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BUTYRYL ADENYLATE AND ITS POSSIBLE FUNCTION IN THE FATTY ACID ACTIVATING SYSTEM

by

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In recent years several enzymes have been discovered and characterized which catalyze the "activation" of a substrate concomitant with a stoichiometric cleavage of ATP* into AMP and PP[1–5]. The "activated" substrate is ultimately found in combination with an additional substance, *e.g.*, CoA. In no case is there evidence that more than a single enzyme is involved. The multiplicity of the reactants in a process catalyzed by one enzyme demands a complex mechanism, which involves a multiple transfer of groups. It is therefore difficult to conceive of a mechanism which is in agreement with all experimental observations and would also satisfy theoretical considerations[6,7].

* The following abbreviations will be used: adenosine mono- and triphosphates, AMP and ATP; acetyl and butyryl adenylates, AcAMP and BuAMP; coenzyme A, CoA; butyryl CoA, BuCoA; butyrate, BuO^-; pyrophosphate, PP; diphosphopyridine nucleotide, DPN; reduced DPN, DPNH; tris(hydroxymethyl)aminomethane, Tris; fatty acid activating enzyme, FAAE.

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The experiments communicated in this paper were performed with the aim of gaining insight into the mechanism of activation of fatty acids by ATP as catalyzed by the fatty acid activating enzyme. Studies on the incorporation of PP into ATP under the conditions of the activating reaction suggested the possibility that BuAMP might be an intermediate in this reaction. When the synthesis of BuAMP was in progress a preliminary report appeared by BERG[8] who adduced evidence that AcAMP participates in the analogous acetate activating system. In view of the excellent and convincing work of BERG[8*], it was decided to avoid duplication and to summarize here briefly the pertinent results obtained so far with the fatty acid system. These results are in agreement with those obtained by BERG with the acetate activating system. They add weight to the conclusion that the mechanism for which BERG first supplied definite evidence may be a general one for activating reactions of the type discussed above.

EXPERIMENTAL

Materials and methods

ATP was obtained from Pabst Laboratories and AMP from Sigma Chemical Company. Labeled pyrophosphate was prepared by heating $Na_2H^{32}PO_4$ to 400° for 1 hour. Pyrophosphate analysis with yeast pyrophosphatase showed that 98% of the phosphate had been converted to pyrophosphate. The fatty acid activating enzyme was prepared by the method of MAHLER *et al.*[4]. The activity of this enzyme declines somewhat on storage. The figures given for the specific activities throughout the paper refer therefore to the activity of the freshly prepared enzyme. Purified yeast hexokinase was a gift from Dr. EARL JACOBS. Highly purified phosphoglycerate kinase from pea seed and phosphoglycerate were gifts from Dr. N. S. LING. Samples of crystallized triosephosphate dehydrogenase were donated by Dr. H. A. LARDY and Dr. D. M. GIBSON. The microtitration of BuAMP was kindly performed for us by Dr. R. M. BOCK with an automatic titration apparatus. Pyrophosphate was separated from adenine nucleotides by the procedure of CRANE AND LIPMANN[9]. Paper chromatography of nucleotides was carried out according to the system of KREBS AND HEMS[10]. Hydroxamic acids were separated according to the octyl alcohol-formic acid system of THOMPSON[11]. Hydroxamates and sulfhydryl groups were estimated by modifications of the known standard methods[12,13].

RESULTS

Preparation and purification of BuAMP

BuAMP was prepared by a modification of AVISONS synthesis of acetyl riboflavin-5-phosphate[14]. One mmole of AMP, suspended in 5 ml of water, was neutralized with 1 ml of 1 *N* NaOH. The resulting solution of sodium AMP was cooled in ice and 2.5 ml of pyridine were added. The solution was stirred mechanically and the temperature was kept at 0°. Excess butyric anhydride (5 mmole) was added slowly under stirring. After 10 minutes 80 ml of cold acetone were added and the precipitate was centrifuged in steel cups at about 20,000 × *g* (high speed head of the International centrifuge) for 30 minutes. The supernatant was decanted and the precipitate was twice washed with cold acetone and once with cold dry ether. About 75% of the AMP was present as BuAMP in the isolated product.

BuAMP was further purified and separated from AMP by paper electrophoresis. 0.5 mg of sample was streaked on paper strips (Whatman, 3MM; 6.3 × 46 cm) and

* Personal communication of Dr. P. BERG to Dr. H. BEINERT.

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exposed to 800 volt for 1 hour at 15°. 0.05 *M* Tris acetate of pH 7.0 was used as a buffer. AMP and BuAMP separated into two distinct bands seen under illumination from an ultraviolet lamp. The paper was dried in a current of warm air. The hydroxamic acid reaction was used to identify the band containing BuAMP. The paper in the area of this band was cut out, divided into small pieces and soaked in cold water. After 30 minutes the fluid was removed on a Buchner funnel and the paper pulp was washed with small quantities of water. The extracts were combined and an excess of ether was added. Tris ions were removed by adjusting the pH to 3 with Dowex 50 (H^+-form) and the bulk of the acetic acid was removed by two ether extractions. The resin was separated by centrifugation and the solution was lyophilized. The remaining acetic acid was volatilized during this operation.

Analysis of BuAMP

After electrophoresis and subsequent elution from paper, BuAMP was assayed with NH_2OH for hydroxamate formation and its extinction at 260 mμ was determined. Acethydroxamic and octanohydroxamic acids, which have an identical molar extinction coefficient at 540 mμ under the conditions of the hydroxamate assay, served as standards. The molar extinction coefficient of AMP (15.4 at pH 7.0[15]) was used for the absorption at 260 mμ. The ratio of AMP to hydroxamate present was 1.01 ± 0.05.

BuAMP is unstable at room temperature at pH values above 8 and below 3 (Fig. 1). At pH 7, however, only 12% is hydrolyzed in 20 minutes at 100°. When BuAMP was hydrolyzed with 0.5 *N* KOH, a compound was formed in equimolar quantities, which was indistinguishable from AMP on paper chromatography. When BuAMP was decomposed by NH_2OH it was possible to demonstrate by paper chromatography the formation of a hydroxamate, which migrated with an R_F of butyrohydroxamic acid and was inseparable from butyrohydroxamic acid. Evidence that the butyryl residue in the new compound is indeed attached to the phosphate residue of AMP was obtained by titration, which showed that the secondary phosphate of AMP was blocked. In the titrated sample 13.2 ± 1 microequivalents of a group with a p*K* of 4.1 (amino group of adenine) was found and only 1 ± 0.5 microequivalent of a group with p*K* near 7 (secondary phosphate). It is likely that this latter group was liberated by hydrolysis of BuAMP on acidification of the sample at the beginning of the titration.

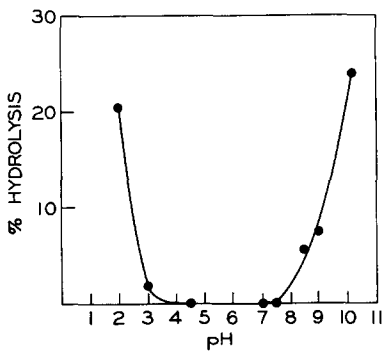

Fig. 1. Hydrolysis of BuAMP at various values of pH. The following buffers were used at 0.1 *M* concentration with respect to the buffering ion: pH 3.1, sodium citrate; pH 4.5, sodium acetate; pH 7.0 and 7.5, Tris chloride; pH 8.5, 9.0 and 10.5, Tris acetate. Hydrolysis was followed by the hydroxamic acid test.

Incorporation of labeled PP into ATP

Table I shows the pattern of incorporation of labeled PP into ATP under a variety of conditions. The results are similar to those published by BERG. It is evident that incorporation was at least as high without CoA as in the complete system. This would be a requirement if the overall reaction studied were occurring in steps the first of which involves only ATP and butyrate but not CoA.

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TABLE I

INCORPORATION OF ^{32}P ^{32}P INTO ATP

Component omitted	ATP recovered		Exchange
	μmoles	counts/min/μmole	%
None	2.16	6,000	60
CoA	2.34	6,600	66
Butyrate	2.40	1,225	12
None*	2.58	107	1

The complete system contained 20 μmoles of Tris chloride, pH 7.5, 3 μmoles of ATP, 3 μmoles of ^{32}P ^{32}P (18,550 counts/min/μmole), 5 μmoles of sodium butyrate, 0.5 μmoles of CoASH, 2 μmoles of KBH_4, 5 μmoles of $MgCl_2$, 20 μmoles of KF and 0.4 mg of FAAE (specific activity 13) in 1 ml volume. The mixture was incubated for 30 minutes at 37°.

* Trichloroacetic acid was added to the enzyme before addition of butyrate.

Participation of BuAMP in two reaction steps

The overall reaction of activation

$$ATP + BuO^- + CoA \xrightleftharpoons{Enzyme} BuCoA + PP + AMP \quad (1)$$

may be conceived of as occurring in two reaction steps:

$$ATP + BuO^- \xrightleftharpoons{Enzyme} BuAMP + PP \quad (2)$$

$$BuAMP + CoA \xrightleftharpoons{Enzyme} BuCoA + AMP \quad (3)$$

Synthetic BuAMP shows the properties expected of the postulated intermediate in reactions (2) and (3). Thus ATP is formed from BuAMP and PP in a reversal of reaction (2) and BuCoA is obtained from BuAMP and CoA in reaction (3).

Formation of ATP

The formation of ATP was shown qualitatively in the following way. 100 μmoles of Tris chloride, pH 7.5, 25 μmoles of PP, 10 μmoles of $MgCl_2$, 20 μmoles of KF, and 5.8 μmoles of BuAMP were incubated with 0.4 mg of the fatty acid activating enzyme in a total volume of 1.0 ml at 37°. After 30 minutes 1 ml of 0.2 *N* $HClO_4$ was added, the protein was separated by centrifugation and the supernatant fluid was neutralized with KOH. The solution was cooled and $KClO_4$ was removed by centrifugation. The nucleotides were separated on a Dowex-1 column (Cl^--form) according to the procedure of COHN AND CARTER[16]. The material which was obtained from the peak corresponding to ATP was further identified by paper chromatography[10].

In a similar system ATP formation was demonstrated by separation of inorganic phosphate and PP from nucleotides by charcoal[9]. After the charcoal was washed free of inorganic phosphates, ATP was hydrolyzed on the charcoal with 1 *N* H_2SO_4 and phosphate was determined in the hydrolysate. No phosphate was found in the hydrolysate, when BuAMP or PP had been omitted in the original reaction mixture. 2.4 and 4.0 μmoles of BuAMP present in the original incubation mixture gave rise to 0.26 and 0.45 μmole of hydrolyzable phosphate, respectively, within 40 minutes at 37°. As will be discussed below, the low yield in conversion is referable to disappearance of BuAMP by hydrolysis.

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ATP formation was also shown qualitatively by coupling reaction (2) (reverse) to the hexokinase and glucose-6-phosphate systems according to KORNBERG[17]. Reduction of TPN was observed when BuAMP was present. Because of some interference in the coupled system a quantitative determination of ATP was not possible.

ATP could, however, be determined quantitatively by coupling reaction (2) (in reverse) to the phosphoglycerate kinase (PGK)[18] and triosephosphate dehydrogenase (TPD) reactions. The oxidation of DPNH was followed spectrophotometrically as the reaction proceeded. The sequence of reactions carried out is the following:

$$\text{BuAMP} + \text{PP} \overset{\text{FAAE}}{\rightleftharpoons} \text{BuO}^- + \text{ATP} \qquad (2a)$$

$$\text{3-Phosphoglycerate} + \text{ATP} \overset{\text{PGK}}{\rightleftharpoons} \text{1,3-diphosphoglycerate} + \text{ADP} \qquad (4)$$

$$\text{1,3-Diphosphoglycerate} + \text{DPNH} \overset{\text{TPD}}{\rightleftharpoons} \text{3-phosphoglyceraldehyde} + \text{DPN} + \text{phosphate} \qquad (5)$$

TABLE II

FORMATION OF ATP FROM BuAMP

Component omitted	*μmoles DPNH oxidized in*	
	20 min	*60 min*
None	0.072	0.12
BuAMP	0	0
PP	0	0.02
Enzyme	0	0

50 μmoles of Tris acetate, pH 7.5, 10 μmoles of $MgCl_2$, 5 μmoles of PP, 10 μmoles of KF, 25 μmoles of 3-phosphoglycerate, 0.25 μmole of DPNH and 1 μmole of BuAMP were incubated at 28° with 0.4 mg of FAAE, 0.03 mg of phosphoglycerate kinase and about 0.05 mg of triosephosphate dehydrogenase in a total volume of 3.0 ml. Oxidation of DPNH was followed spectrophotometrically at 340 mμ.

It was ascertained that under the conditions of the reaction ATP reacted quantitatively and that no 1,3-diphosphoglycerate accumulated. A typical experiment is recorded in Table II. DPNH was not oxidized when BuAMP, PP or FAAE was omitted.

Formation of BuCoA

When reaction (3) was carried out, disappearance of CoASH was followed by the nitroprusside reaction. Appearance of BuCoA was demonstrated by the use of the green fatty acyl CoA dehydrogenase[19], which is specific for short chain fatty acyl derivatives of CoA. In the presence of this enzyme and the electron transferring flavoprotein[20], BuCoA is quantitatively oxidized to crotonyl CoA by indophenol[21]. Dye reduction is a precise measure of the quantity of substrate originally present. Enzymes of high purity from pig liver were used for this assay. The results are shown in Table III.

TABLE III

FORMATION OF BuCoA FROM BuAMP

BuAMP added μmoles	*CoASH disappeared μmoles*	*BuCoA formed μmoles*
0	0	0
1.9	0.1	
3.8	0.21	0.18

The complete system contained 10 μmoles of Tris chloride, pH 7.5, 2 μmoles of $MgCl_2$, 2 μmoles of KBH_4, 0.2 μmole of CoASH, BuAMP as indicated, and 0.40 mg of FAAE (specific activity 9) in a total volume of 0.2 ml. The mixture was incubated under nitrogen for 20 minutes and at 37°.

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Rate of reactions (1) and (3)

Initially, it was observed that BuAMP was rapidly hydrolyzed in presence of the preparation of fatty acid activating enzyme used (specific activity 9). Preparations of higher purity (specific activity 34) showed a lower ratio of the hydrolytic rate to the rate of the overall reaction (1) (Table IV). It is therefore obvious that the hydrolytic activity observed is not an intrinsic property of the fatty acid activating enzyme but is due to a contaminating phosphatase. The presence of a powerful phosphatase, which is not easily separated from the fatty acid activating enzyme, made studies of the quantitative conversion of BuAMP to products in reactions (2) and (3) difficult. A comparison was, however, made of the rate of reaction (1) and that of the half reaction (3). In reaction (3), under otherwise equal conditions, an amount of BuAMP was used equimolar to the amount of ATP used in reaction (1). Butyrate was only supplied for reaction (1). Rates were measured during initial periods of reaction, for which linearity was ascertained. If BuAMP is to qualify as an intermediate in the overall reaction, reaction (3) should under no circumstances occur at a rate lower than that of reaction (1). It was indeed found (Table IV) that reaction (3) could occur at an equal or higher rate than reaction (1). These experiments would therefore not contradict the possible implication of BuAMP as an intermediate.

TABLE IV

COMPARISON OF REACTION RATES

Specific activity of FAAE	*Reaction (1)*	*Reaction (3)*	*Hydrolysis*	*Ratios*	
				reaction (3) / reaction (1)	*reaction (1) / hydrolysis*
	Δμmole CoASH in 5 min	*Δμmole CoASH in 5 min*	*Δμmole BuAMP in 5 min*		
9	0.048	0.047	0.45	1.0	0.11
34	0.041	0.140	0.09	3.4	0.45

The system contained in each case 10 μmoles of Tris chloride, pH 7.5, 2 μmoles of $MgCl_2$, and 1.1 μmole of nucleotide: ATP in reaction (1) and BuAMP in reaction (3) and for hydrolysis. For reaction (1) and (3) 2 μmoles of KBH_4 and 0.2 μmole of CoASH were added. 5 μmoles of sodium butyrate were added in reaction (1). 0.3 mg of FAAE of specific activity 9 was used and 0.1 mg of FAAE of specific activity 34 as indicated. The volume was 0.2 ml. Reactions (1) and (3) were followed by the nitroprusside reaction and hydrolysis with the hydroxamic acid reaction.

DISCUSSION

It has been shown by the experiments communicated in this paper that synthetic BuAMP can be utilized by the fatty acid activating system for the formation of BuCoA or ATP in reactions which may be conceived of as the two constituent reaction steps of the overall reaction (1). The rates observed are not incompatible with the possibility that reaction (1) does indeed occur in two steps, reactions (2) and (3), with BuAMP as the true intermediate. However, it has so far not been possible to show, neither in the present work nor in earlier experiments with the fatty acid or acetate activating systems, that any intermediate accumulates, when experimental conditions are maintained which would be expected to favor accumulation of an intermediate. Hydroxamate formation was observed in the presence of ATP, acetate or butyrate, hydroxyl-

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amine and the respective enzymes and in the absence of CoA (*cf.*[22]). However, the presence of enzyme-bound CoA would have to be rigorously excluded, before this could be accepted as evidence for an interaction of ATP with acetate or butyrate to form an activated intermediate of the type of an acyl adenylate. One may, of course, recur to the postulate that such an intermediate would only exist in an amount stoichiometric with the enzyme. Further work involving considerable amounts of highly purified enzyme, is obviously required, if such a postulate is to be verified. It should be pointed out that Mg^{++}-ions are also part of the activating systems under study and that the true intermediate may be more complex than a simple acyl adenylate.

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SUMMARY

The preparation of butyryl adenylate (BuAMP) from butyric anhydride and AMP and some of the properties of this compound are described. Synthetic BuAMP is able to react with pyrophosphate to form ATP and with CoA to form butyryl CoA when incubated in presence of the fatty acid activating enzyme from beef liver. Butyryl CoA formation from BuAMP occurs at a rate which is compatible with the concept that BuAMP is a true intermediate in the overall reaction of butyrate activation by ATP and CoA.

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