INHIBITION OF TUMOR GLYCOLYSIS BY HYDROGEN PEROXIDE FORMED FROM AUTOXIDATION OF UNSATURATED FATTY ACIDS. [1]

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UV-irradiation of methyl linoleate and of methyl linolenate yields a water-soluble substance(s) which inhibits endogenous oxygen consumption, aerobic and anaerobic respiration of Ehrlich ascites carcinoma (Shuster, 1955). Inhibition has been correlated with thiobarbituric acid (TBA)-reactive material in the extract. However, malonaldehyde, the probable TBA-reactive material, is relatively non-inhibitory (Baker and Wilson, 1961).

The primary reactions induced by UV-irradiation in Shuster's experiments probably involve interaction between activated oxygen and unsaturated fatty acids, since methyl linoleate and linolenate are known not to absorb appreciable energy from UV irradiation. After passing previously activated oxygen (ozone) through solutions of mono-, di-, tri-, and tetra-unsaturated fatty acids dissolved in cold CCl_4, an inhibitor of anaerobic tumor glycolysis was found in the water extract of each oxidized lipid. In the case of those unsaturated fatty acids containing two or more double bonds, TBA-reactive material was also formed; however, no TBA-reactive substance was formed when oleic acid was oxidized in this system. Clearly, an inhibitor of glycolysis having no TBA-reactivity can be formed by oxidation of an unsaturated fatty acid. The present com-

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munication establishes that the sole water-soluble inhibitory product of the brief reaction at -15° between activated oxygen and any unsaturated fatty acid is hydrogen peroxide.

EXPERIMENTAL

Aqueous extracts of oxidized unsaturated fatty acids were prepared as follows: Oxygen was flushed through an ozone generator (Bonner, 1953) for 2 min. (1.40 l./min.), bubbled through concentrated H_2SO_4 and then through a solution of 100 mg unsaturated fatty acid (Hormel) in 10 ml CCl_4 at approximately -15°. Unreacted oxygen was removed by flushing with N_2 for 2 min. The CCl_4 solution was shaken with 3 volumes of water for 20 min. at room temperature and the aqueous extract was washed twice by shaking for 20 min. with 0.7 volumes petroleum ether, placed in a boiling H_2O bath for 5 min. and the volume of aqueous extract reduced to about a tenth by flash evaporation.

The effect of this extract on anaerobic glycolysis was evaluated as follows: 3.0×10^6 Ehrlich ascites tumor cells (7-14 days growth, washed twice with cold Krebs-Henseleit bicarbonate buffer to remove blood) were incubated in 0.60 ml volume of Krebs-Henseleit buffer, pH 7.35 for 15 min. at 37° in the presence of varying concentrations of the aqueous extract of oxidized unsaturated fatty acids. Glucose was then added (0.60×10^{-3} M, final concentration) in 0.10 ml buffer. Tubes were then shaken in a water bath for 2 hours (gas phase, 95% N_2; 5% CO_2). Incubations were carried out in duplicate.

After stopping enzymatic activity by addition of ethanol (70% final concentration) glucose disappearance was measured using a modified glucose oxidase-peroxidase method[2].

Aliquots of the aqueous extract of oxidized fatty acids were assayed for malonaldehyde using thiobarbituric acid (Wolfson et al., 1956) and

[2] Glucostat reagent (Worthington Biochemical Corp., Freehold, N.J.).

for H_2O_2-like material using an enzymatic method which employs peroxidase and dye of a commercial preparation[2]. The presence of H_2O_2 in an extract was determined by exposing an aliquot to crystalline beef liver catalase (Worthington) in Krebs-Henseleit bicarbonate buffer (pH 7.3) and measuring the disappearance of H_2O_2-like material by means of the above peroxidase assay. H_2O_2 [3] was used as a reference standard.

RESULTS AND DISCUSSION

The water extract of oxidized oleic acid which had no detectable malonaldehyde contained H_2O_2-like material. The extract inhibited anaerobic glycolysis of the tumor cells to the same extent as H_2O_2 [3] when added in an equivalent amount based upon a peroxidase enzyme assay for H_2O_2 (Fig. 1A). The peroxidase-reactive material was removed by catalase at the same rate as H_2O_2 in Krebs-Henseleit bicarbonate buffer pH 7.3 (manuscript in preparation). After removal of 95% of the peroxidase-reactive material from the extract by prior treatment with catalase, the extract's capacity to inhibit glycolysis was lost (Fig. 1B). H_2O_2 addition in a concentration equivalent to that removed by catalase restored the original capacity of the water extract to inhibit anaerobic glycolysis (Fig. 1C). No inhibitor of glycolysis was formed when oxygen which had not been activated in the ozone generator was passed through the fatty acid solution.

The water extracts of other oxidized unsaturated fatty acids (linoleic, linolenic and arachidonic) also were shown to contain H_2O_2-like material. Furthermore they inhibited anaerobic glycolysis of tumor cells and to the same degree as an equivalent concentration of H_2O_2. Each of the extracts also had in common with H_2O_2 the following properties: (1) Prior exposure of cells to the extract in the absence of glucose was necessary in order for inhibition to be observed; (2) H_2O_2-like material withstood heating at 100°C for 5 min. and was not lost during flash

[3] Baker and Adamson, C. P. grade, no added stabilizer indicated.

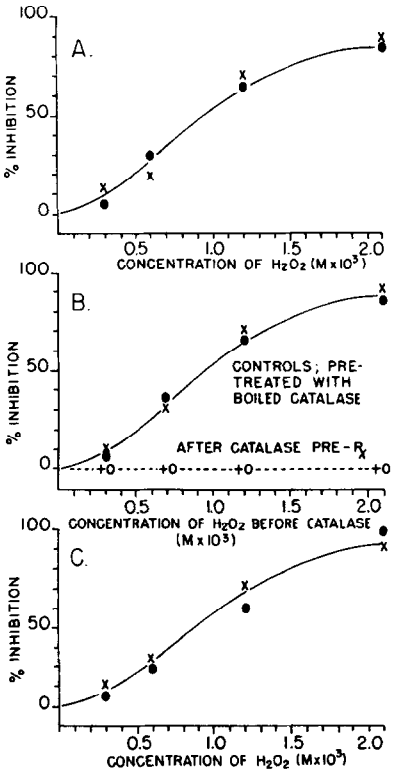

Fig. 1 (A) Equivalent inhibition of anaerobic glycolysis of washed Ehrlich ascites carcinoma cells by the water extract of oxidized H_2O_2 (•) and by oleic acid (x). Concentration of the latter is expressed in equivalent molarity based upon an enzymatic assay using peroxidase.

(B) Complete loss of inhibitory properties of H_2O_2 (o) and of water extract (same as A above) of oxidized oleic acid (+) after prior treatment of the water extracts with catalase. In each case at the end of the catalase treatment over 95% of the peroxidase-reactive material had been removed. The control curves were obtained using catalase which had been boiled before adding either H_2O_2 (•) or the water extract of oxidized oleic acid (x).

(C) Restoration of inhibitory properties of catalase-treated water extract (same as used in A and B above) of oxidized oleic acid by addition of H_2O_2 equivalent to that removed by the catalase treatment (•). Control (x) is oxidized oleic acid plus boiled catalase (cf. also (+) in B above).

evaporation to near dryness. (Virtually all inhibitory action was lost in lyophilization to dryness).

We conclude that under these conditions of autoxidation of unsaturated fatty acids H_2O_2 was the only significant water-soluble inhibitor of anaerobic glycolysis which formed. These observations together with those, for example, of Hochstein and Cohen (1961), Engel and Adler (1961), Pantlitschko and Seelich (1960), Schauenstein et al. (1961, 1962), Weitzel et al. (1961) and Strangeways (1938) indicate that H_2O_2 may form under varying conditions of autoxidation and, in biological systems, would be expected to influence metabolism and structure by virtue of its capacity to oxidize sulfhydryl groups.

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