notes on methodology

Thermal and pH stability of Δ²-isopentenyl pyrophosphate

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Summary The isoprenoid precursors Δ³-isopentenyl pyrophosphate and γ,γ-dimethylallyl pyrophosphate (Δ²-isopentenyl pyrophosphate) have been separated by thin-layer chromatography. The products from Δ²-isopentenyl pyrophosphate incubated under various conditions of pH and temperature have been separated, and the survival of Δ²-isopentenyl pyrophosphate under these conditions has been calculated. The acid-labile Δ²-isopentenyl pyrophosphate can be stored indefinitely at pH 11.5 and −100°C.

Supplementary key words isopentenyl pyrophosphate isomers

Δ²-ISOPENTENYL PYROPHOSPHATE is the precursor of the Δ²-isopentenyl group in N^6-(Δ²-isopentenyl) adenosine, a minor base which has been found in a variety of bacterial, yeast, and mammalian transfer RNAs (1). At low pH Δ²-IPP is very unstable and thus is assumed to be poorly suited for kinetic analysis of the reaction leading to the synthesis of IPA. Thus, in the work by Kline, Fittler, and Hall (2), Δ³-IPP has been used, and the reaction mixture has been supplemented with excess amounts of the Δ³-IPP isomerase (3, 4). I have studied the stability at neutral pH and above of Δ²-IPP at both normal enzyme reaction temperatures and lower temperatures. These data suggest that it is practical to prepare large amounts of the Δ² compound prior to the assay, and the use of Δ³-IPP and isomerase is unnecessary when studying the transferase reaction.

Δ³-IPP was prepared from [³H]mevalonic acid obtained from Amersham/Searle Corp. Reaction mixtures containing a combination of mevalonic phosphate, mevalonic pyrophosphate, and ultimately Δ³-IPP were prepared by incubation of the mevalonic acid with a crude enzyme fraction extracted from yeast by the method of Tchen (5). The reaction mixtures were then chromatographed on Dowex 1 columns by the procedure of Tchen, and the Δ³-IPP peak was collected. Its authenticity was checked by cochromatography with genuine Δ³-IPP (Schwarz/Mann) in several solvent systems. Δ²-IPP isomerase was purified from yeast by the method of Agranoff et al. (3) to a minimum purification of 100-fold. Δ²-IPP was produced by incubation in the following medium: Tris–HCl, pH 7.5, 0.01 M; $MgCl_2$, 0.001 M; Δ³-IPP, 0.1 nmole (10,000–12,000 cpm); and 50–100 μg of isomerase in a total volume of 0.2 ml. After incubation leading to at least 30% conversion of Δ³-IPP to Δ²-IPP (assayed by a modification of the procedure of Agranoff et al. [3]), the reaction was terminated by the addition of 0.001 M iodoacetamide, which inhibits the isomerase. (The identity of the Δ²-IPP was confirmed by its acid lability, its production by isopentenylpyrophosphate isomerase, and its suitability as a substrate for Δ²-IPP transferase to form IPA.) The pH of the resultant mixture was then adjusted to the indicated pH between 7 and 11.5 by the addition of dilute HCl or KOH, and the reaction was heated or cooled to the appropriate test temperature. At times thereafter, from zero time to several hours, aliquots of the reaction mixture were removed and spotted on PEI–cellulose sheets (J. T. Baker Chemical Co.), and the reaction products were chromatographed using isopropanol–isobutanol–ammonia–water 40:20:1:39 (by volume). In this solvent, Δ³-IPP has an R_F of approximately 0.04, and Δ²-IPP has an R_F of approximately 0.72. After development for a minimum of 10 cm, the chromatograms were cut out in sections and the counts in each spot were measured. The concentration of Δ²-IPP was then calculated from the ratio of counts in the Δ²-IPP spot to the sum of the recovered counts from all chromatogram fractions. The survival of Δ²-IPP after various incubation times was calculated by a comparison of the Δ²-IPP concentration relative to the zero time value (100%). The major breakdown products of Δ²-IPP have R_F's of >0.85, and thus are well separated from the Δ²-IPP. Control experiments with purified Δ²-IPP showed that neither the rate nor the extent of Δ²-IPP breakdown was affected by the presence of Δ³-IPP and the other reactants of the enzyme reaction. In general, the breakdown of IPP is exponential to at least 30% survival. However, at higher temperatures (37°C, 45°C) the survival curve sometimes tends to level off slightly after 60–80% breakdown, and the inactivation is no longer strictly exponential.

Each survival curve can be characterized by a "half-life," or the time required to reach 50% Δ²-IPP survival under different conditions. Table 1 reports the half-life of Δ²-IPP under the conditions I have tested. The rate of Δ²-IPP breakdown is decreased by a factor of 4–5 in going from pH 7.0 to 11.5 and is increased at all pH's by a factor of 25–30 when the temperature is raised from 5°C to 45°C.

The physical properties, including stability, of some prenyl pyrophosphates have been discussed by Corn-

Abbreviations: Δ²-IPP, Δ²-isopentenyl pyrophosphate; Δ³-IPP, Δ³-isopentenyl pyrophosphate; IPA, N^6-(Δ²-isopentenyl) adenosine.

TABLE 1. Δ^2-Isopentenyl pyrophosphate: half-life under different pH and temperature conditions

pH	Temperature (°C)					
	5	20	30	32	37	45
			hr			
7.0	60–70	19	8.1		4.5	2.3
7.5	70–80	22	9.3		5.2	2.6
8.5	[a]	28	13	10.5	6.8	3.5
10.0	[a]	45–50	21		12	6.2
11.5	[a]	80–90	35–40		20	10

[a]Half-life > 96 hr.

forth and Popják (6). However, neither Δ^3-IPP nor its labile isomer Δ^2-IPP has been tested extensively for stability. The breakdown products of Δ^2-IPP include methylvinylcarbinol and γ,γ-dimethylallyl alcohol (6); however, the possibility of small amounts of other products cannot be overlooked. The tailing off of the survival curves at low survival may reflect the accumulation of breakdown products other than those mentioned above. If these other products had R_F's similar to that for Δ^2-IPP, they would be included with it, causing the observed leveling out of the survival curve.

The data on the effect of temperature on the inactivation at pH 8.5 were analyzed in an Arrhenius plot, from which the activation energy was calculated to be 6.3×10^4 J (15.0 kcal).

The main interest of this research was to find conditions under which Δ^2-IPP could be stored as a stable compound. Storage of Δ^2-IPP at pH 11.5 and −20°C leads to about 20% breakdown per month, whereas storage at −100°C leads to negligible breakdown over many months. Thus, the preparation of large stocks of Δ^2-IPP for extended storage is quite practical. Preparative-scale production of Δ^2-IPP is performed by simply scaling up the standard reaction mixture, chromatographing the entire mixture on Whatman 3MM paper, and eluting with a basic solvent, pH 10–11. Chromatography on PEI–cellulose is not a useful preparative technique because of the very poor recovery of Δ^2-IPP from the chromatograms.

Finally, under our Δ^2-transferase assay conditions (pH 7.5, 60-min incubation at 37°C) the substrate breakdown is about 13%. It is therefore possible to determine the kinetic parameters of the transferase reactions by applying relatively simple corrections, which are very difficult to make in the presence of large amounts of Δ^3-IPP and isopentenyl isomerase.

I thank Miss G. Hansen for her excellent technical assistance. This work was supported by grants from the National Cancer Institute of Canada and the National Research Council of Canada.

Manuscript received 2 June 1971; accepted 22 July 1971.

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