

ACYL ADENYLATES: THE SYNTHESIS AND PROPERTIES OF ADENYL ACETATE*

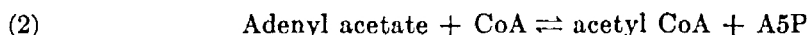
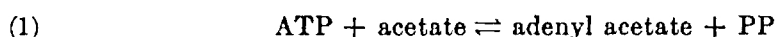
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A study of the mechanism of acetyl CoA¹ formation from ATP, acetate, and CoA by yeast aceto-CoA-kinase has revealed that the reaction occurs in two steps (1, 2). The evidence suggests that ATP and acetate react to form PP and the acetyl derivative of A5P, which is then utilized in the formation of acetyl CoA. This formulation is supported by the finding that synthetic adenyl acetate and PP are enzymatically converted to ATP (Reaction 1), and that acetyl CoA is formed from adenyl acetate and CoA (Reaction 2).



The present communication deals with a description of the synthesis and properties of adenyl acetate.

Materials and Methods

DPN, TPN, A5P, and glucose-6-phosphate dehydrogenase (0.8 unit per mg. (3)) were products of the Sigma Chemical Company, and CoA (75 per cent pure) was obtained from the Pabst Brewing Company. Hexokinase and P³²P³² were prepared as previously described (2), and citrate-condensing enzyme (4) containing malic dehydrogenase was kindly supplied by Dr. S. Ochoa. A5P deaminase was prepared from rabbit muscle according to the method of Kalckar (5), and 5'-nucleotidase was obtained from bull semen according to Heppel and Hilmoe (6), by using the "simplified preparation."

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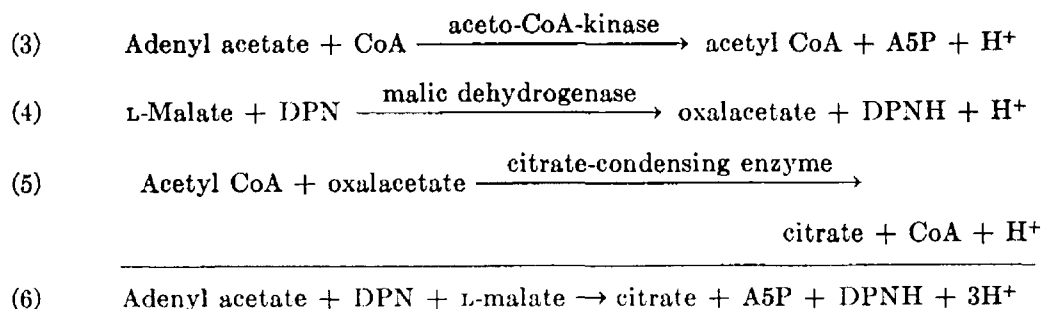
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¹ The following abbreviations have been used throughout. Acetyl coenzyme A, acetyl CoA; adenosine triphosphate, ATP; inorganic pyrophosphate, PP; adenosine-5'-phosphate, A5P; diphosphopyridine nucleotide, DPN; triphosphopyridine nucleotide, TPN; trichloroacetic acid, TCA; tris(hydroxymethyl)aminomethane, Tris.

Free A5P was determined by deamination with A5P deaminase (7) and by the liberation of inorganic phosphate (8) with 5'-nucleotidase (6), while acetate was measured with a partially purified preparation of acetokinase by the method of Rose *et al.* (9).

Determination of Adenyl Acetate—Adenyl acetate was determined either colorimetrically as the ferric complex of acethydroxamate, or enzymatically by conversion to ATP or acetyl CoA.

ATP synthesis from adenyl acetate was measured in two ways. In the first, adenyl acetate and $P^{32}P^{32}$ were incubated with the enzyme, and the ATP formed was determined after adsorption and elution from Norit (2). ATP formation was also determined by measurement of TPN reduction in the presence of aceto-CoA-kinase, hexokinase, glucose, glucose-6-phosphate dehydrogenase, and TPN (2). Acetyl CoA synthesis from adenyl acetate and CoA was measured by DPNH formation in the following series of reactions.



In the colorimetric procedure, 0.1 ml. of freshly neutralized 2 M hydroxylamine was added to the sample in 0.9 ml., and after 5 minutes at 37°, or at room temperature, 0.5 ml. of 10 per cent ferric chloride containing 0.2 N TCA and 0.66 N HCl was added. After 5 minutes the optical density at 540 m μ was determined in a cuvette with a light path of 1 cm. by using a Beckman DU spectrophotometer. A sample to which no adenyl acetate had been added served as the blank. 1 μ mole gave an optical density of 0.630 under these conditions. There was good agreement between the values obtained in this manner and those determined enzymatically (Table I). During these experiments it was found that incubation of the adenyl acetate hydroxylamine mixture at 100° for 5 minutes, instead of at 37°, gave higher values for adenyl acetate than those found enzymatically. This discrepancy will be discussed later.

Synthesis of Adenyl Acetate—Adenyl acetate was prepared in two ways. The first was the reaction of acetyl chloride and silver adenylate by a modification of the method used by Lipmann and Tuttle for acetyl phosphate (10), and the second was a modification of the method of Avison (11) with acetic anhydride and A5P in aqueous pyridine.

Procedure A—360 mg. of A5P were suspended in 20 ml. of water and the pH was adjusted to pH 6.5 with KOH. 4.0 ml. of 1.0 M silver nitrate were added and after 1 hour at 4° the precipitate was removed by centrifugation, washed twice with 25 ml. portions of cold water, twice with 50 ml. portions of cold ethanol, and twice with 50 ml. portions of ether. The precipitate was dried *in vacuo* over phosphorus pentoxide and paraffin. The yield of silver adenylate was 435 mg.

430 mg. of the silver adenylate were suspended in 5 ml. of anhydrous ether (from a freshly opened container) in a three-necked flask fitted with a dropping funnel and two drying tubes containing calcium chloride. The dropping funnel was also fitted with a drying tube at the top. The flask was immersed in an ice bath and the suspension stirred with a magnetic stirrer. 15 ml. of an ether solution containing 10 mg. of freshly distilled acetyl chloride per ml. were added dropwise with vigorous stirring over a

TABLE I
Enzymatic and Colorimetric Determination of Synthetic Adenyl Acetate

Acethydroxamate formation	ATP formation	Acetyl CoA formation
<i>μmoles per ml.</i>	<i>μmoles per ml.</i>	<i>μmoles per ml.</i>
75.2	73.9* 76.0†	74.9

* Measured by conversion of $P^{32}P^{32}$ to ATP (2).

† Measured spectrophotometrically by TPNH formation (2).

period of about 20 minutes. The stirring was continued for an additional 15 minutes and then 5 ml. of cold water were added. The suspension was carefully adjusted to pH 6.5 by the addition of 0.2 M potassium carbonate, and then the ether layer was removed and washed twice with 5 ml. portions of cold water. The aqueous fractions were combined and the residual ether removed by blowing a stream of air over the solution at room temperature.

To the above solution (30 ml.) were added 3.3 ml. of 1 N HCl and, after centrifugation, the silver chloride precipitate was washed with 5 ml. of cold water and the wash and supernatant fluids were combined and carefully neutralized to pH 6.5 with KOH. This solution (38 ml.), based on its optical density at 260 m μ (extinction coefficient $16 \times 10^3 \text{ cm.}^{-1} \text{ M}^{-1}$ (7)), contained 20.3 μ moles of total A5P per ml. By measurement of acethydroxamic acid formation at 37°, this solution contained 2.8 μ moles of labile acetyl groups per ml.

Procedure B—2.0 gm. of A5P (5.81 millimoles) were suspended in 16 ml. of water and the pH was adjusted to about 7 with 8.5 M KOH. Pyridine (4 ml.) was added and the solution was diluted to 24 ml. with water. The

solution was cooled to -5° , and acetic anhydride (3.4 ml., 35 mmoles) was added with vigorous stirring over a period of 3 minutes. 5 minutes later 375 ml. of acetone (-15°) were added and after 15 minutes at -15° the solution was centrifuged. The precipitate formed at this stage was gelatinous, and washing was difficult. Therefore, the precipitated material was dissolved in 10 ml. of cold water and reprecipitated with 150 ml. of cold acetone. After 10 minutes the precipitate was removed by centrifugation and washed with 150 ml. of an acidified mixture of acetone and ether (1:1 by volume containing 0.001 N HCl). This treatment converted the gelatinous precipitate to a somewhat granular one which was then washed with 50 ml. of ether and then air-dried. The dried precipitate was dissolved in 35 ml. of cold water and the pH adjusted to 6.5. This solution contained 3.92 mmoles of total A5P as determined by the optical density, and 2.6 mmoles of adenylyl acetate. The yield of adenylyl acetate was 45 per cent, based on the amount of A5P used, but 66 per cent according to the A5P recovered. The supernatant fluid of the acetone precipitation contained the remainder of the A5P and some adenylyl acetate, but no attempts were made to recover this material. The ratio of adenylyl acetate to free A5P depends to a large extent on the rapidity with which the material is precipitated from the pyridine-containing mixture since pyridine catalyzes a rapid hydrolysis of adenylyl acetate.

Purification of Adenylyl Acetate—Adenylyl acetate was purified by anion exchange chromatography with Dowex 1 resin. In those preparations in which the ratio of adenylyl acetate to A5P was 1 or less, a preliminary barium fractionation was employed. One typical fractionation experiment was carried out as follows: To 20 ml. of the crude adenylyl acetate solution obtained in Procedure A were added 30 ml. of cold water, then 0.7 ml. of a saturated solution of barium chloride, followed by 175 ml. of 95 per cent ethanol. After 4 hours at -15° , the precipitate of barium adenylylate was removed by centrifugation and washed with 60 per cent ethanol. The combined wash and supernatant fluids were adjusted to pH 6.5 to 7.0 and concentrated *in vacuo* to about 20 ml. to remove most of the ethanol. This solution was diluted to 150 ml. with cold water and put on a 2.5×5 cm. column of Dowex 1 Cl^- (200–400 mesh, 2 per cent cross-linked). The adenylyl acetate was eluted almost immediately with 0.015 N HCl (peak at 2 resin bed volumes). This procedure was followed when eluates suitable for enzymatic and chemical analysis were needed. By starting with 56 μ moles of adenylyl acetate, 45.7 μ moles were eluted between 38 and 53 ml. of eluate. These three fractions contained 57.8 μ moles of A5P, indicating a purity of 79 per cent or a purification of almost 6-fold. The purity of the best preparations obtained by the combined barium fractionation and chromatographic separation ranged between 75 and 85 per cent, based on the optical density at 260 $m\mu$ and on the enzymatic activity.

Results

All of the preparations of adenylyl acetate obtained as described above had an absorption spectrum indistinguishable from A5P. At pH 7 the absorption maximum was at 259 $m\mu$ and the $\lambda_{280}/\lambda_{260}$ and $\lambda_{250}/\lambda_{260}$ ratios were 0.18 and 0.86, respectively. In the samples of adenylyl acetate prepared by Procedure B, only a small fraction of the A5P, determined from the absorption spectrum, was present as free A5P. This was shown by the use of A5P deaminase which is specific for the 5'-phosphate ester of adenosine (5, 12) and by 5'-nucleotidase which is relatively specific for the 5'-phosphate esters of nucleotides (6). Table II shows that only 18 per cent of the total A5P present was deaminated or dephosphorylated by these enzymes.

TABLE II
Enzymatic Analysis of Adenylyl Acetate Prepared by Procedure B

	Original	Hydrolyzed*
	$\mu\text{moles per ml.}$	$\mu\text{moles per ml.}$
Total A5P by optical density	4.5	
Adenylyl acetate by enzymatic assay	3.2	0.0
P_i liberated by 5'-nucleotidase	0.86	4.6
A5P deaminated by A5P deaminase	0.84	4.7

* The analyses were made on both untreated and hydrolyzed aliquots. Adenylyl acetate was hydrolyzed by incubation with 0.01 N KOH for 5 minutes at room temperature. Total A5P was calculated from the measurement of optical density at 260 $m\mu$, and adenylyl acetate was determined by enzymatic conversion to ATP. Free A5P was measured by phosphate liberation after treatment with 5'-nucleotidase under the conditions previously described (6) and by the decrease in optical density at 265 $m\mu$ in the presence of A5P deaminase (7).

Treatment of the adenylyl acetate with 0.01 N KOH at room temperature for 5 minutes or with neutral hydroxylamine at 37° resulted in the destruction of its enzymatic activity and the liberation of free A5P. Of the A5P which is not susceptible to A5P deaminase and 5'-nucleotidase ("bound" A5P), approximately 70 per cent was accounted for as adenylyl acetate by its enzymatic activity. The remainder of the "bound" A5P, 0.6 $\mu\text{mole per ml.}$, can best be accounted for as the diacetyl derivative of A5P. This can be seen in the following experiment (Table III). 5 ml. of a solution containing 100 μmoles of total A5P and 53 μmoles of adenylyl acetate per ml. were fractionated with Ba^{++} as described earlier and chromatographed on a Dowex 1 Cl^- column (2.5 \times 5 cm.). The adenylyl acetate was eluted with 0.0035 N HCl at 4° and approximately 0.75 resin bed volume of eluate was collected per fraction. Ultraviolet-absorbing material started to appear in the eluate at about 6 resin bed volumes and continued to be eluted until 37 resin bed volumes had passed through the column, with the peak being at

25 resin bed volumes. Over this entire range the optical density ratio at 280 to 260 $m\mu$ remained between 0.20 and 0.22. The fractions comprising the major portion of the peak (14 to 32 resin bed volumes) were analyzed as described in Table III. It can be seen that there is good agreement between the enzymatically determined adenylyl acetate and the amount of acethydroxamic acid formed at 37°, but each of these is lower than the total amount of A5P present, even though in the early fractions free A5P

TABLE III
Analysis of Chromatographed Adenylyl Acetate

Fraction No.	Free A5P	Adenylyl-acetate	Acethydroxamate		Acetate	Total A5P	
			Formed at 37°	Formed at 100°		Determined	Calculated
19	0.00	0.34	0.33	0.56	0.58	0.43	0.45
21		0.44	0.40			0.54	
23	0.00	0.48	0.47	0.75	0.78	0.63	0.62
25		0.54	0.50			0.71	
27	0.00	0.60	0.57	0.93	0.95	0.79	0.78
29		0.63	0.62			0.84	
31	0.02	0.67	0.66	1.01	1.04	0.91	0.86
33		0.72	0.70			0.97	
35	0.03	0.74	0.73	1.10	1.09	0.99	0.95
37		0.73	0.70			0.96	
39	0.05	0.66	0.66	1.02	1.04	0.92	0.91
41		0.65	0.63			0.84	
43	0.02	0.56	0.53	0.82	0.80	0.70	0.72

All of the values are expressed as micromoles per ml.

Free A5P + adenylyl acetate + (acethydroxamate, 100° - acethydroxamate, 37°/2) = total A5P. The fractions were analyzed for total A5P (by optical density at 260 $m\mu$), acethydroxamic acid formed at 37° and 100°, adenylyl acetate (by conversion to acetyl CoA), free A5P (by A5P deaminase), and acetate (by acetokinase after incubation of the adenylyl acetate in 0.01 N KOH for 5 minutes at room temperature).

could not be detected. Moreover, there is more acetate liberated on treatment with dilute alkali than can be accounted for as adenylyl acetate or acethydroxamate (37°). The data show, however, that all of the acetate present is converted to acethydroxamate at 100°. If it is assumed that the extra acethydroxamate formed at 100° is derived from adenylyl diacetate, then all of the A5P can be accounted for. Thus, if one adds to the sum of free A5P and adenylyl acetate one-half the difference between acethydroxamic acid formed at 100° and 37°, then there is good agreement with the value of total A5P.

By Procedure B, it has been found that variable amounts of adenylyl di-

acetate are formed and usually comprise 10 to 20 per cent of the total A5P of purified adenylyl acetate preparations. The present evidence suggests that the diacetyl derivative is not enzymatically active in the formation of ATP or acetyl CoA, but more extensive studies with this compound separated from the monoacetyl derivative would be required to establish this point conclusively.

Stability of Adenylyl Acetate—Fig. 1 shows the kinetics of destruction of adenylyl acetate under various conditions of temperature and pH. A sig-

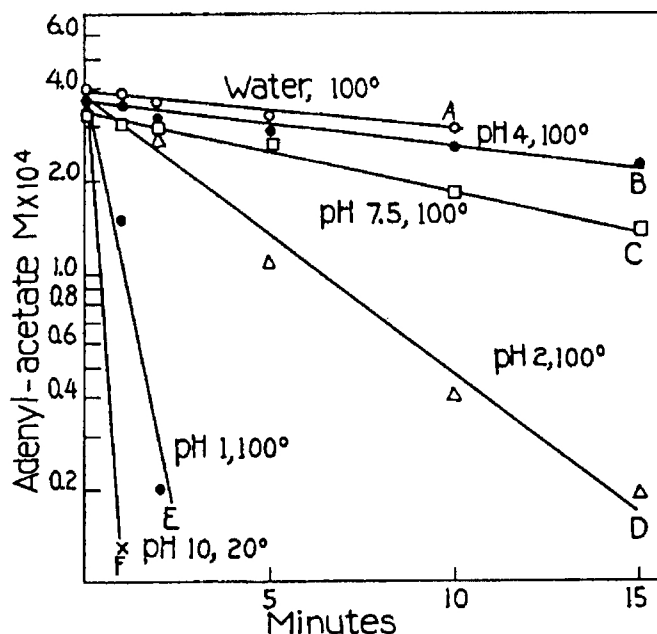


FIG. 1. Hydrolysis of adenylyl acetate. Curve A, unbuffered aqueous solution, 100°; Curve B, 0.10 M sodium formate, pH 4.0, 100°; Curve C, 0.10 M Tris buffer, pH 7.5, 100°; Curve D, 0.01 N HCl, 100°; Curve E, 0.10 N HCl, 100°; Curve F, 0.10 M glycine buffer, pH 10.0, 20°, and 0.01 N KOH, 20°. The first order hydrolysis constants calculated from Curves A, B, C, D, E, and F are 0.026, 0.030, 0.061, 0.209, 1.37, and 3.40 min.^{-1} , respectively.

nificant feature of this experiment was the comparative stability of adenylyl acetate at pH 4.0 at 100°, and the rapid destruction at pH 10 at 20°. In the course of chromatographing adenylyl acetate, little or no detectable disappearance of the adenylyl acetate was observed when it was kept at 0–4° in 0.01 N HCl for as long as 12 hours, but even a minute's exposure at this temperature to a pH of about 10 or above results in the complete hydrolysis of the compound. When adenylyl acetate was incubated at 37° in 0.10 M potassium phosphate buffer, pH 7.5, or in 0.10 M Tris buffer, pH 7.5, there was less than 10 per cent destruction in 30 minutes. However, in 0.10 M Tris buffer, at pH 8.0, 8.5, or 9.0, at 37°, there was approximately 15, 30, and 50 per cent destruction in 30 minutes.

DISCUSSION

In the present report the preparation and some of the properties of adenylyl acetate have been described. All of the evidence available thus far indicates that adenylyl acetate is an anhydride of acetic and adenylic acids joined by an acyl-phosphate linkage. In support of this conclusion are the following observations. The absorption spectra of adenylyl acetate preparations of up to 85 per cent purity are almost identical to that of free A5P. However, the lack of reaction with A5P deaminase shows that the A5P moiety exists in a modified or "bound" form. Moreover, adenylyl acetate is not degraded by 5'-nucleotidase, suggesting that the acetyl moiety is on the 5'-phosphate group. Treatment of adenylyl acetate with hydroxylamine at neutral pH results in the liberation of A5P and the formation of acetylhydroxamic acid. The acetylhydroxamic acid formed at neutral pH and room temperature is equivalent to the amount of acetyl groups which can be enzymatically transferred to CoA. The stability of adenylyl acetate under various conditions of pH and temperature closely resembles that of acetyl phosphate (13), the major difference being the greater stability at pH 4.0 at 100°. All of these findings taken together are in agreement with the formulation of adenylyl acetate as the phosphoacetyl derivative of A5P.

The method of synthesis of adenylyl acylates described by Avison (11), and used here for adenylyl acetate, appears to offer a convenient method for the preparation of a number of similar derivatives. Peng² has prepared adenylyl butyrate from butyric anhydride and A5P and has shown it to be converted to ATP and butyryl CoA with the butyrate-activating system of liver (14). Comparable studies with the higher fatty acid adenylylates are worthy of further investigation.

SUMMARY

Adenylyl acetate has been synthesized by the reaction of acetic anhydride and A5P in pyridine and from acetyl chloride and silver adenylylate. Studies with A5P deaminase and 5'-nucleotidase indicate that adenylyl acetate is the phosphoacetyl derivative of A5P. In agreement with this conclusion was the finding of acetylhydroxamate and A5P formation on treatment of adenylyl acetate with neutral hydroxylamine. Adenylyl acetate is relatively stable at acid pH at 0-4°, but is rapidly split at pH 2 and below, at 100°. At pH 10 and above, it is rapidly hydrolyzed even at 0°.

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² Personal communication from Dr. H. Beinert and Dr. C. H. Lee Peng.

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