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PYROPHOSPHATE AS A SELECTIVE INHIBITOR OF MACROMOLECULE SYNTHESIS IN NORMAL AND LEUKEMIC LEUKOCYTES*

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Abstract—Pyrophosphate (PPi) was shown to inhibit RNA, DNA and protein synthesis of intact human leukocytes and HeLa cells and to inhibit such enzymes as DNA polymerase and amino acyl synthetase. The results with intact leukocytes and the demonstration in inhibition of bacterial growth suggest that PPi is readily transported into the cell and is not efficiently degraded by pyrophosphatase. A possible regulatory role for PPi is suggested.

PYROPHOSPHATE (PPi) is an end product of key enzyme reactions in the synthesis of RNA, DNA, protein and fats. As such it might be a candidate to exert feedback control if the intracellular level of pyrophosphate were not excessive. Because of the ubiquitous presence of pyrophosphatase there have been no studies to indicate inhibitory effects of PPi on intact cells. This paper describes the inhibition of RNA, DNA and protein synthesis in the absence of effects on energy metabolism of intact human leukocytes, and suggests a mechanism of action for pyrophosphate. A possible physiologic regulatory role for pyrophosphate in cell metabolism is discussed.

MATERIALS AND METHODS

Chemicals and isotopes. Sodium pyrophosphate (reagent grade) was purchased from J. T. Baker Chemical Co. Thymidine-methyl-^{3}H, uridine-^{3}H and phenylalanine-^{14}C were products of New England Nuclear Corp. and L-arginine-^{3}H and tritiated deoxytriphosphates were from Schwarz BioResearch.

Intact cell studies. Normal and leukemic cells were obtained by withdrawing 50 ml of heparinized blood from patients having granulocytosis of infection or chronic granulocytic leukemia (CGL). The tube of blood settled for 45 min and the leukocyte-rich plasma was removed by aspiration and adjusted to a cell count of 100,000 cells/mm^3. Aliquots were incubated with uridine-^{3}H, thymidine-^{3}H or arginine-^{3}H for measurement of RNA, DNA and protein synthesis as previously described.[1] Protein content was determined by the Lowry method,[2] RNA by orcinol[3] and DNA by diphenylamine.[4]

Energy metabolism. Respiration and anaerobic glycolysis of intact cells were measured by standard Warburg manometry as previously described.[5]

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DNA polymerase (*DNA nucleotidyltransferase*; *EC* 2.7.7.7). Leukocyte DNA polymerase was prepared by the method described by Mantsavinos[6] for rat liver enzyme. Leukocytes were disrupted by sonication rather than homogenization, since leukocytes are not readily disrupted by the latter method. Purification of DNA polymerase was carried out to the pH 5·0 step, since the specific activity of this preparation was sufficiently high to permit enzyme quantitation and the reaction was linear for 60 min. The assay conditions for polymerase were similar to those described,[6] with thymidine triphosphate-^{3}H being used as the radioactive substrate. Radioactivity was determined in a Beckman scintillation spectrometer.

Ribosomal protein synthesis. Protein synthesis *in vitro* was studied by modification of the method of Nirenberg and Matthei.[7] The formation of poly-U-directed polyphenylalanine-^{14}C in a ribosomal system was measured in the presence or absence of sodium pyrophosphate (PPi). Aliquots of the reaction mixture were placed on 2·3 cm filter paper discs and precipitated in 5% trichloroacetic acid (TCA) at 4°. The discs were washed in 5% TCA at 90° for 7 min, then washed in ethanol, then in ethanol:chloroform:ether (2:2:1) and finally in ether. Counting was performed by placing the discs in a vial and overlaying with 10 ml of toluene–PPO–POPOP* scintillation solution.

Amino acid transfer RNA. Assay of amino acid transfer RNA formation is based upon the trapping of labeled amino acid as the amino acid hydroxamate.[8] The experimental flasks contained a total volume of 0·5 ml including : 0·1 ml of 0·1 M Mg^{++}ATP, pH 7·4; 0·05 ml of 30 M NH_2OH; 0·025 ml phenylalanine-^{14}C (366 mc/m-mole); and 0·1 ml of pH 5 enzyme. The control flask was identical except that 0·05 ml of medium A substituted for NH_2OH. Reaction tubes were incubated at 37° and the reaction was initiated by the addition of pH 5 enzyme. Aliquots (0·1 ml) were removed at 0, 10 and 20 min and applied to Amberlite cation-exchange paper (SA-2) on a line 25 mm from one end. The enzyme was destroyed in a jet of steam and the strips were eluted by dipping the bottom end into 150 ml of 0·05 M Na_2HPO_4 (pH 7·0) in a mason jar. When the buffer reached the mark at the top, the strips were dried and the amino acid hydroxamate was cut out and counted in Spectrofluor PPO–POPOP. The values of the control strips were subtracted from the experimental values.

Bacterial and mammalian cell cultures. Escherichia coli B cells were cultured into exponential growth in Bacto-tryptone. Aliquots were diluted with fresh media and pyrophosphate was added (time zero). Bacterial growth was estimated in a Bausch-Lomb spectrophotometer at 450 mμ. HeLa cells were cultured for 48 hr in Tc 199 and cells were scraped off the glass, washed, counted and analyzed as above.

RESULTS

The effects of pyrophosphate on RNA, DNA and protein synthesis of intact leukemic granulocytes are shown in Table 1. Similar inhibition of macromolecule synthesis was noted when normal granulocytes were studied, except that total synthesis was proportionately lower in the control and pyrophosphate-treated flasks. These concentrations of PPi did not change the rate of leukemic granulocyte respiration (Q_{O_2} *ca.* 5·0) or anaerobic glycolysis ($Q^{N}_{A_2}$ *ca.* 25·0).

* PPO = 2,5-diphenyloxazolyl; POPOP = 1,4-*bis*-2-(4-methyl-5-phenyloxazole)benzene.

TABLE 1. THE EFFECT OF PYROPHOSPHATE ON DNA, RNA AND PROTEIN SYNTHESIS OF INTACT GRANULOCYTES TAKEN FROM AN UNTREATED PATIENT WITH CHRONIC GRANULOCYTIC LEUKEMIA*

Pyrophosphate (μmoles/ml)	DNA (% of control)	RNA (% of control)	Protein (% of control)
0	100	100	100
4	80	93	100
10	72	70	71
20	60	46	43

* The cell concentration was adjusted to 100,000/mm³ in autologous plasma and incubated with radioactive precursors for 60 min as described under Methods. In each instance the precursor incorporation obtained in the absence of pyrophosphate is listed as 100 per cent.

Pyrophosphate was tested on a partially purified DNA polymerase system (Fig. 1). Pyrophosphate (3×10^{-3}M) was required to cause near complete inhibition of DNA synthesis. Pyrophosphate (3×10^{-3}M) also inhibited poly-U-directed polyphenylalanine synthesis (Fig. 2). Similar degrees of inhibition were noted with endogenous ribosomal polypeptide synthesis (data not detailed). These effects were probably due to the inhibition of amino acyl-transfer RNA formation. Before

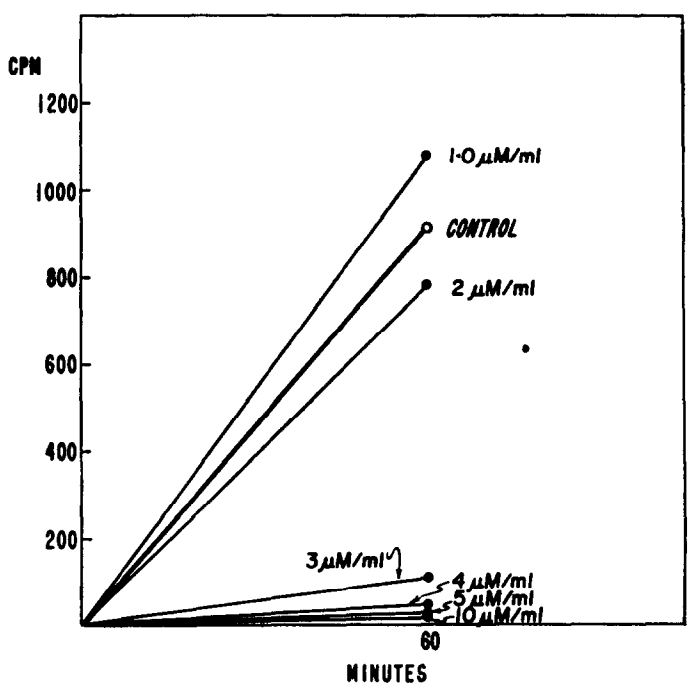

FIG. 1. Effect of pyrophosphate on DNA polymerase. Partially purified DNA polymerase was obtained from leukemic granulocytes, and denatured leukemic cell DNA was used as the primer (similar inhibition was also obtained with native DNA). Pyrophosphate was added at time zero and the reaction was started by raising the temperature from 4° to 37°.

reversal studies with ATP were attempted, it was necessary to determine the minimum requirement of the reaction for ATP (2×10^{-3}M) and the concentration of pyrophosphate causing approx. 50–70 per cent inhibition of amino acyl-transfer RNA formation (6×10^{-3}M). By using these starting concentrations, the effect of increasing ATP in the reaction mixture was measured (Fig. 3). There did occur a partial reversibility of PPi inhibition after the addition of ATP.

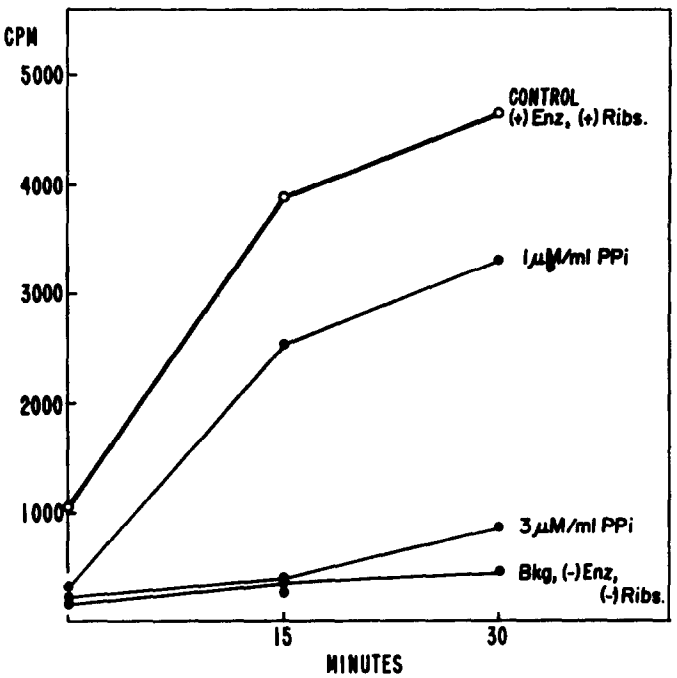

FIG. 2. Effect of pyrophosphate on poly-U-directed polypeptide synthesis. A time zero sample was obtained when the reaction mixture was completed and the incubation was begun. The control flasks regularly had a higher "background" than the inhibited experimental flasks.

Pyrophosphate did inhibit the growth of *E. coli* B and HeLa cells in culture (Figs. 4, 5). In repeated experiments the minimal inhibitory concentration of PPi was approx. 5×10^{-3}M.

DISCUSSION

Cleavage of pyrophosphate from triphosphates is required for ester formation in the synthesis of polynucleotides and for activation of transfer RNA and fatty acids. The subsequent exergonic hydrolysis of pyrophosphate is thought to be responsible for the irreversibility of these reactions. If pyrophosphate were to inhibit its own formation it might do so by allosteric enzyme interaction, by nonspecific chelation of divalent cations or by mass action.

Pyrophosphate inhibited DNA polymerase and ribosomal protein synthesis at a concentration of *ca.* 3×10^{-3}M under the conditions tested. These inhibitions were not overcome by the addition of Mg^{++} in concentrations as high as 4×10^{-2}M.

To further define the mechanism of pyrophosphate inhibition, we selected the initial reaction of protein synthesis, the charging of transfer RNA. This reaction has two steps which are catalyzed by a single enzyme, amino acyl synthetase:

(1) amino acid + ATP + enzyme ⇌ amino acyl — AMP — enzyme + PPi;

(2) amino acyl — AMP — enzyme + tRNA ⇌ amino acyl — tRNA + AMP + enzyme.

Sum: amino acid + ATP + tRNA → amino acyl — tRNA + AMP + PPi.

The present experiments demonstrate a pyrophosphate inhibition of amino acyl–

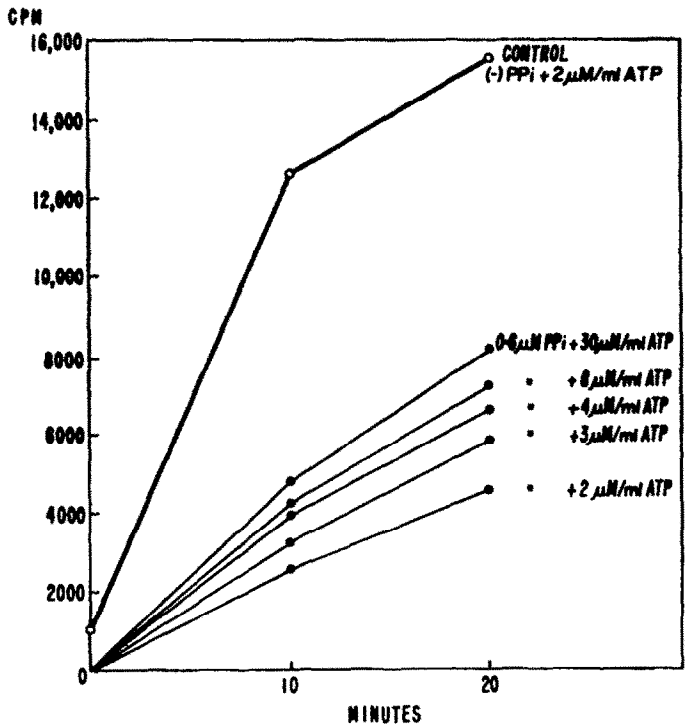

FIG. 3. Effect of varying ATP concentrations on the inhibition of amino acyl–transfer R NA formation caused by pyrophosphate. PPi and ATP were added at time zero and the assays were performed as described under Methods.

tRNA formation sufficient to explain the observed effects on poly-U-directed and endogenous ribosomal polypeptide synthesis. Studies of the reversibility of this effect by increased concentrations of ATP suggest that pyrophosphate interacts with the enzyme (step 1) and that this interaction is only partially reversible. The inhibitory effect of pyrophosphate can be removed upon dialysis of the pyrophosphate–enzyme complex. Experiments are in progress to define the nature of binding to the enzyme.

The inhibitory effects of pyrophosphate on RNA, DNA and protein synthesis of intact cells were quite surprising. In each instance, the inhibitory concentration of PPi was approximately 5×10^{-3}M in the medium (similar to the inhibitory concentration in isolated enzyme reactions) and both normal and leukemic leukocytes were affected

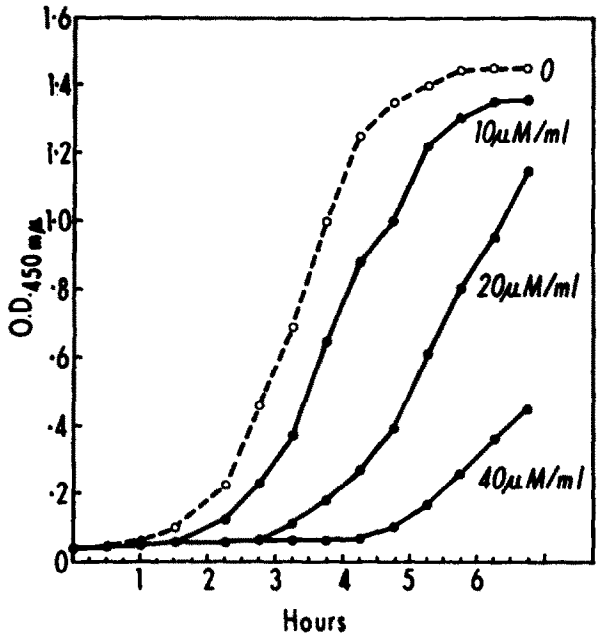

FIG. 4. Effect of pyrophosphate on the growth of *E. coli* B.

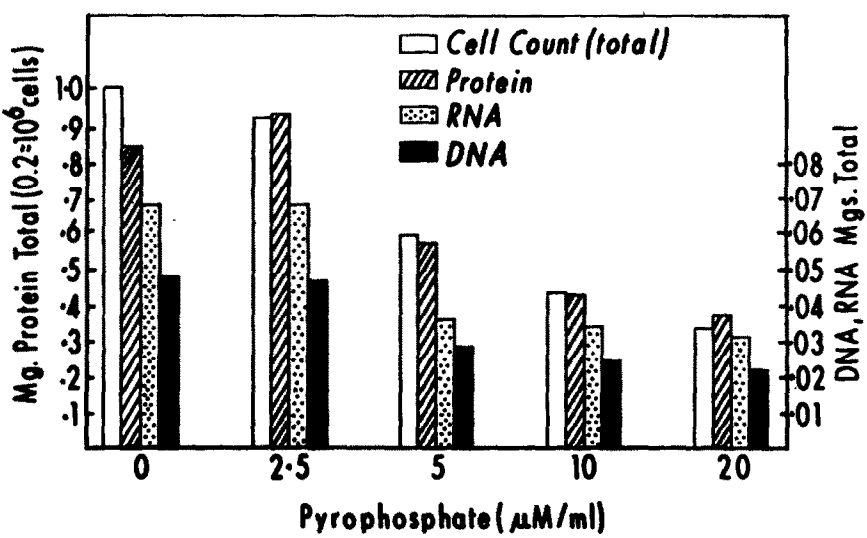

FIG. 5. Effect of pyrophosphate on HeLa cells in tissue culture. Cells were grown in flasks for 48 hr then removed for cell count determination and analysis of protein, RNA and DNA content. All results are expressed as the quantity present in the total cell culture.

equally. Furthermore, the growth of *E. coli* B and HeLa cells was inhibited by pyrophosphate concentrations of 5×10^{-3}M or greater. RNA, DNA and protein synthesis were inhibited to a similar extent by a given concentration of PPi. These results would imply ready transport of PPi into the cell and ineffective intracellular pyrophosphatase activity. The specificity of PPi effects are demonstrated by the lack of inhibition of leukocyte respiration and glycolysis. It is not known whether these effects of "pharmacologic" concentrations of PPi on intact cells and on specific enzyme reactions are applicable to any physiologic role of PPi in normal cells. However, the possibility clearly exists that PPi generation has a regulatory role in limiting further macromolecule synthesis. This hypothesis is currently being tested by attempting to control the intracellular concentration of pyrophosphate.

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