OXIDATION OF OXYPHENBUTAZONE BY SHEEP VESICULAR GLAND MICROSOMES AND LIPOXYGENASE

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Received January 30,1975

SUMMARY

Oxyphenbutazone was oxidized when incubated with acetone powder prepared from sheep vesicular gland microsomes or with lipoxygenase at pH 4 or 5. Oxidation also occurred at pH 8 or 9, if arachidonate or linoleate was added to either of the incubation mixtures. The oxygenated product was found to be identical with 4-hydroxyoxyphenbutazone, which was synthesized and analyzed by gas liquid chromatography and mass spectrometry. The oxygenated compound was not an inhibitor of prostaglandin biosynthesis.

INTRODUCTION Although prostaglandin synthetase has been investigated extensively concerning its ability to oxygenate various unsaturated fatty acids (1), there is only one report (2) pertaining to the oxidation of other classes of compounds during the action of prostaglandin synthetase.

In this communication we wish to report one such example involving oxyphenbutazone (3), a clinically used antiinflammatory agent. Since there is a substantial body of evidence linking the mode of action of non-steroidal antiinflammatory agents with inhibition of prostaglandin biosynthesis (4, 5), we have investigated the constitution of the oxidation product and tested it against prostaglandin synthetase derived from sheep vesicular glands. In addition, the common mechanistic features shared by prostaglandin synthetase and lipoxygenase (6) have prompted us to determine whether the latter enzyme catalyzes a similar oxidation of oxyphenbutazone.

MATERIALS AND METHODS

Materials: [3,5-^{14}C]-Oxyphenbutazone was kindly provided by Ciba-Geigy, Basel, Switzerland. The radiolabeled material was diluted with unlabeled oxyphenbutazone (Hässle-Ciba-Geigy AB, Mölndal, Sweden) to a specific activity of 0.03 Ci/mol. [1-^{14}C]-Arachidonic acid was prepared as described by Stoffel (7) and diluted with unlabeled acid (Hormel Institute, Austin, Minn., USA) to a specific activity of 0.36 Ci/mol. Indomethacin was obtained from Merck, Sharp and Dohme; aspirin and soybean lipoxygenase was purchased from Sigma, St. Louis, Minn., USA.

Preparation of acetone powder from sheep vesicular gland microsomes: Frozen glands (about 150 g) were minced with a meat-grinder and transferred directly into 500 ml of buffer containing 0.25 M sucrose, 10^{-4}M glutathione (reduced form), 0.05 M Tris, and 10^{-3}M EDTA (adjusted with 2 N HCl to pH 7.4). The suspension was blended for one minute in an Ultra-Turrax mixer and homogenized in a Potter-Elvehjem homogenizer. All operations were carried out at 4°C.

The cell debris, nuclei and mitochondria were removed by centrifugation for 12 minutes at 10,000 g. The supernatant was centrifuged further at 100,000 g for 75 minutes. The sedimented microsomes were transferred with an additional 50 ml of sucrose buffer into a glass homogenizer and homogenized. The suspension was transferred into a separatory funnel and added dropwise into 1,000 ml cold (-40°C) acetone, with constant, strong magnetic stirring. Stirring was continued until the temperature of the mixture rose to -23°C. The suspension was then filtered through a Büchner funnel connected to a water pump and washed with 3 portions (50 ml each) of cold (-20°C) butanol. After exhaustive washing with 1,000 ml diethyl ether at -20°C, the powder was

collected, dried in a dessiccator under vacuum for 1/2 to 1 hour and stored at -20°C.

Incubations: Incubations of [3,5-^{14}C]-oxyphenbutazone were conducted in a 1 ml chamber (37°) fitted with a Clark oxygen electrode and accomplished as follows: to 1 ml of 0.1 M Tris-HCl or acetate buffer containing 4 mg (when not specified otherwise) of acetone powder, or 20 µg of soybean lipoxygenase, there was added a sufficient quantity of oxyphenbutazone to make a final concentration of 10^{-3}M. In some experiments, unlabeled substrate (20 µg arachidonate of 100 µg linoleate, respectively) was added after 3 minutes to the mixture containing radioactive oxyphenbutazone and incubation continued for an additional 3 minutes. The incubation was terminated by extracting with 10 ml of ethyl acetate.

Experiments were also carried out with labeled arachidonate. These incubations were performed as described above but in the presence of unlabeled oxyphenbutazone or 4-hydroxyoxyphenbutazone and the termination was accomplished by adding 7 ml of $CHCl_3$-CH_3OH (1:1, v/v).

Extraction and Thin Layer Chromatography: If the incubation contained labeled oxyphenbutazone, the extracted material was applied to silica gel plates buffered to pH 6 with McIlvaine buffer (8) and run in $CHCl_3$-HOAc (8:2, v/v). After detection with a Berthold Dünnschicht II scanner, the radioactive zones were scraped from the plates and counted in a Packard Tri-carb model 3375 liquid scintillation spectrometer. If labeled arachidonate was utilized, the extraction was continued by removing the precipitated protein and adding 3 ml $CHCl_3$ and 2 ml 1% HCOOH. The chloroform phase was applied to silica gel plates and run in benzene-dioxane-acetic acid (80:20:2, v/v/v) to separate uncoverted arachidonate from products.

Synthesis of 4-Hydroxyoxyphenbutazone: A mixture of oxyphenbutazone $\cdot H_2O$ (0.342 g, 1 mmole), 30% H_2O_2 (0.7 ml), 1 N NaOH (0.1 ml) and CH_3OH (3.5 ml) was allowed to stand at 22°C for 13 hours. After that period the reaction mixture was poured into 5% HCl (20 ml) and extracted with ethyl acetate, (2x20 ml). The ethyl acetate phase was separated, dried over Na_3CO_3, and the solvent removed in vacuo without heating. The residue was subjected to gradient elution column chromatography (SilicAR CC-4, 34 g) starting with $CHCL_3$ and terminating with $CHCl_3$-HOAc (96:4). The product (94 mg) was eluted using a solvent which varied in composition from 99:1 to 98:2. The material was recrystallized from ethyl acetate. Its melting point was 177.5-179.5°C and it had an R_f = 0.47 on silica gel plates, run in $CHCl_3$-HOAc (8:2,v/v). The elemental analysis showed following values: C, 66.8; H, 5.9; N, 8.3; which well agreed with the formula $C_{19}H_{20}N_2O_4$ and its calculated values: C, 67.1; H, 5.9; N, 8.3.

Mass spectrum was taken using a LKB 9000 instrument, the electron energy was maintained at 70 eV and the trap current was 60 μA.

The spectrum m/e (% intensity): 41(30); 51(18); 52(32); 55(11); 57(94); 65(15); 77(40); 79(19); 85(100); 92(21); 93(55); 107(25); 119(31); 120(36); 135(72); 199(16); 205(48); 340(6) showed a molecular ion at m/e 340, which is 16 units higher than the molecular ion from oxyphenbutazone (9). Ions at m/e 57 containing the butyl group and ions at m/e 119, 135, 199 with the aromatic rings were identical to those obtained from oxyphenbutazone. This indicates that the newly introduced OH group cannot be attached to the butyl group or located in either of the aromatic rings.

The synthesized compound was also converted into the trimethyl-

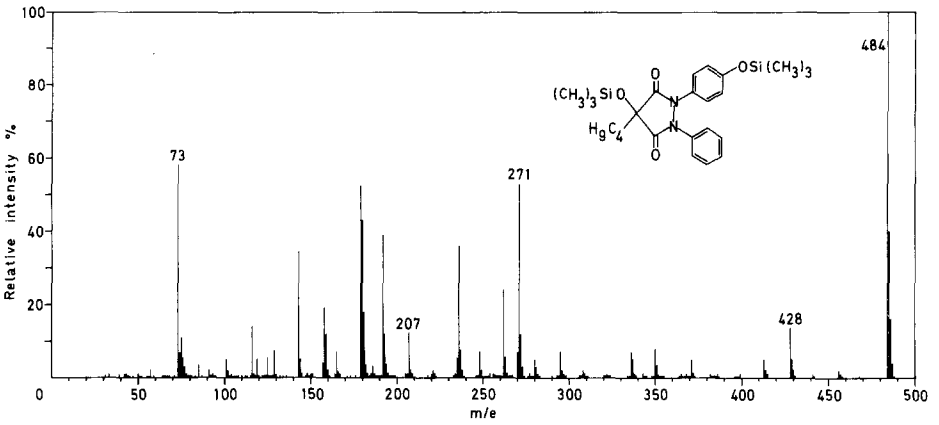


Fig. 1. The mass spectrum of 4-hydroxyoxyphenbutazone as trimethylsilyl ether.

silyl ether and analyzed by the mass spectrometer combined with a gas chromatograph equipped with a 1 % SE-30 column. The retention time of the compound was converted into the C-value, 23.6 using methyl esters of normal fatty acids as standards (10). The mass spectrum (Fig. 1) was obtained with the electron energy at 22.5 eV and the trap current 60 μA. It had a molecular ion at m/e 484, which is 2x72=144 units higher then 340, due to the presence of two trimethylsilyl groups. The m/e values of ions containing aromatic rings were shifted 72 units upward, compared with the spectrum of oxyphenbutazone. The fragmentation pattern indicates that the introduced OH groups is located in the 4 position. This would be a particularly susceptible site for oxidation because the free radical intermediate would be stabilized by both carbonyl groups.

Identification of [3,5-^{14}C] 4-Hydroxyoxyphenbutazone in Incubation Mixtures: After separation on TLC, the radioactive zone at R_f=0.47 was scraped from the plate and eluted with ethyl acetate. An aliquot was converted into the trimethylsilyl ether and analyzed by gas chromatography using a Barber Colman model

5000 instrument with simultaneous registration of mass and radioactivity. The mass spectrum was obtained as described for the trimethylsilyl derivative of the synthesized compound.

RESULTS AND DISCUSSION: When oxyphenbutazone was incubated at pH 4 or 5 with 25 mg acetone powder prepared from sheep vesicular glands, there was full uptake (185nmoles)of oxygen in the chamber within five minutes. No oxygen consumption occurred at pH 6 or above. In the absence of enzyme or with boiled enzyme there was no oxygen uptake. The oxygen uptake was not inhibited significantly by aspirin or indomethacin, nor were these agents capable of effecting oxygen uptake on their own at pH 4 or 5.

Because of the uncertainty as to which component in the acetone powder was responsible for oxygen uptake, similar experiments were carried out with soybean lipoxygenase since it bears a resemblance (5) to the dioxygenase component enzyme in prostaglandin synthetase. It was found that at pH 5 oxygen uptake was induced by oxyphenbutazone, while at pH 9 this did not occur.

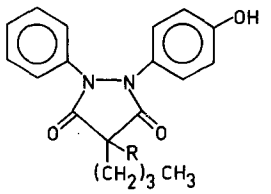
As it was of interest to determine whether oxygen consumption was related to oxidation of oxyphenbutazone or merely to a triggering of oxygen uptake by the enzyme, experiments were carried out with [3,5-^{14}C]-oxyphenbutazone. During the course of these experiments it was found that this compound is succeptible to air oxidation after application to TLC plates, but this could be inhibited by utilizing silica gel G buffered to pH 6. After three minutes incubation with the acetone powder at pH 5, 15 % of [3,5-^{14}C]-oxyphenbutazone was converted to a single product, whose R_f=0.47 is identical to that produced by air oxidation on unbuffered silica gel G. Incubation of [3,5-^{14}C]-oxyphenbutazone at pH 6 under otherwise identical conditions resulted only in a 3% conversion. These results therefore indicate that oxygen up-

take at pH 5 is related to enzymatic oxidation of oxyphenbutazone. Similarly, the same oxidation product (13%) was produced at pH 5 when the drug was incubated with lipoxygenase.

Of particular significance was the finding that substantial conversion (15%) of [3,5-^{14}C]-oxyphenbutazone to the same oxidation product also occurred at pH 8 in the presence of the acetone powder or lipoxygenase when arachidonate or linoleate,respectively, were added to the incubation mixture. Since it is possible that co-oxidation of this type could take place in vivo as well, it was of interest to investigate the structure and properties of the oxidation product.

Its identity was determined by converting both the oxidation products from the incubation mixtures and the synthetically prepared 4-hydroxyoxyphenbutazone into trimethylsilyl ethers. The derivatives were subjected to gas chromatography- mass spectrometry which showed identical C-values and mass spectra. 4-Hydroxyoxyphenbutazone incubated with acetone powder at pH 5 did not promote oxygen uptake and this fact also suggests that the rest of the molecule is resistent to oxidation under these conditions.

Oxyphenbutazone is an inhibitor of prostaglandin biosynthesis (11, 12). When arachidonate was incubated at pH 8 with the acetone powder in the presence of oxyphenbutazone, there was an in-



R=H, Oxyphenbutazone
R=OH, 4-Hydroxyoxyphenbutazone
R=OOH, 4-Hydroperoxyoxyphenbutazone

Fig. 2. The structure of oxyphenbutazone and its oxygenated derivatives.

hibition of the oxygen uptake. When oxyphenbutazone was replaced by 4-hydroxyoxyphenbutazone, the oxygen uptake was enhanced. The transformation of [1-^{14}C]-arachidonate by the acetone powder was also inhibited by oxyphenbutazone but not by 4-hydroxyoxyphenbutazone. The oxygenations observed seem to be of importance for future work on the mechanisms of action of prostaglandin synthetase and its inhibitiors.

ACKNOWLEDGEMENT: One of the authors (P.S.P.) wishes to thank the American Foundation for Pharmaceutical Education for support through a Gustavus A. Pfeiffer Memorial Research Fellowship. This work was supported by a grant from the Swedish Medical Research Council (03X-217).

1. Samuelsson, B. (1972) Fed. Proc. 31, 1442-1450.

2. Marnett, L.J., Wlodawer, P., and Samuelsson, B. (1974) Biochem. Biophys. Res. Commun. 60, 1286-1294.

3. "The Pharmacological Basis of Therapeutics", fourth ed., Goodman, L.S., and Gilman, A., editors. The Macmillan Co., N.Y., 1970, p. 337.

4. Ferreira, S.H., and Vane, J.R. (1974) Ann. Rev. Pharmacol. 14, 57-73.

5. Flower, R.J. (1974) Pharmacol. Rev. 26, 33-67.

6. Hamberg, M., and Samuelsson, B. (1967) J. Biol. Chem. 242, 5329-5335.

7. Stoffel, W. (1964) Am. Chem. 673, 26-36.

8. Awang, D.V.C., Vincent, A., and Matui, F (1973) J. Pharm. Sci. 62, 1673-1676.

9. Unterhalt, B. (1972) Arch. Pharmaz. 305, 334-337.

10, Bergström, S., Ryhage, R., Samuelsson, B., and Sjövall, J. (1963) J. Biol. Chem. 238, 3555-3564.

11. Lands, W.E., LeTellier, P.R., Rome, L.H. and Vanderhock, J. (1972) Fed. Proc. 31, 476 A.

12. Ku, E.C., and Wasvary, J.M. (1973) Fed. Proc. 32, 3302.