Benzene-Induced Inhibition of Erythroid Colony Formation in Vitro

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ABSTRACT: The erythroid colony forming unit assay technique was used to study the inhibitory effect of benzene on mouse bone marrow cell culture in vitro. The ability of vitamin B_6 to prevent benzene-induced inhibition of erythroid colony formation was also tested. At concentrations equal to or higher than the lowest inhibitory concentration of benzene, pyridoxine HCl significantly prevented benzene-induced inhibition of erythroid colony formation in vitro. However, when benzene concentration was higher, the added pyridoxine HCl was ineffective in preventing benzene-induced inhibition of erythroid colony formation in vitro, even when it was preincubated with the bone marrow cells for 30 minutes prior to adding benzene. On the basis of this finding, it is proposed that benzene-induced inhibition of erythroid colony formation may involve two different mechanisms, one of which involves interaction with vitamin B_6 added as pyridoxine HCl. **KEY INDEXING** TERMS: Pyridoxine; Bone Marrow Toxicity; Anemia; Vitamin B_6. [Am J Med Sci *1986;292(6):356–362.*]

The toxic effect of benzene on the hematopoietic cells has been reported by several investigators.[1–3] These reports, which included laboratory experiments as well as case studies, have demonstrated that chronic benzene exposure of both animals and man leads to progressive degeneration of bone marrow, aplastic anemia, and leukemia.

Although the exact mechanism by which benzene produces hematopoietic toxicity is as yet unknown, many recent studies have examined its inhibitory effects of heme synthesis.[4–6] Freedman et al[7] demonstrated that benzene inhibits in vitro heme synthesis at the δ-aminolevulinic acid synthetase step.

Other studies have also looked at benzene inhibition of protein synthesis in reticulocytes as well as in liver cells.[4,8] The inhibition in this case was accompanied by disappearance of polyribosomes followed by the accumulation of ribosomal monomer-dimers and the appearance of an intermediate electrophoresis peak that was previously absent.

More recently, other studies have suggested that metabolites of benzene, produced mostly by liver and bone marrow microsomal enzyme systems, are able to bind to mitochondrial DNA covalently. This covalent binding subsequently leads to inhibition of transcription and protein synthesis. The enzyme(s) and/or cofactor(s) involved in the covalent binding of these metabolites have not been identified.

In the present study, we have used in vitro erythroid colony forming unit (CFU-E) assay technique to investigate the hematopoietic toxicity of benzene and to evaluate the possibility that vitamin B_6 is able to prevent benzene-induced inhibition of erythroid colony formation by mouse bone marrow cells in vitro. The results suggest that benzene-induced inhibition of erythroid colony formation may involve two different mechanisms.

Materials and Methods

Animals. The 40 mice used in this study were a pool of control groups obtained from other experiments conducted in the department at an earlier time; as such the mice (6–12 weeks old) consisted of mixed strains of Swiss Webster (Balb/c NIH, and C57B1.BiGW). They were maintained in a standard animal care facility of the university until use.

Bone Marrow Suspension. Mononuclear cells of mouse bone marrow were obtained from the two femoral bones according to the technique described by Oliver and Goldstein.[9] Minimum essential medius with Hank's balanced salt solution (BSS, Grad Island Biological Company, New York, NY) supplemented with basal medium amino acids (Microbiological Associates, Bethesda, MD) was used to harvest and wash the cells. All cell suspensions were washed twice before cell counts were performed. The details of the techniques used to wash, count, and evaluate cell viability are as previously described.[9] The final cell volume was adjusted to give 2×10^7 nucleated cells per milliliter in each case.

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Analytical grade benzene (Thiophene free, Mallinckrodt Chemical Works, St. Louis, MO) was used by making a 1.3M stock solution in pure ethyl alcohol. Aliquots of this were then added to the culture system in microliter amounts to achieve the concentration level determined for each experimental condition. The concentrations tested were 1×10^{-3}, 2×10^{-3}, and 3×10^{-3} M, respectively. The starting concentration of 1×10^{-3} M was chosen on the basis of the results of three separate dose–response curve experiments preliminarily carried out using five levels of benzene concentration (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M, Table 1). In subsequent experiments the concentration range tested was limited to 3× the starting concentration (1×10^{-3} M) because no significant difference in the number of CFU-Es/10^5 cultured cells could be demonstrated for 2× and 3× the starting concentration under the experimental conditions described above.

Similarly, vitamin B_6 in the form of pyridoxine HCl (Sigma Chemical Company, St. Louis, MO) was prepared as a 1.3M stock solution in supplemented Ham's F–12 modified medium (GIBCO, New York, NY) and microliter aliquots were added to the culture system as needed. Vitamin B_6 solution was protected from direct light by wrapping the container in aluminum foil. Both the benzene and the pyridoxine solutions were made up fresh every 2 weeks and kept at 4°C at all times.

Erythroid Colony Cultures. The cells were cultured in plasma clots as described by Stephenson, et al[11] with minor alterations. In brief, approximately 0.1 ml of the cell suspensions (approximately 2×10^6 nucleated cells) was pipetted into prelabeled test tubes that also contained the following ingredients: 0.3 ml Ham's F-12 modified medium (GIBCO, New York, NY), 0.1 ml, each, bovine citrated Plasma (Sigma Chemical Company), beef embryo extract (50% in Gey's BSS, GIBCO), thrombin (10 units/ml, Parke-Davis), erythropoietin (Step 111, 5.0 units/ml, Connaught Medical Res. Laboratories), and bovine serum albumin (Fraction V Powder, 10% BSA, Sigma Chemical Company). Other ingredients included in the mixture were fetal calf serum (heat inactivated, 0.2 ml), sodium bicarbonate (7.5% solution, 0.01 ml), L-glutamine (200 mM, 0.1 ml): all purchased from Microbiological Associates of Bethesda, Maryland. The plasma was added to the mixture immediately before aliquoting into the microwells.

One tenth of the resulting mixture in the tube was quickly pipetted into each of three 6-mm diameter plastic microwells (Linbro Chemical Company, Inc.), allowed to clot and then incubated for 48 hours at 37°C in a highly humidified environment with 5% CO_2 and 95% air.

At the end of 48 hours, the plasma clots were removed from the wells (three clots per glass slide). The clots were fixed according to the technique described by Stephenson et al[11] with slight modifications. The clots were blotted with a 5 × 2.5-cm piece of Whatman No. 1 filter paper, flattened and fixed with a 10% glutaraldehyde solution. The cells were then stained for hemoglobin using 1% solution of 3,3′-dimethoxybenzidine (Eastman Chemical Company) in methanol and counterstained with Harris' hematoxylin (Harleco, Gibbstown, NJ). To make

TABLE 1
CFU-E Counts/10^5 Cultured Cells for Three Separate Dose Response Curve Experiments

Experimental Condition	Expt. No. 1	Expt No. 2	Expt No. 3	Mean ± SE	% of Control
Medium [control]	115	120	118	118 ± 1.5	100
Ethyl alcohol (10^{-2}M)	113	109	117	113 ± 2.3	95.8
Benzene					
1×10^{-2}M	11	24	33	23 ± 6.4	19.5
1×10^{-3}M	84	63	71	73 ± 6.0	61.9
1×10^{-4}M	95	90	81	89 ± 4.1	75.4
1×10^{-5}M	106	111	102	106 ± 2.6	89.8
1×10^{-6}M	119	109	113	114 ± 2.9	96.6

These were preliminarily carried out to establish the appropriate starting concentration of benzene used under the assay procedure adopted in this study. 1×10^{-3} M was considered to be appropriate as starting concentration in all subsequent experiments because this concentration appears to more closely approximate the 50% inhibiting Concentration.

The concentration of ethyl alcohol (1×10^{-2} M) is the maximum concentration that was used under these experimental conditions.

permanent slide preparations, the slides were air-dried, and cover slips applied using permount. Each clot was examined and the number of colonies containing eight or more benzidine-positive cells was counted as CFU-E's. The average number of CFU-E's from the three clots of each slide was expressed as the number of CFU-E's/10^5 cultured cells for the specific experimental condition per experiment.

Overall, there were 32-40 experiments carried out, and in each case all the experimental conditions specified above were tested in triplicates. The student's t-test for paired and unpaired data was used to evaluate the results of all the experiments performed. All calculations were carried out on a programmable hand calculator (model HP 33C).

Results

Figure 1A and 1B show photomicrographs (obtained at low and high magnifications, respectively) of typical erythroid colonies formed by mononuclear bone marrow cells after incubating for 48 hours at 37°C in highly humidified environment with 5% CO_2—95% air and stained with 3,3′-dimethoxybenzidine, followed by Harris' hematoxylin counter stain. Morphologically, most of the cells have the appearance of erythroblasts and are deeply stained.

A few contaminating granulocytes with typical doughnut-shaped nuclei (data not shown) could be seen in association with some of the erythroid colonies. Potentially, these contaminating cells could confound the counting process and invalidate the results. However, in the present study, the use of 3.3′-dimethoxybenzidine as stain to identify the erythroid colonies (groups of cells that appear to have brown-orange color) greatly minimized these problems. Furthermore, only colonies with eight or more benzidine-positive cells were counted as CFU-Es.

In general, the group of cells cultured in media that had no added benzene or pyridoxine HCl (control) showed a mean value (± SE) of 166 ± 5.94 CFU-Es/10^5 nucleated cells cultured. The comparable value for the group of cells cultured in media that had added pyridoxine HCl alone was 177 ± 3.21. This suggests that added pyridoxine alone at a concentration of 1×10^{-3} M improved erythroid colony formation by
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Figure 1A. Photomicrograph of erythroid colonies after 48 hours incubation. Erythroid colony cells were stained with 3.3′-dimethoxybenzidine and counter stained with Harris' hematoxylin. Under this condition cells with hemoglobin show brown–orange color and only colonies with eight or more such cells were counted as CFU-Es (original magnification. x 200).

Figure 1B. A repr
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ormation by 6.6 ± 0.12%. This improvement, however, is not statistically significant.

Conversely, the group of cells cultured in the media that had added benzene alone at concentration of 1×10^{-3} M showed a mean value of 62 ± 1.99 CFU-Es/10^5 cells cultured. This value is significantly lower (by 37.3 ± 2.01) than the mean value for control ($t = 20.149$, $p < 0.001$).

Similarly, the mean value (62 ± 1.99 CFU-Es) for cells cultured in the presence of 1×10^{-3} M benzene is significantly lower than the mean value (99 ± 5.40 CFU-Es/10^5 cultured cells) obtained for the group of bone marrow cells cultured in medium with added benzene and pyridoxine together at equal concentration of 1×10^{-3} M, respectively ($t = 8.162$, $p < 0.001$). This suggests that pyridoxine HCl may have provided some protection against benzene-induced inhibition of erythroid colony formation under these experimental conditions.

Figure 2 shows graphically the percent inhibition of erythroid colony formation by bone marrow cells cultured in media that had 1×10^{-3}, 2×10^{-3} and 3×10^{-3} M benzene added simultaneously with a constant concentration (1×10^{-3} M) of pyridoxine HCl in each case. Overall, the inhibition of erythroid colony formation increased (from 36.4 ± 1.99% to 53.8 ± 2.5% and 54.4 ± 3.84%, respectively) as the concentration of benzene increased from 1×10^{-3} M to 2×10^{-3} M and 3×10^{-3} M, respectively in spite of the added (1×10^{-3} M) pyridoxine in each case. The difference between the percent inhibition at 2×10^{-3} M and 3×10^{-3} M benzene is not statistically significant. However, the difference between percentage inhibition at 1×10^{-3} M and at 3×10^{-3} M benzene was statistically significant ($t = 3.992$, $p < 0.001$).

The group of bone marrow cells cultured in media that had pyridoxine added at a concentration of 2×10^{-3} M, and 3×10^{-3} M in the presence of a constant amount (1×10^{-3} M) of benzene showed 16.5 ± 0.53% and 24.8 ± 0.71% inhibition respectively. Again, the differences between the percent inhibition at the two experimental conditions are not statistically significant.

To test for possible benefit of preincubation of bone marrow cells with pyridoxine, the cells were first incubated for 30 minutes in culture media that contained 1×10^{-3} M pyridoxine HCl and then cultured for 48 hours.

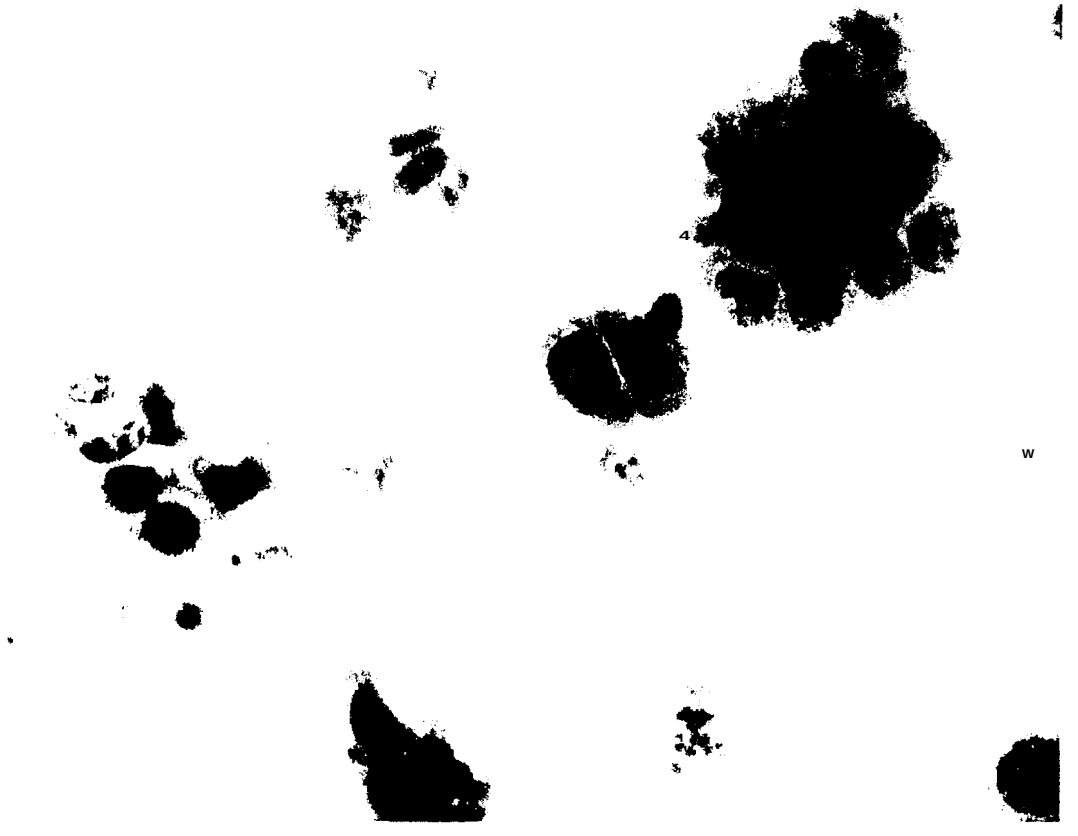

Figure 1B. A representative erythroid colony at higher magnification to show typical appearance of the cells under our experimental condition (original magnification x 1000).

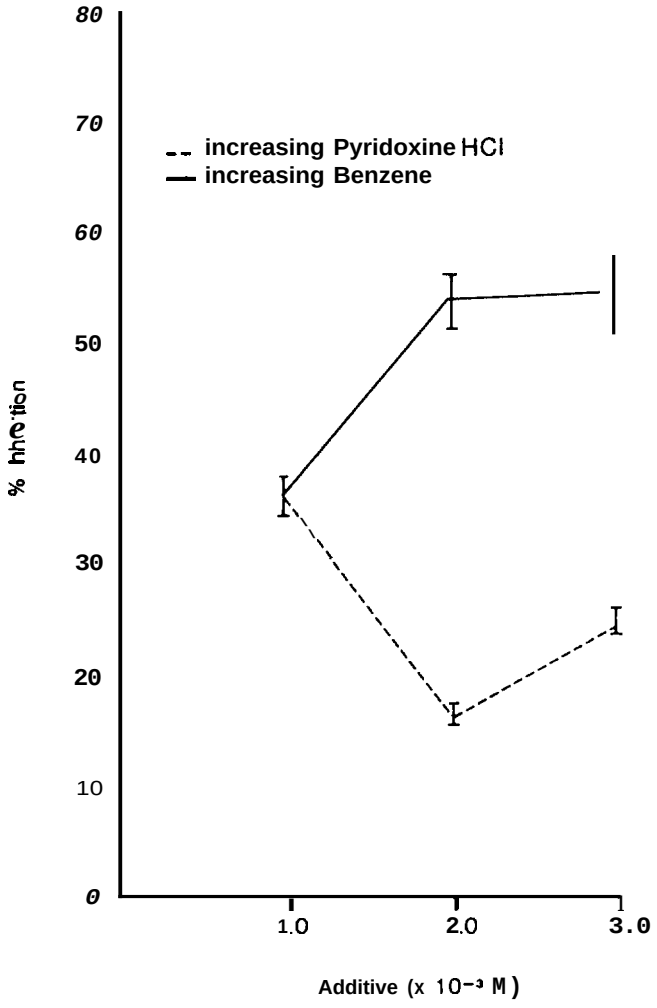

Figure 2. Percentage inhibition of erythroid colony formation under increasing pyridoxine (solid line) and increasing benzene concentration (broken lines). To correct for the effect of pyridoxine alone, % Inhibition was calculated as $\left[1 - \left(\text{Treatment/control} \times \text{pyridoxine}\right)\right] \times 100$.

Figure 3 shows a histograph of the mean values (± SE) of CFU-Es/10^5 cells cultured (aspercent control) under these experimental conditions. Overall there is no statistically significant difference between the mean values of CFU-Es/10^5 cells determined for the group of bone marrow cells cultured in media which had benzene added simultaneously with pyridoxine and the mean values obtained for the cells preincubated with pyridoxine at all three levels of benzene concentration tested.

Discussion

The erythroid colony forming unit assay technique is based on the principle that the number of colonies formed is directly proportional to the number of nucleated marrow cells incubated in the individual plasma clot. The data presented in this study are for tests carried out on the bone marrow cells obtained from 40 mice. The data indicate that added benzene at a concentration of 1×10^{-3} M significantly inhibits erythroid colony formation under the in vitro conditions described.

When pyridoxine HCl is added together with benzene at equal concentration (1×10^{-3} M each) or higher than benzene (2×10^{-3} M pyridoxine, respectively, added with 1×10^{-3} M benzene), there was significant protection against benzene-induced inhibition of erythroid colony formation as indicated by the significant increase in the number of CFU-Es/10^5 cells cultured under this condition. However, when the concentration of pyridoxine is maintained at 1×10^{-3} M while the concentration of benzene is increased to 2×10^{-3} and 3×10^{-3} M, respectively, the added pyridoxine was ineffective in preventing benzene-induced inhibition of erythroid colony formation. Furthermore, preincubation of the bone marrow cells with pyridoxine HCl (at 1×10^{-3} M) for

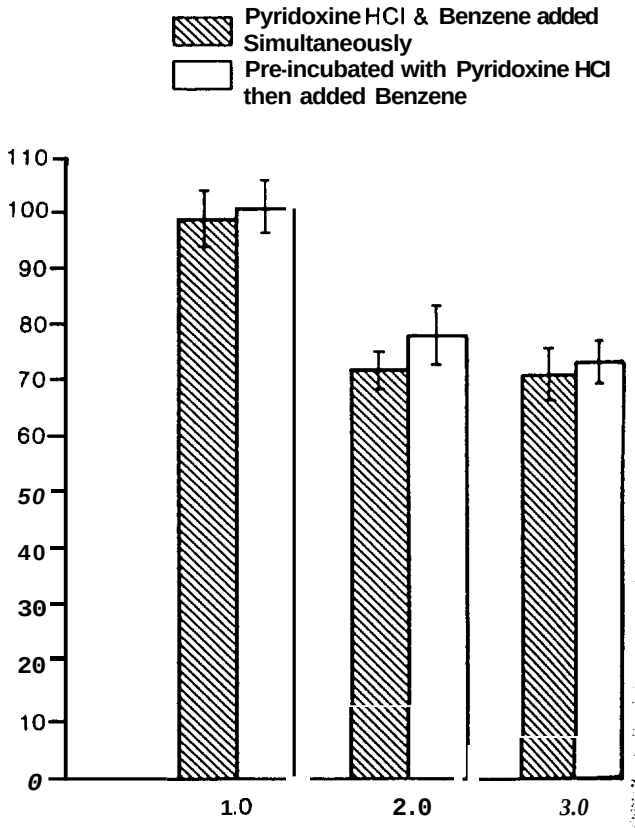

Figure 3. A histograph of the mean values (± SE) of CFU-E per 10^5 cells cultured under added pyridoxine and benzene simultaneousiy, and added pyridoxine 30 minutes prior to adding benzene. Preincubation with pyridoxine did not provide added protection against benzene inhibition of erythroid colony formation.

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30 minutes prior to adding benzene (at 2×10^{-3} and 3×10^{-3} M, respectively) did not result in improved protection against benzene-induced inhibition of erythroid colony formation.

In general, the finding that benzene significantly inhibits erythroid colony formation is in occordance with similar data reported by several other investigators.[7,12,13]

Since the classical experiment of Selling,[14] which studied the sequence of hemopoietic changes following the subcutaneous injection of benzene, several studies have been conducted to evaluate the mechanism by which benzene produces hematopoietic toxicity. Most of these studies agree that in the bone marrow the cells in early stages of development (pronormoblasts and normoblasts of the myeloid line) are the most sensitive to benzene hematopoietic toxicity.',[15]

At the molecular level, experimental data have demonstrated that one or more of benzene metabolites may be involved in its hematopoietic toxicity. It is suggested that the putative metabolite(s) may mediate benzene-induced hematopoietic toxicity by direct interaction with the nucleus and the chromosomes of these cells. This in turn could result in the arrest of maturation of bone marrow cells and/or inhibition of cell division in the erythrocytic sys-

However, other studies have demonstrated that benzene (possibly through the action of its metabolites) suppresses not only DNA and RNA synthesis, but also inhibits heme and cytoplasmic protein synthesis in differentiating bone marrow cells of mice and rabbit reticulocytes respectively. Forte et al[4] have reported data that demonstrate that delta-amino levulinic acid (ALA) or hemin reversed benzene-induced inhibition of protein synthesis in reticulocytes. This has led F'reedman et al[7] to postulate that the specific molecular site of benzene-induced inhibition of heme synthesis is at or before the ALA synthetase step. The group also demonstrated that 1mM pyridoxine added to a rabbit reticulocyte suspension significantly protected against and reversed benzene inhibition of ^{14}C-glycine incorporation into haemin that was used as an index of heme synthesis. More recently, evidence has been presented that suggests that some metabolic product(s) of benzene may bind to mitochondrial DNA covalently; such covalent binding could lead to benzene-induced inhibition of protein synthesis.''

In the present study, it is possible that protein synthesis was inhibited by covalent binding of the metabolites of the added benzene to mitochondrial DNA and/or RNA; this in turn could have led to the observed decrease in the number of CFU-Es under the experimental condition described. On the basis of the data observed for the added pyridoxine HCl experiments, however, it appears that there may be an additional mechanism by which benzene inhibits erythroid colony formation in vitro. It appears also that this other mechanism operates at a moderate (1×10^{-3} M) level of benzene concentration and could be prevented by the added pyridoxine HCl.

Several studies have been reported that demonstrate the regulatory role of vitamin B_6 (as pyridoxal-5-phosphate) in heme and protein synthesis.[19,20] The protective effect of the added pyridoxine in the present study may be due to its regulatory role in heme and protein syntheses. Under these conditions, added pyridoxine HCl should prevent benzene-induced inhibition of erythroid colony formation at all levels of benzene concentration. However, the preceding data appear to suggest otherwise.

The data reported in this study have not fully explained all of the known effects of benzene on hemopoietic systems. Nevertheless, they do support the view that one of the effects of benzene apparently involves the inhibition of erythroid colony formation in vitro. In addition, the data suggest that the apparent benzene-induced inhibition of erythroid colony formation may involve two different mechanisms. However, it would be interesting if these findings are investigated further in more primitive precursor cells such as the erythyroid burst forming cells (BFU-Es) and in vivo experiments to investigate further the relationship between the two mechanisms and the possible involvement of vitamin B_6.

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