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Adenosine 5′-Tetraphosphate and Adenosine 5′-Pentaphosphate Are Synthesized by Yeast Acetyl Coenzyme A Synthetase†

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Yeast (*Saccharomyces cerevisiae*) acetyl coenzyme A (CoA) synthetase (EC 6.2.1.1) catalyzes the synthesis of adenosine 5′-tetraphosphate (p_4A) and adenosine 5′-pentaphosphate (p_5A) from ATP and tri- or tetrapolyphosphate (P_3 or P_4), with relative velocities of 7:1, respectively. Of 12 nucleotides tested as potential donors of nucleotidyl moiety, only ATP, adenosine-5′-*O*-[3-thiotriphosphate], and acetyl-AMP were substrates, with relative velocities of 100, 62, and 80, respectively. The K_m values for ATP, P_3, and acetyl-AMP were 0.16, 4.7, and 1.8 mM, respectively. The synthesis of p_4A could proceed in the absence of exogenous acetate but was stimulated twofold by acetate, with an apparent K_m value of 0.065 mM. CoA did not participate in the synthesis of p_4A (p_5A) and inhibited the reaction (50% inhibitory concentration of 0.015 mM). At pH 6.3, which was optimum for formation of p_4A (p_5A), the rate of acetyl-CoA synthesis (1.84 μmol mg^{-1} min^{-1}) was 245 times faster than the rate of synthesis of p_4A measured in the presence of acetate. The known formation of p_4A (p_5A) in yeast sporulation and the role of acetate may therefore be related to acetyl-CoA synthetase.

Adenosine 5′-tetraphosphate (p_4A) and adenosine 5′-pentaphosphate (p_5A) were detected as contaminants of adenosine 5′-triphosphate preparations (21, 27), and p_4A was shown to be present in muscle (16, 33), liver (38), and yeast (*Saccharomyces cerevisiae*) spores (9). The physiological role of nucleoside 5′-tetraphosphates (p_4N) is largely unknown. Adenosine-, guanosine-, and uridine-tetraphosphates (p_4A, p_4G, and p_4U) are strong competitive inhibitors (nanomolar K_i values) of asymmetrical dinucleoside tetraphosphatase (EC 3.6 1.17) (15, 17, 24, 36), an enzyme cleaving dinucleoside tetraphosphates to the corresponding nucleoside tri- and monophosphates. Since Ap_4A may be important in metabolic regulation (for a review, see reference 23), changes in the level of p_4N could modulate its concentration and physiological effect. Other enzymes known to be inhibited (micromolar K_i values) by p_4N are the soluble guanylate cyclase (EC 4.6.1.2) (8) and phosphodiesterase I and nucleotide pyrophosphatase (EC 3.1.4.1 and EC 3.6.1.9) from rat liver (2).

The level of p_4A depends on its rate of synthesis and degradation. A specific enzyme (EC 3.6.1.14) which hydrolyzes p_4A to ATP and P_i exists in muscle (32), and the following enzymes have been reported to synthesize p_4N: aminoacyl-tRNA synthetases and, particularly, the lysyl-tRNA synthetase (EC 6.1.1.6), which catalyzes the synthesis of p_4A from lysine, ATP, and pentasodium tripolyphosphate (P_3) (37); yeast phosphoglycerate kinase (EC 2.7.2.3), which forms p_4A (33) and p_4G (6) from 1,3-bis-phosphoglycerate and ATP or GTP, respectively; adenylate kinase (EC 2.7.4.3), which was shown to transfer phosphate from ADP to ATP (14); a mutated *Escherichia coli* succinyl-coenzyme A (CoA) synthetase (EC 6.2.1.5) that was unable to catalyze the overall reaction (i.e., the synthesis of succinyl-CoA from ATP, succinate, and CoA) but could synthesize p_4A from ATP through phosphorylation of the enzyme and transfer of the phosphate moiety from the enzyme-phosphate complex to ATP (19); and, finally, firefly luciferase (EC 1.13.12.7), which catalyzes the synthesis of p_4A or p_5A by using luciferin, ATP, and P_3 or hexaammonium tetraphosphate (P_4), respectively (25).

Because of the similarities between the reactions catalyzed by aminoacyl-tRNA synthetases, luciferase, and acyl-CoA synthetases (7, 22, 25, 31), we checked whether yeast acetyl-CoA synthetase (EC 6.2.1.1) could also catalyze synthesis of p_4A from ATP and P_3. Here, we describe the experimental conditions for the synthesis of both p_4A and p_5A by acetyl-CoA synthetase.

MATERIALS AND METHODS

Three commercial preparations of yeast acetyl-CoA synthetase were used: lot 89F8180 from Sigma (catalog no. A-5269) and two batches of a lyophilized synthetase, lot 127F0650 from Sigma (catalog no. A-1765) and lot 11496526-29 from Boehringer Mannheim GmbH (catalog no. 161675). p_4A-degrading activity was undetectable in those synthetase preparations. As auxiliary enzymes, inorganic pyrophosphatase from yeast cells (catalog no. 108987), alkaline phosphatase from calf intestine (catalog no. 108138) (both purchased from Boehringer), and potato apyrase from Sigma (catalog no. A-6132) were used. All of the enzymes were solubilized and/or diluted before use in 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-KOH buffer (pH 7.6) containing 5% glycerol, 0.1 mM dithiothreitol, and 1 mg of bovine serum albumin per ml.

Adenosine-5′-*O*-[1-thiotriphosphate] *S*-isomer (ATPαS) and adenosine-5′-*O*-[3-thiotriphosphate] (ATPγS) were from Boehringer Mannheim GmbH. Adenosine 5′-(α,β-methylenetriphosphate) ($ppCH_2pA$), adenosine 5′-(β,γ-methylenetriphosphate) (pCH_2ppA), adenosine N^1-oxide 5′-triphosphate, adenine 9-β-D-arabinoside 5′-triphosphate, and other nucleotides were from Sigma. Acetyl-AMP was synthesized as described earlier (10). P_3, P_4, and sodium phosphate glass with a chain length of 5 ± 2P were from Sigma (catalog no. T5633, T5758, and S5878, respectively). Buffers, salts, and organic

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solvents were of analytical grade. Labeled [2,8-^{3}H]adenosine 5'-triphosphate (catalog no. TRK.622 [42 Ci/mmol]) was from Amersham International plc, and [^{32}P]tetrasodium pyrophosphate (catalog no. NEX 019 [20 mCi/ml]) was from Dupont de Nemours GmbH, NEN. Enzyme assays are described in the legend to Fig. 1.

RESULTS AND DISCUSSION

In Fig. 1, a reaction mixture containing ATP, $MgCl_2$, acetate, inorganic pyrophosphatase and acetyl-CoA synthetase is shown to accumulate p_4A, dependent on addition of P_3, or at a lower rate, p_5A, dependent on P_4. The reaction could be monitored either by thin-layer chromatography (TLC) (Fig. 1A) or high-performance liquid chromatography (HPLC) (Fig. 1B). The identity of p_4A and p_5A was established by comigration with standards, UV spectra identical with those of ATP, and sensitivity to alkaline phosphatase (Fig. 1C) and apyrase (data not shown). Smaller amounts of Ap_5A (in the presence of P_3) and traces of Ap_6A (in the presence of P_4) and Ap_4A were also formed and similarly identified (Fig. 1B).

Modifying the standard incubation mixture by adding 100 mM MES (morpholineethanesulfonic acid), HEPES, and CHES (2-[*N*-cyclohexylamino]ethanesulfonic acid) buffers covering the pH range from 5.1 to 9.5 ([^{3}H]ATP and TLC method) showed a pH optimum for p_4A synthesis between 6 and 6.5 (MES-KOH buffer), with half-maximum activity at pH 5.2 and 7.4 and no synthesis observed above pH 8. Dependence on divalent cations is shown in Fig. 2.

The following compounds were tested at 3 mM as potential donors of a nucleotidyl moiety onto P_3 by using the (nonradioactive) HPLC assay method: ADP, ATP, dATP, ATPαS and ATPγS, $ppCH_2pA$, pCH_2ppA, adenosine N^1-oxide 5'-triphosphate, adenine 9-β-D-arabinoside 5'-triphosphate (ara-ATP), CTP, GTP, UTP, and acetyl-AMP. Only ATP, ATPγS, and acetyl-AMP were active, relative rates of p_4A synthesis being 100, 62, and 80, respectively. Using the [^{3}H]ATP assay with TLC, rates with P_3 and P_4 were 7.5 and 1.0 nmol mg^{-1} min^{-1}, respectively; P_5, even with 24 h of incubation, was not identified as a substrate. The rates of acetyl-CoA formation (in the presence of 5 mM acetate and 5 mM CoA) at pH 6.3 and 7.5 (pH optimum of the reaction) were, respectively, 1.84 and 7.4 nmol mg^{-1} min^{-1}. The ATP-PP_i exchange reaction, using 2 mM [^{32}P]pyrophosphate, (1 pmol is 112 cpm) (in the absence of pyrophosphatase), gave a rate of 0.72 μmol mg^{-1} min^{-1} in the presence of 1 mM acetate and about half that value in the absence of added acetate.

For p_4A synthesis the K_m value for ATP was 0.16 mM; a range of [^{3}H]ATP from 0.15 mM to 2.4 mM was used, with TLC determination of p_4A. The K_m for P_3 was 4.7 mM, employing the same assay with 3 mM [^{3}H]ATP and 0.3 to 5 mM P_3. The K_m for acetyl-AMP, was 1.8 mM; a range of 0.15 to 2.4 mM acetyl-AMP was used along with HPLC determination of p_4A.

p_4A synthesis occurred in the absence of added acetate, but the rate was increased twofold in the presence of 0.5 mM acetate with half-stimulation at 0.065 mM. The possibility that the enzyme preparation contributed acetate was apparently excluded by the lack of effect of addition of preheated enzyme solution. No effect was observed for formate or propionate. Added CoA itself was inhibitory to the standard reaction ([^{3}H]ATP, TLC method) with a 50% inhibitory concentration of 0.015 mM, and such inhibition was prevented by inclusion of 0.5 mM acetate, showing that acetyl-CoA is not inhibitory.

We have recently suggested that enzymes (mainly synthetases and some transferases) which catalyze the transfer of a nucleotidyl moiety via nucleotidyl-containing intermediates and release of PP_i, may produce dinucleoside polyphosphates (7). Aminoacyl-tRNA synthetases (26, 37) and luciferase (7, 25, 31) are examples of this type of enzymes known to catalyze the synthesis of nucleoside 5'-polyphosphates ($n > 3$) and diadenosine tetraphosphate. Acetyl-CoA synthetase is another enzyme theoretically able to catalyze the synthesis of nucleoside 5'-polyphosphates ($n > 3$) or Ap_4A (7). The results presented in this paper arose from the observation made very recently with firefly luciferase (25) that the enzyme can catalyze the transfer of the adenylyl moiety from the enzyme-luciferin-AMP complex onto various polyphosphates. The formation of p_4A or p_5A with acetyl-CoA synthetase might analogously involve P_3 or P_4 as the acceptor of the adenylyl moiety of the enzyme–acetyl-AMP complex as follows:

$$\text{enzyme–acetyl-AMP} + P_n \leftrightarrow P_{n+1}A + \text{acetate} + \text{enzyme} \quad \text{(reaction 1)}$$

An alternative mechanism, in view of the apparent absence of involvement of acetate in the reaction, would be

$$\text{enzyme} + \text{ATP} \leftrightarrow \text{enzyme-AMP} + PP_i \quad \text{(reaction 2)}$$

$$\text{enzyme-AMP} + P_3 \leftrightarrow p_4A + \text{enzyme} \quad \text{(reaction 3)}$$

In contrast to various aminoacyl-tRNA synthetases (26, 37) and firefly luciferase (7, 25, 31), which require the cognate amino acid and luciferin, respectively, to form an enzyme-X-AMP complex (in which X stands for an organic acid able to form an anhydride bond with AMP), the yeast acetyl-CoA synthetase can catalyze the transfer of adenylate coming from ATP to a polyphosphate without forming, apparently, an acyl-AMP intermediate. In addition to that, however, the yeast enzyme can use the specific acyl-AMP intermediate acetyl-AMP as a source of adenylate.

Although it is not the object of this work to elucidate the mechanism of the synthesis of p_4A, whether it goes through reaction 1 or reaction 2, (implying the formation of enzyme–acetyl-AMP or enzyme-AMP intermediates, respectively), some comments could be raised on this point. Contradictory reports concerning the acetate dependence or independence of the ATP-PP_i exchange date from the early fifties (compare references 12 and 20 with reference 1). Variability in the results has been ascribed, among other causes, to important mechanistic differences between the members of this type of enzymes (acyl-CoA synthetases included), to the occurrence of a hypothetical factor required for the formation of enzyme-AMP complex, which could be lost in the process of the enzyme purification, and to the presence of the contaminant acetate (18). Direct interaction of ATP with the enzyme is also supported by the stabilizing effect exerted by that nucleotide on the acetyl-CoA synthetase through an unknown mechanism (5, 29). Since the molecular weights obtained in the presence and absence of ATP are identical (29), the possibility that adenylylation of the synthetase by ATP contributes to its stability should also be considered. Also, because the enzyme has an α_2 structure (5) and, as is shown in this study, the exogenous acetate doubles the rate of p_4A synthesis observed in the absence of acetate, it seems plausible that each subunit of the synthetase participates in that reaction in a different manner—according to reaction 1 or 3. The existence of two interacting substrate sites for ATP was proposed earlier for the acetyl-CoA synthetase from *Methanothrix soehngenii* (11).

As shown, tripolyphosphate was a very good adenylyl acceptor. In contrast to the findings with luciferase, which employs polyphosphate chains of up to 20 phosphate residues, yeast acetyl-CoA synthetase was not able to produce even p_6A, from ATP and P_5. Also, unlike the reaction with luciferase, the

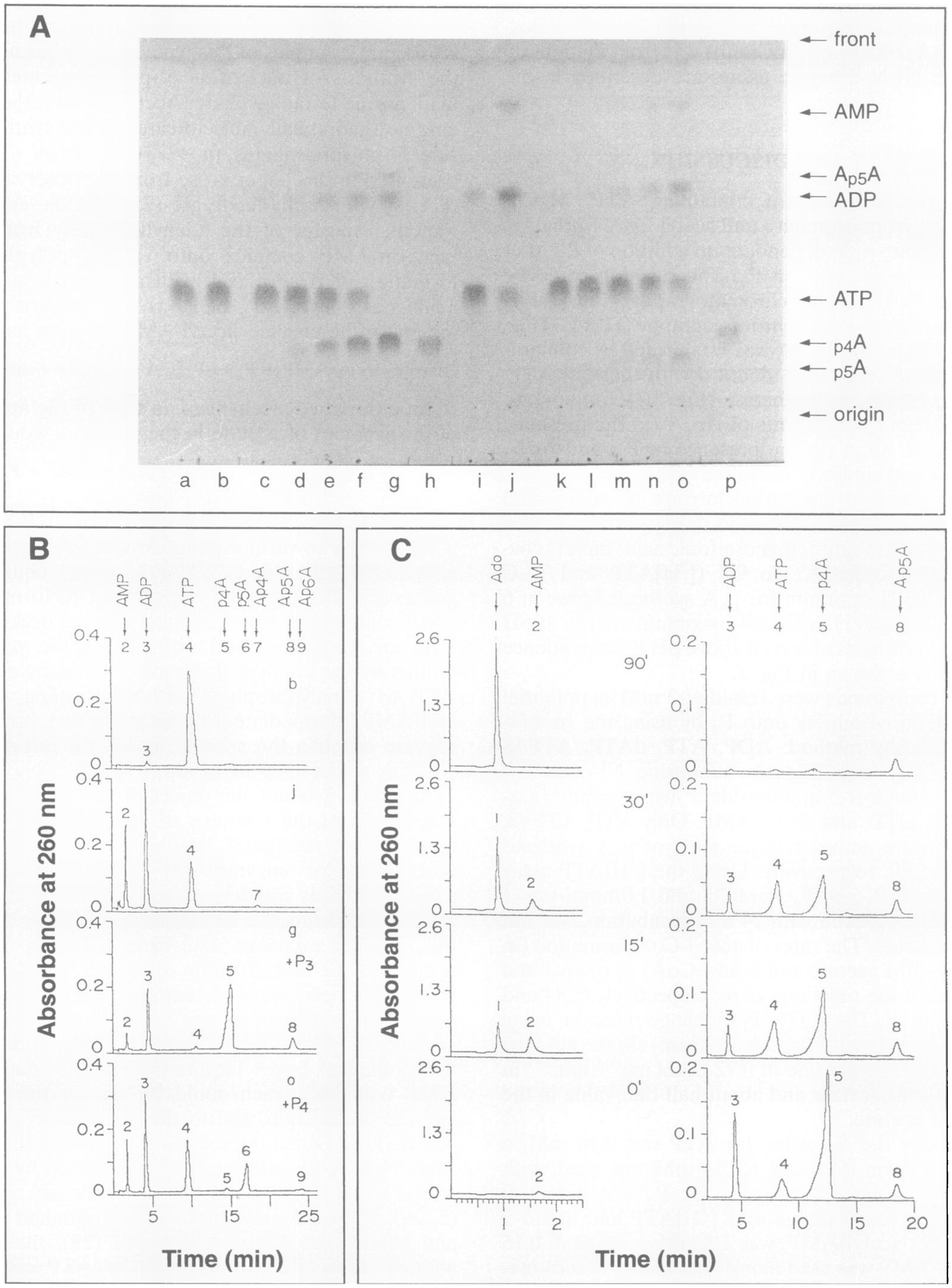

FIG. 1. Synthetic products from ATP, P_3, and P_4. (A) TLC. The complete assay mixture (0.05 ml, final volume) contained 50 mM MES-KOH (pH 6.3), 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 5 mM potassium acetate, 3 mM ATP, 0.05 U of inorganic pyrophosphatase, 5 mM polyphosphate, and 16 μg of acetyl-CoA synthetase. Incubation was carried out at 30°C. At the times mentioned below, 0.002-ml aliquots were spotted on silica gel sheets containing a fluorescent indicator (Merck, Darmstadt, Germany). The chromatogram was developed for 90 min in dioxane-ammonia-water (6:1:6 by volume). This chromatographic system was specifically developed to better separate p_4A (R_f = 0.17) and p_5A (R_f = 0.11) from ATP (R_f = 0.31). The picture was taken under shortwave UV light. The lanes represent the following: a and b, the mixture without enzyme taken after 5 and 24 h, respectively; c to g, the complete mixture containing tripolyphosphate taken after 0, 1, 5, 10, and 24 h, respectively; h and p, authentic standard of p_4A; i and j, the mixture without polyphosphate taken after 5 and 24 h, respectively; k to o, complete mixture containing tetrapolyphosphate taken after 0, 1, 5, 10, and 24 h, respectively. (B) HPLC. Reaction mixtures corresponding to lanes b, j, g, and o from panel A were diluted 10-fold and analyzed by HPLC as previously described (25). (C) Effect of alkaline phosphatase. A reaction mixture with P_3 (as described above) was incubated overnight at 30°C, heated at 100°C for 3 min, and centrifuged, and 0.05-ml portions of the supernatant were treated with alkaline phosphatase (2 U) for the indicated times and analyzed by HPLC as described in reference 25. Ado, adenosine.

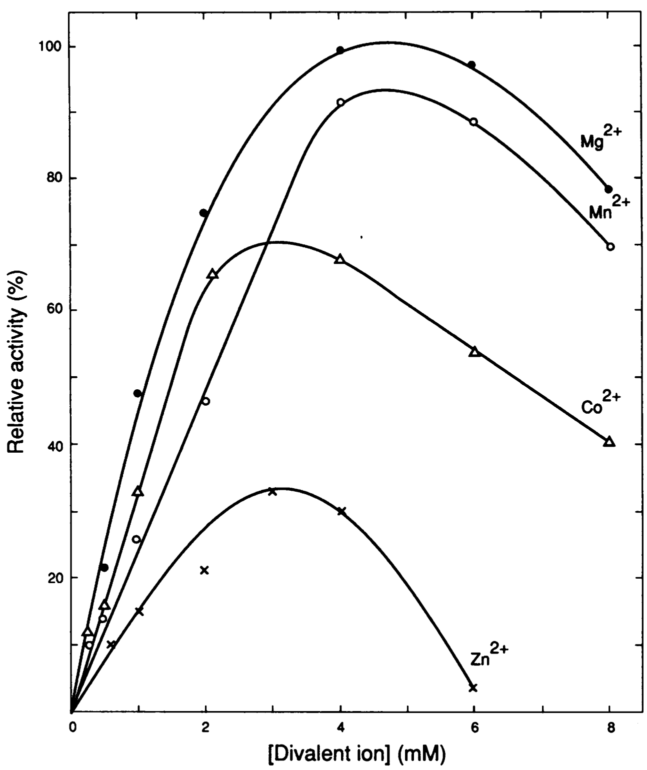

FIG. 2. Dependence of the synthesis of p_4A, catalyzed by yeast acetyl-CoA synthetase, on divalent cations. The standard incubation mixture (Fig. 1) containing 3 mM [^{3}H]ATP was modified by substituting 5 mM M_gCl_2 with chloride of the indicated cation and was analyzed by TLC. Aliquots of 0.005 ml were transferred at intervals onto silica gel sheets, p_4A or p_5A standard was added (~5 nmol), and the chromatograms were developed as described in the legend to Fig. 1. Spots corresponding to p_4A were cut, and the radioactivity was counted. In our assay conditions, 1 pmol of ATP is 4.3 cpm.

synthesis of Ap_4A is not yet clearly established. We do believe that p_4A synthesis is an intrinsic property of yeast acetyl-CoA synthetase; it was observed in four different batches of enzyme. Only one preparation (lot 42H8025, catalog no. A-5269 from Sigma) showed an insignificant capability for the synthesis of p_4A. Among others, one can speculate on the following reasons for the insignificant capability: (i) mutational changes in some essential amino acids of the enzyme (a similar example for succinyl-CoA synthetase is described in reference 19), (ii) phosphorylation or dephosphorylation of the native enzyme (phosphorylation of threonyl- and seryl-tRNA synthetases) increased the Ap_4A synthesis up to six- and twofold, respectively (3), (iii) the possible occurrence of different isozymic forms, depending upon the yeast growth conditions (13, 28, 29). In an attempt to clarify this point, the commercial preparations of acetyl-CoA synthetase used in this work were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme from Boehringer Mannheim had a main band of 72 kDa. All samples from Sigma had two important bands of 72 kDa (main band) and 82 kDa. The preparation from Sigma (lot 42H8025) with negligible capacity for the synthesis of p_4A presented a protein electrophoretic pattern indistinguishable from the Sigma batches which were active in the synthesis of that nucleotide.

Which enzymes contribute most to the p_4A and p_5A production observed during yeast sporulation (9) is not known. Yeast 3-phosphoglycerate kinase catalyzes p_4A and p_4G synthesis at rates of about 10^{-4} times the rate of the transfer of a phosphate group from 1,3-bisphosphoglycerate to ADP (6, 33). The activity of this enzyme is circa 47 U/mg of protein in crude extract (30). Aminoacyl-tRNA synthetases (9, 26, 37) also can make p_4A and p_5A. As we show here, acetyl-CoA synthetase may also contribute. Evidence for its possible role in the sporulation-related production of p_4A (p_5A) is strengthened by the facts that (i) acetate is an inducer of sporulation (4), (ii) the level of the enzyme in yeast cells is relatively high (0.2 U/mg of protein in crude extract (5), and (iii) yeast cells contain high levels of polyphosphates, mainly P_3 and P_4 (34, 35). The possibility that acetate could stimulate p_4A (p_5A) formation from ATP and P_3 (P_4) could then be considered as one of the reasons for the efficiency of acetate as an inducer of sporulation. Acetate has been used for that purpose, on an empirical basis, since 1916 (4). As previously stated (7), when the normal metabolic equilibria are unbalanced (as could happen in yeast cells growing in a sporulating medium), the level of such cosubstrates as specific tRNA or CoA may decrease, favoring the synthesis of p_4A and/or p_5A through reactions between various enzyme-X-AMP complexes and polyphosphates.

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