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INHIBITION OF PROTEIN AND RNA SYNTHESIS BY 5-MERCAPTOPYRIDOXINE*

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Abstract– The effect of 5-mercaptopyridoxine (vitamin B_6SH) on nucleic acid and protein synthesis in HeLa cells was studied with radioactive precursors. The compound inhibited both RNA and protein synthesis, independently and simultaneously. In these studies, vitamin B_6SH did not interfere with amino acid transport or the uptake of nucleic acid precursors and vitamin B_6. The addition of pyridoxine or pyridoxal phosphate did not prevent the inhibitory effect of the compound on protein synthesis. The previously reported reduction in antibody titers in patients with auto-immune disease treated with this compound might be a reflection of its inhibitory effect on RNA and protein synthesis, as demonstrated in these studies.

5-Mercaptopyridoxine (vitamin B_6SH), a vitamin B_6 analogue, was first used *in vivo* as a protective agent against ionizing radiation in mice [1]. Further investigation confirmed this observation and also showed the compound to be similarly protective in *Escherichia coli* strain B/r [2]. In 1961 Ritzmann and Levin [3] reported a reduction in the cold agglutinin and the acid cold hemolysin titers in a patient with cold agglutinin disease, after treatment with this compound.

In the course of investigating the effect of D-penicillamine on poliovirus replication in HeLa cells [4], a variety of other mercaptan compounds including vitamin B_6SH was studied. In order to assess the potential antiviral properties of these compounds, it was necessary to determine their effect upon the metabolic integrity of the host cells. Vitamin B_6SH was found to be an inhibitor of the metabolism of noninfected HeLa cells, and the following studies were performed to elucidate further the mechanism of that inhibition.

MATERIALS AND METHODS

HeLa cells were grown in either monolayer or suspension culture in Eagle minimum essential medium (MEM) supplemented with 7% calf serum and 2 mM glutamine. Tissue culture plates (60 × 15 mm) were seeded with $1{\cdot}5 \times 10^6$ cells/ml and incubated for 72 hr at 37° in 5% CO_2 to obtain confluent monolayers. Rabbit kidney cultures were prepared by trypsinization of minced renal cortex. The tissue was washed and trypsinized twice with 0·25% trypsin in Earle solution (EBSS) at 37° for 15 min. Trypsinization was stopped by the addition of 10% calf serum, and the dispersed cells were centrifuged and washed. The packed cells were diluted with MEM, supplemented with 10% fetal calf serum at a ratio of 1 to 200. Tissue culture plates were seeded with 5 ml of the cell suspension and incubated in 5% CO_2 at 37° to obtain confluent monolayers.

Measurement of protein, RNA and DNA synthesis. Suspension cultures of HeLa cells were washed with EBSS and suspended in MEM supplemented with glutamine and 5% calf serum at a density of 5×10^6 cells/ml. Vitamin B_6SH was dissolved in EBSS, buffered with Tris–HCl, pH 7·3, and added to the cell culture at appropriate concentrations. The accumulation of cell protein and RNA was measured by the addition of [^{14}C]leucine (0·1 μCi/ml, 350 mCi/m-mole, Amersham) or [^{14}C]uridine (0·1 μCi/ml, 450 mCi/m-mole, Amersham) respectively. The isotopes were added at the same time as the vitamin B_6SH. At appropriate times, 0·2-ml samples of the cultures were removed and diluted with 2 ml of cold EBSS. Samples were centrifuged, supernatants were discarded and cells were lysed in 1 ml of distilled water. The lysates were precipitated with 2 ml of cold 10% trichloroacetic acid (TCA) and filtered on Whatman GF/C glass-fiber filters. The filters were washed with cold 5% TCA, and the acid-insoluble material was analyzed for radioactivity in a Beckman scintillation spectrometer.

HeLa and rabbit kidney cell monolayers were washed with EBSS, and 3 ml MEM containing vitamin B_6SH and the isotope was added. Plates were incubated at 37° in 5% CO_2, and at appropriate intervals the supernatants were removed. Plates were washed with EBSS, and cells were lysed with 1 ml of distilled water and treated as were the suspension cultures.

To determine the rate of protein synthesis, suspension cultures of HeLa cells were prepared as above and vitamin B_6SH was added. At appropriate time intervals, 0·5-ml aliquots of cell cultures were removed and pulsed with 0·5 μCi of [^{14}C]leucine for 10 min at 37°. The pulse was terminated by the addition of ice-cold EBSS, and the cells were centrifuged and treated as above. Monolayers of HeLa and rabbit kidney cells were similarly pulsed and incubated at 37° in 5% CO_2.

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DNA synthesis was measured by incubation of 2.5×10^7 HeLa cells (5 ml) with 5 μCi of [^{3}H]thymidine and 500 μg/ml of vitamin B_6SH. The cell cultures were incubated at 37° for 1 and 4 hr. Cell suspensions were centrifuged, washed, lysed and precipitated with TCA. The precipitates were centrifuged, washed with 5% TCA and dissolved in 1 ml Hyamine and counted.

Uptake of amino acids, nucleosides and vitamin B_6SH. HeLa cells were washed and suspended in EBSS at a density of 1×10^7 cells/ml and incubated at 37°. ^{14}C-amino acids, uridine, thymidine and [^{3}H]pyridoxine and unlabeled vitamin B_6SH were added to the cell suspensions in a volume of EBSS to give a final density of 5×10^6 cells/ml. The uptake of nucleosides and amino acids was measured at 1, 2 and 5 min, and at 5-min intervals thereafter for 20 min. Pyridoxine uptake was measured at 5-min intervals for 20 min. The reaction was terminated by the addition of 5 ml of ice-cold EBSS, and the cells were filtered, washed and transferred to counting vials. They were freeze-thawed twice, dried and counted. When the uptake of amino acids and nucleosides was measured, samples were also analyzed for incorporation of label into TCA-precipitable materials.

RESULTS

Cytotoxic effects of vitamin B_6SH. When confluent monolayers of HeLa and rabbit kidney cells were incubated with vitamin B_6SH (300 μg/plate), the cytotoxic effect of this compound was apparent within 24 hr for HeLa cells and within 36–48 hr for rabbit kidney cells. When semiconfluent monolayers of both cell types were incubated with vitamin B_6SH, the cytotoxic effect was evident within 24 hr for both. Incubation of HeLa suspension cells with the same concentration of vitamin B_6SH resulted in 50 per cent cell death in 24 hr. The surviving cells excluded trypan blue and appeared normal in morphology when examined under the light microscope.

Effect of vitamin B_6SH on protein synthesis. HeLa suspension cells incubated simultaneously with [^{14}C]leucine and concentrations of vitamin B_6SH up to 400 μg/ml gave approximately 35 per cent inhibition of protein synthesis, which was evident within the first 10 min (Fig. 1). Above this concentration the inhibitory effect did not increase significantly. The effect of vitamin B_6SH on the rate of protein synthesis in HeLa cells is shown (Fig. 2). Vitamin B_6SH caused 30 per cent inhibition of protein synthesis in the first 60–90 min, which then increased linearly with time. By 2·5 hr, the rate of synthesis was inhibited approximately 65 per cent. Comparable results were obtained with HeLa and rabbit kidney cell monolayers. The inhibition was fully reversible for at least 2 hr following a 30-min lag period (Fig. 3).

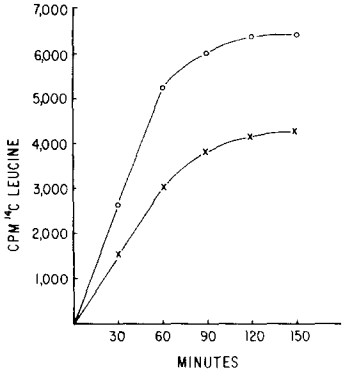

Fig. 1. Inhibitory effect of vitamin B_6SH on protein synthesis. The compound was added simultaneously with [^{14}C]leucine, and incorporation of the isotope was measured at 30-min intervals. ○—○, Control; ×—×, vitamin B_6SH (400 μg/ml).

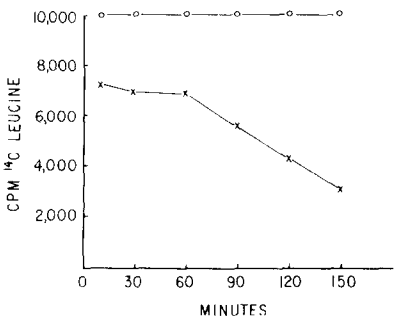

Fig. 2. Inhibitory effect of vitamin B_6SH on the rate of protein synthesis. Half the cell culture was treated with vitamin B_6SH, and the other half served as control. At the times indicated, aliquots were removed and pulsed with [^{14}C]leucine for 10 min, and the incorporation of the isotope was measured. ○—○, Control; ×—×, vitamin B_6SH (400 μg/ml).

To determine whether the inhibitory effect of vitamin B_6SH was due to alteration of amino acid transport or interference with synthetic processes, cell suspensions were pulsed for 2 min with [^{14}C]leucine, followed by a chase of a 1000-fold concentration of cold leucine. Vitamin B_6SH did not alter leucine transport but did inhibit protein synthesis.

Effect of vitamin B_6SH on RNA and DNA synthesis. Vitamin B_6SH in concentrations up to 500 μg/ml produced a 30 per cent inhibition of RNA synthesis (Fig. 4). This inhibition was evident within the first 10 min (Fig. 4) and was also reversible. DNA synthesis was not significantly inhibited by vitamin B_6SH

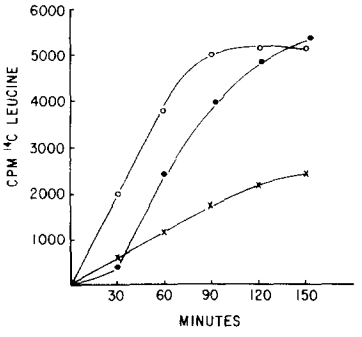

Fig. 3. Protein synthesis following reversal of vitamin B_6SH. Two-thirds of a HeLa cell culture was treated with vitamin B_6SH (400 μg/ml) for 90 min. In half the treated culture, the compound was removed by centrifugation and washing, and cells were resuspended in fresh medium. [^{14}C]leucine (0·1 μCi/ml) was added to untreated, treated and reversed cultures, and the incorporation of the isotope was measured. ○—○, Control; ×—×, vitamin B_6SH; ●—●, vitamin B_6SH removed after 90 min.

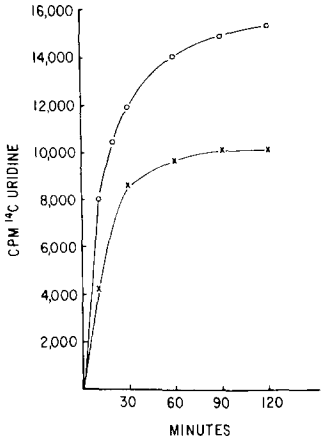

Fig. 4. Effect of vitamin B_6SH on RNA synthesis. HeLa cells (5×10^6 cells/ml) were suspended in MEM. and [^{14}C]uridine (0·1 μCi/ml) and vitamin B_6SH (500 μg/ml) were added. At the indicated intervals, incorporation of uridine into acid-insoluble material was measured. ○ ○, Contral; × × , vitamin B_6SH (500 μg/ml).

(500 μg/ml) within the first hr, but there was 45 per cent inhibition following a 4-hr incubation.

Uptake of amino acids and nucleosides. In order to determine if the inhibitory effect of vitamin B_6SH on protein and nucleic acid synthesis was due to impairment of membrane function, HeLa cells were exposed to ^{14}C-labeled amino acids and nucleosides in the presence of vitamin B_6SH. The compound had no effect on the uptake of glycine, leucine, isoleucine, valine, lysine, arginine, histidine, phenylalanine, proline and methionine. The uptake of [^{14}C]uridine and thymidine was also not altered by vitamin B_6SH.

An antivitamin B_6 effect. Since vitamin B_6SH is an analogue of vitamin B_6, its inhibitory effect could be due to analogue inhibition of vitamin B_6 function(s). The effect of this compound on protein synthesis was determined in the presence of a 100-fold excess of either pyridoxine or pyridoxal phosphate. Neither of these compounds altered the inhibition produced by vitamin B_6SH on protein synthesis. To determine whether vitamin B_6SH interfered with the uptake of vitamin B_6, its effect on the uptake of [^{3}H]pyridoxine was studied in a pyridoxine-free solution. The uptake of radioactive pyridoxine was not altered.

DISCUSSION

Vitamin B_6SH inhibits both protein and RNA synthesis simultaneously, but not completely (Figs. 1 and 4). The gradual cytotoxic effect of this compound is apparently due to amplification of this inhibition through the interdependence of RNA and protein synthesis. This is indicated in the enhanced, time-dependent and linear reduction of the rate of protein synthesis (Fig. 2). A possible effect of vitamin B_6SH on the integrity and function of the cell membrane was investigated. A decreased availability of protein and RNA precursors would explain the simultaneous inhibition of the synthesis of both of these macromolecules. The data indicate that the compound had no effect on the uptake of uridine or the transport of the amino acids which were tested.

Vitamin B_6 is active in a variety of biochemical functions [5], among which is its participation in amino acid transport [6] and protein synthesis [7]. Since vitamin B_6SH is a structural analogue of vitamin B_6, it is possible that the compound could function as an analogue inhibitor of the vitamin. As indicated above, vitamin B_6SH had no effect on the transport of amino acids, and the addition of pyridoxine or pyridoxal phosphate to the cell cultures did not reverse the inhibitory effects of this compound on protein synthesis. In addition, vitamin B_6SH did not interfere with the uptake of vitamin B_6 by the cell. Despite these negative results, the possibility remains that an antivitamin B_6 effect may be produced by this structural analogue in other vitamin B_6-dependent reactions.

It is suggested that the inhibitory effect of vitamin B_6SH on protein and RNA synthesis may be due to conformational alteration of enzymes required for the synthesis of these macromolecules. The non-covalent binding of this compound [as evidenced by the reversibility of the inhibition (Fig. 3)] to sites other than substrate binding sites would result in a rate of synthesis which is slowed but not stopped (Figs. 1 and 4). The effect may be on the affinity of enzymes for their substrates (hyperbolic competitive inhibition) or on the rate of formation of the products (non-competitive inhibition) [8].

The reduction of circulating antibody in patients with cold agglutinin disease could be explained by an inhibitory effect of this compound on the synthesis of the antibodies. While depolymerization *in vivo* of macroglobulin antibodies by this mercaptan was the explanation originally offered for the fall in antibody titers, the persistent reduction in circulating antibody after discontinuance of vitamin B_6SH suggests an effect on the cellular level. Inhibition of the synthesis of antibody would be entirely consistent with the reported observations. It is suggested that vitamin B_6SH may be an inhibitor of protein synthesis when administered to man. This inhibition might be most readily manifest in a cell system engaged in increased protein synthesis, such as immunoglobulin synthesis by lymphocytes in auto-immune diseases. Further studies are warranted to determine if this compound might represent a clinically useful immunosuppressant drug.

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