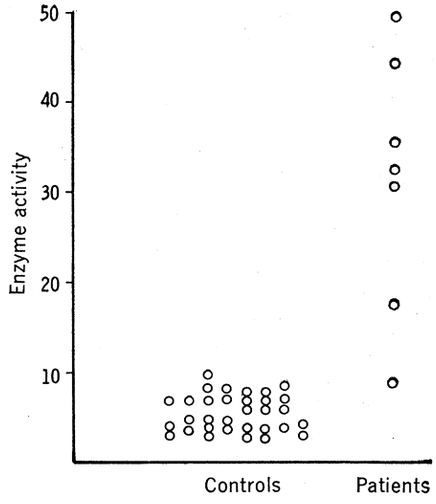

Fig. 2. Comparison of orotate phosphoribosyltransferase in the lysed red cells of normal subjects with that in patients receiving allopurinol. Enzyme levels are expressed as nanomoles of orotic acid converted to orotidine 5′-phosphate per 10^9 red blood cells per hour. Each circle represents a single estimation of activity in individual controls or patients.

greater quantities of orotidine, about 200 to 400 mg/day. These patients had renal insufficiency. Utilizing the thin-layer chromatographic system described above and the colorimetric test, we found that most patients receiving allopurinol also excrete excess orotic acid in their urine, but to a lesser degree than orotidine.

Subsequent investigations indicated that allopurinol is an inhibitor of uridine 5′-phosphate biosynthesis. Using the assay procedure of Smith *et al.* (*6*), we found that, in a lysed erythrocyte system, allopurinol appears to act as a competitive inhibitor (the inhibition constant K_i is about $10^{-5}M$) with respect to orotic acid in the phosphoribosyltransferase reaction. However, we were unable to detect any inhibition by allopurinol ($10^{-4}M$) of the decarboxylase reaction.

The results appear to be paradoxical insofar as the metabolite excreted in the urine is orotidine, the dephosphorylated product of the enzyme apparently inhibited. They are, however, somewhat analogous to results of Čihak and Šorm (*7*) with 5-azaorotate. These workers demonstrated that although 5-azaorotate itself was an inhibitor of orotate phosphoribosyltransferase, the inhibitor in vivo was the ribonucleotide. Thus orotate phosphoribosyltransferase converted 5-azaorotate to the ribonucleotide which then inhibited the decarboxylase reaction.

An experiment designed to test the possibility that a similar mechanism operated in the case of allopurinol (Table 1) indicated that, although allopurinol itself does not inhibit the decarboxylase reaction, prior incubation with phosphoribosyl pyrophosphate converts it to an inhibitor of this enzyme. This is most likely to be the ribonucleotide of allopurinol and presumably reflects the situation in vivo. Further investigation has revealed that the inhibition is competitive with respect to orotidine 5′-phosphate.

Thus we conclude that allopurinol in vivo is converted by a phosphoribosyltransferase to the ribonucleotide, which inhibits orotidine 5′-phosphate decarboxylase and leads to the accumulation of orotidine 5′-phosphate. The dephosphorylated product, orotidine, is then excreted in the urine.

We have also found that orotate phosphoribosyltransferase activity in the erythrocytes of patients receiving allopurinol increased severalfold (Fig. 2). This could arise from derepression of enzyme synthesis because of decreased formation of uridine 5′-phosphate during the period of erythrocyte maturation. However, Pinsky and Krooth (*8*) have demonstrated, in cultured human cells, increases of activity of orotate phosphoribosyltransferase when the cells are grown in the presence of 6-azauridine and other inhibitors of pyrimidine biosynthesis. They presented evidence that this increase in activity was due to accumulation of a precursor of uridine 5′-phosphate rather than depletion of this nucleotide.

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References and Notes

1. R. W. Rundles, J. B. Wyngaarden, G. H. Hitchings, G. B. Elion, *Annu. Rev. Pharmacol.* **9**, 345 (1969).
2. M. Lotz, H. J. Fallon, L. H. Smith, Jr., *Nature* **197**, 194 (1963).
3. R. M. Fox, W. J. O'Sullivan, B. G. Firkin, *Amer. J. Med.* **47**, 332 (1969).
4. L. E. Rogers and F. S. Porter, *Pediatrics* **42**, 423 (1968).
5. R. M. C. Dawson, D. C. Elliott, W. H. Elliott, K. M. Jones, *Data for Biochemical Research* (Oxford Univ. Press, London, ed. 2, 1969), p. 176.
6. L. H. Smith, Jr., M. Sullivan, C. M. Huguley, Jr., *J. Clin. Invest.* **40**, 656 (1961).
7. A. Čihak and F. Šorm, *Biochim. Biophys. Acta* **149**, 314 (1967).
8. L. Pinsky and R. S. Krooth, *Proc. Nat. Acad. Sci. U.S.* **57**, 925, 1267 (1967).
9. We thank Dr. W. Hensley, Royal Prince Alfred Hospital, for access to a Unicam SP800 scanning spectrophotometer and to Burroughs Wellcome and Co. (Australia) Ltd. for allopurinol. Supported by the National Health and Medical Research Council of Australia and, in part, by a Ciba donation to the Department of Medicine. We thank Professor C. R. B. Blackburn for interest.

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Endotoxin: Stimulation of Bone Resorption in Tissue Culture

Abstract. *Bacterial endotoxins can stimulate the release of previously incorporated calcium-45 and tritiated proline from fetal rat bone in tissue culture. Endotoxin from* Bacteroides melaninogenicus, *an organism regularly found in the gingival crevice of man, produces a response similar to parathyroid hormone and is effective at doses as low as 0.1 microgram per milliliter. This response is inhibited by serum and dependent upon the presence of albumin. Endotoxins may play a role in the bone loss characteristic of human periodontal disease.*

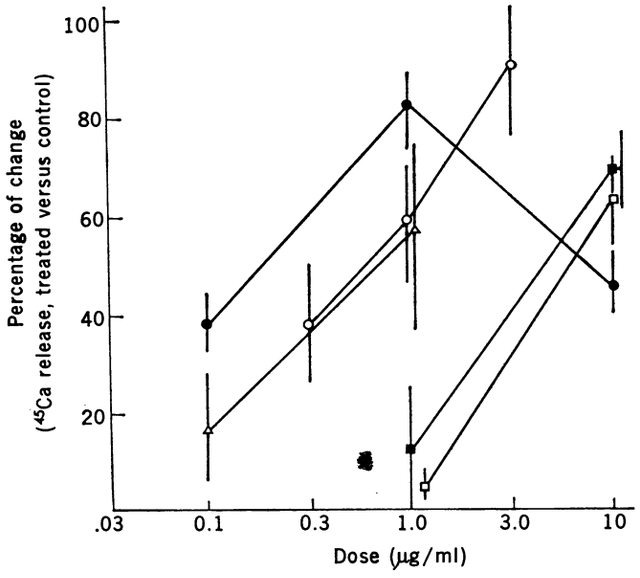
More teeth are lost in the adult population of the United States over 35 years of age as a consequence of chronic periodontal disease than as a result of dental caries (*1*). Resorption of the supporting bone that surrounds the roots of teeth is characteristic of human chronic periodontal disease (*2*). In epidemiological studies, the incidence of severity of bone destruction can be related to the amount of bacteria and microbial products adjacent to and in the gingival crevice (*1*). We, therefore, studied the effect of purified endotoxin from *Bacteroides melaninogenicus,* a microorganism regularly found in the gingival crevice of man (*3*), on bone resorption in tissue culture. It stimulated significant resorption at a dose as low as 0.1 μg/ml.

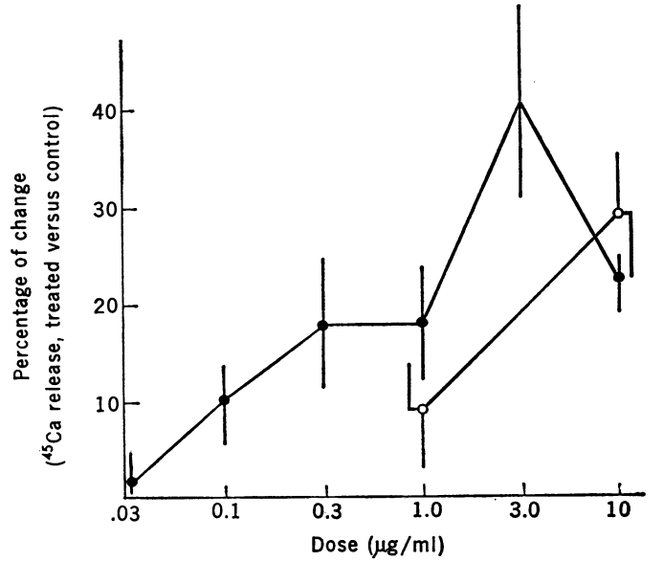
Resorption was assessed quantitatively by the release of previously incorporated ^{45}Ca from paired test and control bones from 19-day-old fetal rats, cultured in chemically defined medium (*4*). In one experiment, the pregnant rats were injected with 2 mc of tritiated proline instead of calcium on the day before they were killed. Endotoxin from two separate lots of *Bacteroides melaninogenicus*, strain CR-2A (*5*), as well as endotoxins from *Escherichia coli* and *Salmonella typhi* (*6*), could stimulate bone resorption in a manner similar to parathyroid hormone (Fig. 1). Morphologically, endotoxin caused a proliferation of osteoclasts and removal of matrix (Fig. 2). The endotoxin from *B. melaninogenicus* appeared to be more potent than that from the other bacteria and on a weight basis was similar to parathyroid hormone. However, with large doses of endotoxin (10 μg/ml) the response actually decreased, which suggests a possible toxic component. Several

months later, the two lots of endotoxin from *B. melaninogenicus* were reassayed (Fig. 3). Both showed somewhat smaller effects than those observed in the early experiments, which suggests that these preparations may deteriorate. The decreased response to 10 μg was seen again. The dose response was similar when the release of previously incorporated proline, instead of ^{45}Ca, was measured (Table 1).

Because of the complex nature of the endotoxin molecule (*7*), it will be important to determine what components may be responsible for stimulating resorption. Whole serum (*8*) and serum albumin (*9*) have also been implicated as stimulators of bone resorption. In the present system the response to endotoxin from *B. melaninogenicus* appears to be inhibited by heat-treated serum and dependent on the presence of albumin in the tissue culture system (Table 2). It is quite possible that whole serum contains antibodies to this ubiquitous material.

The observation that vitamin A (*10*) and endotoxin (*11*) can both release lysosomal enzymes and that vitamin A (*12*) and parathyroid hormone (*13*) cause such enzyme release during stimulated bone resorption suggests a possible mechanism. Recently, inhibition of proline incorporation was reported in cultures of bones from young rats in the presence of 30 μg/ml of endotoxin from *E. coli* (*14*). Although this suggests that endotoxin could also affect bone formation, such an effect could not account for our observations, since the system employed is one in which formation is minimal or absent.





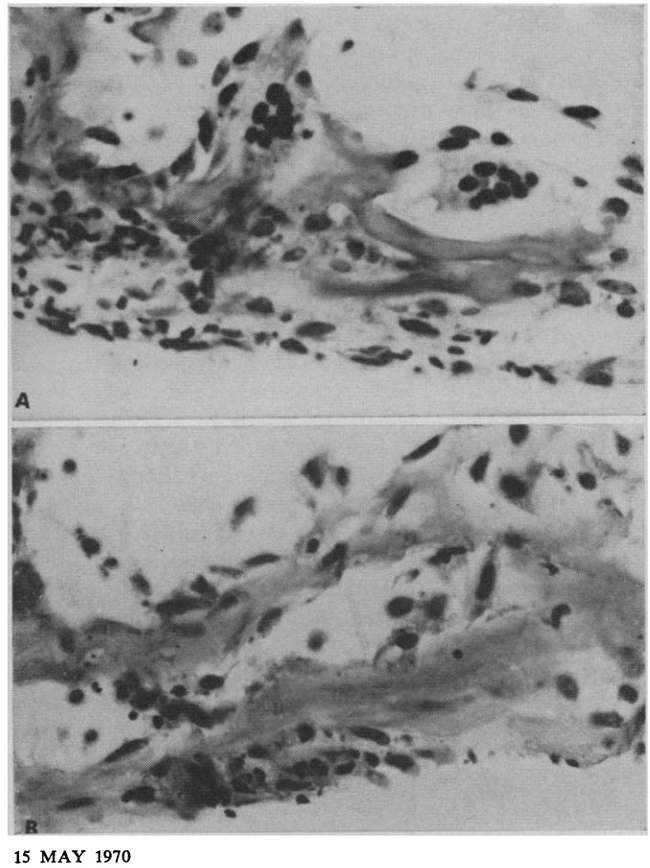


Fig. 1 (upper left). Effect of changing endotoxin or parathyroid hormone concentration on the release of previously incorporated ^{45}Ca from fetal bones cultured for 48 hours.

$$\text{Percentage of change} = \left(\frac{^{45}\text{Ca exp.} - ^{45}\text{Ca control}}{^{45}\text{Ca control}}\right) \times 100$$

The ^{45}Ca refers to ^{45}Ca in the mediums of experimental and control bone cultures. ●——●, Endotoxin from lot No. 1 of strain CR-2A, *Bacteroides melaninogenicus*; △——△, endotoxin from lot No. 2 of strain CR-2A, *B. melaninogenicus*; ■——■, endotoxin from *E. coli* O127:B8; □——□, endotoxin from *S. typhi* 0901; and ○——○, purified parathyroid hormone. Points are mean and vertical lines indicate standard errors of four pairs of bone cultures. Fig. 2 (lower left). Histologic sections of fetal bones cultured for 48 hours. (A) Bone cultured in medium containing 3 μg/ml of endotoxin from *Bacteroides melaninogenicus*. Note the presence of osteoclasts and paucity of bone matrix (× 300). (B) Bone cultured in control medium (× 300). Both sections are oriented with the periosteum situated at the lower edge. Fig. 3 (upper right). Effect of changing endotoxin concentration from strain CR-2A of *Bacteroides melaninogenicus* on the release of previously incorporated ^{45}Ca from fetal bones cultured for 48 hours. ●——●, Endotoxin from lot No. 1; ○——○, endotoxin from lot No. 2.

Table 1. Effect of endotoxin from *Bacteroides melaninogenicus* on release of previously incorporated [^{3}H]proline from cultured bones. Values are expressed as the ratio of the release from treated bones to that from control bones (± the standard error of the mean).

Dose (μg/ml)	[^{3}H]Proline release
Endotoxin	
0.1	0.95 ± 0.06
1.0	1.57 ± .19*
10.0	1.20 ± .07*
Parathyroid hormone	
1.0	1.56 ± .15*

*Significantly different from 1.0, $P < .05$.

Table 2. Effect of albumin and serum on the release of ^{45}Ca from cultured bones stimulated by endotoxin from *Bacteroides melaninogenicus*. Endotoxin concentration in culture medium of all experimental bones was 1 μg/ml. Culture conditions, except for addition of endotoxin or serum, or deletion of albumin, as described in (*4*), $n = 4$. The serum was heated for 30 minutes at 60°C. Values are expressed as the ratio of the release from treated bones to that from control bones (± the standard error of the mean).

Culture conditions	^{45}Ca release
Albumin (0.1%)	1.56 ± 0.19*
Minus albumin	0.93 ± .05
Normal rabbit serum (1.0%)	1.01 ± .07

*Significantly different from 1.0, $P < .05$.

It is not surprising that endotoxins from several bacterial sources can stimulate bone resorption, in view of the similarities of their chemical and biological properties (*7*). Other genera of bacteria which produce endotoxin have been isolated from the human mouth besides *B. melaninogenicus* (*15*). The endotoxin derived from *B. melaninogenicus* is unique in that it does not contain 2-keto-3-deoxyoctonate, a component found in other endotoxins (*16*); however, it produces reactions typical of endotoxin in rabbits (*17*), such as elevation in body temperature and local Shwartzman reactions in the skin. On the basis of these studies, it is possible that endotoxins released by bacteria in the gingival crevice play a significant part in bone resorption seen in human periodontal disease.

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References and Notes

1. A. L. Russell, in *Periodontal Therapy*, H. M. Goldman and D. W. Cohen, Eds. (Mosby, St. Louis, 1968), p. 64.
2. H. M. Goldman and D. W. Cohen, in *ibid.*, p. 75.
3. T. Rosebury, *Microorganisms Indigenous to Man* (McGraw-Hill, New York, 1962), pp. 162–165.
4. L. G. Raisz and I. Niemann, *Endocrinology* **85**, 446 (1969).
5. Strain CR-2A of *Bacteroides melaninogenicus* was obtained from Dr. Ronald Gibbons, Forsyth Dental Center, Boston, Mass. The bacteria used as our source of endotoxin were grown by Dr. Elias Kaufman, Department of Pedodontics, School of Dentistry, State University of New York at Buffalo. Two separate lots of bacteria were grown as described by R. Gibbons and J. B. MacDonald [*J. Bacteriol.* **81**, 614 (1961)]. Endotoxin was purified as described by H. Tauber and W. Garson [*J. Biol. Chem.* **234**, 1391 (1959)]. The ultraviolet absorption spectrum in the range of 220 to 300 nm showed no peaks.
6. Purified endotoxins from *E. coli* O127 : B8 and *S. typhi* 0901 were obtained from Difco Laboratories, Detroit, Mich.
7. A. Nowotny, *Bacteriol. Rev.* **33**, 72 (1969).
8. H. B. Fell and L. Weiss, *J. Exp. Med.* **121**, 551 (1964).
9. P. Stern and L. G. Raisz, *Exp. Cell Res.* **46**, 106 (1967).
10. J. T. Dingle, *Biochem. J.* **79**, 509 (1961).
11. G. Weissmann and L. Thomas, *J. Exp. Med.* **116**, 433 (1962).
12. H. B. Fell and J. T. Dingle, *Biochem. J.* **87**, 403 (1963).
13. G. Vaes, *J. Cell Biol.* **39**, 676 (1968).
14. L. A. Norton, W. R. Proffit, R. R. Moore, *Nature* **221**, 469 (1969).
15. S. W. Mergenhagen, *J. Dent. Res.* **46**, 46 (1967).
16. T. Hofstad, *Arch. Oral Biol.* **13**, 1149 (1968).
17. S. E. Mergenhagen, E. G. Hampp, H. W. Scherp, *J. Infec. Dis.* **108**, 304 (1961).
18. Supported by PHS grants DE-01932-06 and AM-06205. [^{3}H]Proline is a gift from Dr. S. Steven Brodie.

2 January 1970

Teratogenic Evaluation of 2,4,5-T

Abstract. *The herbicide 2,4,5-trichlorophenoxyacetic acid is teratogenic and fetocidal in two strains of mice when administered either subcutaneously or orally and in one strain of rats when administered orally. The incidences of both cystic kidney and cleft palate were increased in the C57BL/6 mice as well as the incidence of cleft palate in the AKR mice. The incidence of cystic kidney was also increased in the rats. In addition, an increase in the ratio of liver weight to body weight in the mouse fetus and the occurrence of hemorrhagic gastrointestinal tract in the rat fetus suggest that this compound also has fetotoxic properties.*

The chlorinated herbicide 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) is used extensively for weed control (*1*). There have been few reports concerning its pharmacologic and toxicologic properties in animals (*2*, *3*), and there are no data available concerning the effects of this compound on the developing embryo and fetus. Therefore, we evaluated the teratogenic and fetotoxic potential of 2,4,5-T in mice and rats.

Breeding colonies of C57BL/6 and AKR strains of mice were established to supply the mice. Breeding was by random mating. Detection of a vaginal plug indicated day zero of pregnancy. Pregnant rats were procured (Holtzman) with known insemination dates. Detection of sperm indicated day zero of pregnancy. All animals were given free access to chow and water.

The herbicide 2,4,5-T (*4*) was administered either subcutaneously or orally. A solution of 2,4,5-T in 100 percent dimethylsulfoxide (DMSO) in a volume of 100 μl per mouse was used for each subcutaneous administration. For oral administration by stomach tube, 2,4,5,-T was suspended in a honey solution (honey to water, 1:1), volumes of 100 μl per mouse and 200 μl per rat were used.

In the studies with the C57BL/6 strain, 2,4,5-T was administered daily beginning on day 6 of pregnancy and continuing through day 14 or from day 9 through day 17. The mice were killed on day 18 of gestation. In the studies with the AKR strain, 2,4,5-T was administered daily beginning on day 6 of pregnancy and continuing through day 15. These mice were killed on day 19 of gestation. The rats were treated on day 10 through day 15 and killed on day 20 of gestation.

After the animals were killed both mothers and fetuses were examined. In addition, about one-third of the mouse fetuses from each litter were stained with alizarin red S to detect skeletal anomalies.

The following conventions were observed in compiling the data in Tables 1 to 3. If a fetus was either dead or resorbed, it was regarded as a dead fetus. A fetus was classified abnormal if it was alive and had at least one anomaly (regardless of type). Similarly, a litter was classified as abnormal if it contained one or more abnormal fetuses. A fetus was said to have a cystic kidney if at least one of its kidneys was affected. In calculating the ratios of liver to body weight in the mother, maternal body weight was defined as the difference between the weight of the mother on the day it was killed and the gravid uterus weight. Finally, the maternal weight gain was defined as the difference in the corrected maternal body weight on the day it was killed and its weight on day zero of pregnancy.

The percentages for fetal mortality,