presence of H_2NOH, or the reagent added after a period of incubation. It may be that the equilibrium of equation 2′ lies far in the direction of butyrate and ATP, and that although H_2NOH is capable, theoretically, of shifting the equilibrium, it is, in fact, unable to do so because of its toxicity to the enzyme, as previously suggested.[15]

A possible mechanism for the action of FAAE would be to assume that BuAMP, as such, is not on the direct pathway of butyryl CoA formation, but is capable of interacting with the enzyme to form an intermediate, wherein the butyryl and AMP groups are bound to the enzyme,[33] although not necessarily at the same site. The over-all mechanism could then be formulated as

$$\text{ATP} + \text{E} \rightleftharpoons \text{E}\begin{matrix}\text{AMP}\\\text{PP}\end{matrix} \xrightarrow{\text{Bu}} \text{E}\begin{matrix}\text{AMP}\\\text{Bu}\end{matrix} \xrightarrow{\text{CoA}} \text{E} \uparrow \text{BuAMP}$$

$$\text{E}\begin{matrix}\text{CoA}\\\text{Bu}\end{matrix} \longrightarrow \text{E} + \text{Butyryl CoA} \quad (4)$$

It should be emphasized that the present scheme differs from Berg's original formulation only through the introduction of the enzyme[7,8,34,35] as a carrier of the various groups, and the formation, at least in a transient sense, of enzyme-group complexes. The failure to accumulate free acyl adenylates in systems represented by reaction (2) has occasioned a modification[36,37] of Berg's original scheme wherein the intermediate, acyl adenylate, is now assumed to be enzyme-bound.

Regardless of the mechanism involved, it is of interest to note the order with which the various functional groups displace one another during the over-all reaction. In reaction (2′), butyrate forms a mixed anhydride with the phosphoric acid group

(32) Peng[14] has observed likewise that the FAAE system will catalyze equation 2′ in reverse, producing ATP, and that the ATP may be estimated by coupling the system with phosphoglycerate kinase and 3-phosphoglyceraldehyde dehydrogenase. Again, the hexokinase system did not estimate ATP satisfactorily in the presence of the FAAE system. However, even with Peng's improved assay for ATP, only a small fraction of the BuAMP was converted to ATP.

(33) Acyl-enzyme complexes have been prepared and isolated from 3-phosphoglyceraldehyde dehydrogenase by E. Racker (*Physiol. Rev.*, **35**, 1 (1955)), and from *p*-nitrophenyl acetate and chymotrypsin by Balls and Aldritch (*Proc. Nat. Acad. Sci.*, **41**, 190 (1955)).

(34) M. B. Hoagland, *Biochim. Biophys. Acta*, **16**, 288 (1955).

(35) P. D. Boyer, O. J. Koeppe and W. W. Luchsinger, THIS JOURNAL, **78**, 356 (1956).

(36) M. B. Hoagland, E. B. Keller and P. C. Zamecnik, *J. Biol. Chem.*, **218**, 345 (1956).

(37) P. Berg and G. Newton, *Federation Proc.*, **15**, 219 (1956).

of AMP, and the latter group is then displaced by the thiol group of CoA in reaction (3′). The acyl CoA derivative is readily decomposed by H_2NOH. Thus, the increasing order of affinity for the acyl group is $-PO_3OH$, $-SH$ and $-NHOH$, *i.e.*, in order of increased basicity.

Acknowledgment.—The authors are indebted to Mrs. Enid Vercamer for her assistance in carrying out the enzymatic assays, and to Dr. Gordon Kilgour for helpful discussions regarding the chemical synthesis of BuAMP.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF ROCHESTER]

The Chemistry of Fumagillin. IV. The Presence of an Epoxide Grouping and Other Observations on the Nature of the Oxygen Functions[1]

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The alcohol moiety of fumagillin, alcohol-I ($C_{16}H_{26}O_4$), has been obtained crystalline and has been shown to contain an epoxide grouping. Reduction of alcohol-I with lithium aluminum hydride under mild conditions opens the epoxide ring to give a crystalline diol, dihydroalcohol-Ib ($C_{16}H_{28}O_4$), which can be reduced catalytically to give the previously described crystalline tetrahydroalcohol-Iab. Also formed in this reduction are tetrahydroalcohol-Ib, a triol ($C_{16}H_{30}O_4$), in which both the epoxide ring and another cyclic ether grouping have been opened and tetrahydronoralcohol-I ($C_{15}H_{28}O_4$), a triol which has lost one carbon. Reduction of alcohol-I, dihydroalcohol-Ib and tetrahydroalcohol-Iab by lithium aluminum hydride in refluxing tetrahydrofuran gives as main products the C_{16} triols formed by reductive cleavage of the second cyclic ether group, as well as the C_{15} "nor" triols. The ketone ($C_{16}H_{28}O_4$) obtained by oxidizing tetrahydroalcohol-Iab with chromium trioxide–pyridine has been shown to contain one free methylene adjacent to the carbonyl group, by the preparation of a crystalline monofurfurylidene derivative.

Alcohol-I ($C_{16}H_{26}O_4$), which, along with decatetraenedioic acid, is obtained by the alkaline hydrolysis of the antibiotic fumagillin,[3-5] has now been obtained crystalline, $[\alpha]^{23}D$ −68.0, m.p. 55.5–56°. Earlier studies[5] showed that catalytic reduction of alcohol-I with hydrogen and platinum in ethanol yielded a crystalline diol, tetrahydroalcohol-Iab[6] ($C_{16}H_{30}O_4$), in which the double bond in the side chain had been reduced, and a cyclic ether linkage had been hydrogenolyzed to generate a new hydroxyl group. Alcohol-I also consumed one equivalent of lithium aluminum hydride, in addition to the equivalent which reacted with the hydroxyl group. This behavior suggested the presence of an epoxide group in alcohol-I; we have been able to establish the presence of an epoxide group in alcohol-I, and its absence in tetrahydroalcohol-Iab, by the following quantitative methods: the thiosulfate method,[7] the ethereal hydrogen chloride procedure[8] and the periodate–perchloric acid method.[9] The quantitative results showed 70–95% of the theoretical amount of epoxide in alcohol-I; however, the epoxide function in alcohol-I must be fairly highly substituted,[5] and it has been shown that these methods do not yield the theoretical values when applied to highly substituted ethylene oxides.[10] These results, in conjunction with those below, effectively rule out other possible cyclic ether groupings, such as a trimethylene oxide ring.[11]

Treatment of alcohol-I with lithium aluminum hydride in ether at 0° for 24 hr. gave a 66% yield of the crystalline diol, dihydroalcohol-Ib ($C_{16}H_{28}O_4$), m.p. 53°, in addition to the triols, tetrahydroalcohol-Ib ($C_{16}H_{30}O_4$), and tetrahydronoralcohol-I ($C_{15}H_{28}O_4$) discussed below. One of the two hydroxyl groups in dihydroalcohol-Ib must have resulted from hydrogenolysis of the epoxide group. Crystalline dihydroalcohol-Ib and tetrahydronoralcohol-I were obtained in 41 and 33% yield, respectively, by the action of sodium borohydride on alcohol-I in boiling methanol; no dextrorotatory material (*i.e.*, no tetrahydroalcohol-Ib) was obtained. Sodium borohydride thus can open the epoxide ring but not the second ether linkage. Catalytic reduction of the double bond in the side chain of dihydroalcohol-Ib yielded the same crystalline tetrahydroalcohol-Iab, m.p. 89°, which was previously[5] obtained by catalytic reduction of alcohol-I. This proves that the opening of the epoxide ring catalytically, to form tetrahydroalcohol-Iab, and by lithium aluminum hydride to form dihydroalcohol-Ib takes place in the same direction.[12]

The same reactions can be run in the reverse order. Alcohol-I, reduced catalytically with one

(1) Presented in part at the Summer Seminar on Natural Products, University of New Brunswick, Fredericton, New Brunswick, August 17, 1955.

(2) Inquiries regarding this paper should be addressed to this author.

(3) J. R. Schenck, M. P. Hargie, D. S. Tarbell and P. Hoffman, THIS JOURNAL, **75**, 2274 (1953).

(4) J. R. Schenck, M. P. Hargie and A. Isarasena, *ibid.*, **77**, 5606 (1955).

(5) D. S. Tarbell, P. Hoffman, H. R. Al-Kazimi, G. A. Page, J. M. Ross, H. R. Vogt and B. Wargotz, *ibid.*, **77**, 5610 (1955).

(6) It has seemed advisable, in the interests of brevity and clarity, to adopt the naming system employing letters. The material now designated tetrahydroalcohol-Iab was previously[5] called tetrahydroalcohol-I.

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(12) This two-step procedure is a better preparative method for tetrahydroalcohol-Iab than the one-step catalytic reduction because many samples of platinum catalyst are not effective[5] in producing the crystalline tetrahydroalcohol-Iab.