Short Communications

The Decarboxylation of Retinoic Acid by Horseradish Peroxidase and an Acetone-Butanol-Ether-Dried Liver Powder

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The search for metabolites of vitamin A has resulted in the localization of a system in the microsomal fraction that is capable of decarboxylating retinoic acid (Roberts & DeLuca, 1968; Lin, 1969). On the basis of the reported cofactors, inhibitors and other parameters of this reaction, there are a number of oxidative pathways present in liver microsomal fraction that could decarboxylate retinoic acid. A microsomal electron-transport system is responsible for the hydroxylation of many drugs, steroids and phenols. Tam & McCay (1970) have further defined an NADPH oxidase-catalysed oxidation of the polyunsaturated fatty acids present in the microsomal membrane. Wills (1969) has proposed that the electron-transport system normally concerned with hydroxylation might be switched to oxidize unsaturated lipids and that the two processes of hydroxylation and lipid peroxidation may be closely linked in microsomal fraction. Phytol, a C_{20} branched-chain saturated diterpene with a β -methyl group, is degraded after conversion into its carboxylic acid, phytanic acid (Steinberg *et al.* 1967). With the methyl group at the β -position (as in retinol and related compounds), normal degradation of phytanic acid by β -oxidation is blocked. However, Mize *et al.* (1969) concluded that mammals are capable of α -oxidation of phytanic acid with α -hydroxyphytanic acid as an intermediate and the resulting formation of CO_2 and pristanic acid. Pristanic acid can be further degraded by successive β -oxidations. The decarboxylation of α -hydroxy fatty acids by brain microsomal fraction has been described by MacDonald & Mead (1968).

Lin (1969) observed that a partially purified enzyme from liver microsomal fraction required only O_2 and Fe^{2+} (1 mM-ferrous chloride) to achieve nearly 50% decarboxylation of retinoic acid. Other biological reducing agents such as NADPH, L-cysteine and ascorbic acid also enhanced the enzymic activity, but a combination of these reducing agents with Fe^{2+} did not increase the

enzymic activity. Roberts & DeLuca (1968) reported that liver and kidney microsomal fractions required NADPH in addition to O_2 , Fe^{2+} and PP_i for maximum decarboxylation. The mechanism of these decarboxylations has not been elucidated, but the properties described are similar to peroxidation-type reactions, as has been noted (Roberts & DeLuca, 1968; MacDonald & Mead, 1968; Lin, 1969). In the present communication we describe the decarboxylation of retinoic acid by horseradish peroxidase and an acetone-butanol-diethyl ether-dried powder obtained from chick liver.

Methods. Crystalline horseradish peroxidase (type VI, RZ-3.1; Sigma Chemical Co., St Louis, Mo., U.S.A.) was suspended in 0.1 M-sodium phosphate buffer, pH 6.4. Retinoic acid was recrystallized from methanol and checked for purity by t.l.c. and mass spectrometry (Lin, Waller, Mitchell, Yang & Nelson, 1970). Unless stated otherwise, incubation mixtures were composed of horseradish peroxidase (2.6 μM), [$^{15-14}C$]retinoic acid (10 μM ; approx. 10^4 c.p.m., assayed for each set of incubations) and ferrous chloride (1 mM), diluted to a final volume of 2 ml with 0.1 M-sodium phosphate buffer, pH 6.4. Retinoic acid and ferrous chloride were added separately at zero time and the mixture was incubated at 37°C for the desired time. At the end of the incubation period, the reaction mixture was acidified with 2 M-hydrochloric acid, and $^{14}CO_2$ was collected in a glass vial containing a filter-paper wick and 0.3 ml of ethanolamine-ethylene glycol monomethyl ether (1:2, v/v). At 2 h after the acid was added, the trapping solution was transferred to a counting vial, 10 ml of scintillation fluid [2-methoxyethanol-toluene (1:2, v/v) containing 2,5-diphenyloxazole (5.5 g/l)] was added and the radioactivity was counted on a liquid-scintillation counter (Packard Tri-Carb model 3320; Packard Instrument Co., Downers Grove, Ill., U.S.A.). Other parameters measured were the effects of Fe^{2+} and Fe^{3+} , horseradish peroxidase and ascor-

bate concentrations, heat, other haematin compounds, inhibitors, O_2 and pH.

Acetone–butanol–ether-dried powder was prepared by homogenizing 280 g of fresh chick liver in 10 vol. of acetone (all solvents were kept at $-15^\circ C$) in a large Waring blender for 1 min and partially drying by filtration over a Buchner funnel. The precipitate was rehomogenized in 10 vol. of butan-1-ol, filtered and rehomogenized twice with 3 vol. of acetone and finally with 3 vol. of diethyl ether. The powder was rapidly air-dried and stored at $-20^\circ C$ over a desiccant. The liver powder was resuspended (1:100, w/v) in 0.1 M-sodium phosphate buffer, pH 6.4, just before use.

Results and discussion. Retinoic acid was decarboxylated to the extent of 48% by horseradish peroxidase and 40% by acetone–butanol–ether-dried liver powder when each was assayed in the presence of 3 mM- Fe^{2+} and phosphate buffer. Under these conditions the minimum peroxidase concentration that gave maximum decarboxylation in 1 h was 2.6 μM . Since maximum decarboxylation was desired, substrate was not in excess and the reaction was linear with time and proportional to peroxidase concentration only during the first few minutes of the assay. Blank reaction mixtures containing neither peroxidase nor Fe^{2+} resulted in 0.5–1.5% of the radioactivity being collected as $^{14}CO_2$. When the reaction mixture lacked Fe^{2+} but contained peroxidase, the $^{14}CO_2$ collected ranged from 0.6 to 3.5%. When peroxidase was omitted but Fe^{2+} was present, 0.9–3% was collected as $^{14}CO_2$. These represent very low amounts when one considers that in most sets of assays the tubes containing both peroxidase and Fe^{2+} resulted in 35–48% decarboxylation.

The ferrous chloride concentration was varied from 0.2 μM to 2 mM. Maximum decarboxylation (48.4%) occurred at 1 mM and remained near 90% of maximum down to 10 μM , but decreased to 6% at 1 μM and 8–13% at 2 mM. Fe^{3+} (1 mM) gave approx. 50% of the activity of Fe^{2+} . Fe^{3+} in combination with Fe^{2+} at concentrations varying from 1 mM to 1 μM each always resulted in less activity than that with 1 mM- Fe^{2+} alone. The addition of more Fe^{2+} after the incubation had proceeded for some time did not result in an increased decarboxylation. The reaction was completely inhibited (4% decarboxylation) at 3 mM-EDTA. Ascorbate (1 mM) completely inhibited $^{14}CO_2$ formation in the presence or absence of Fe^{2+} , but 10% decarboxylation occurred when 1 μM -ascorbate was used. The present reaction, then, is probably not the non-enzymic lipid peroxidation reaction described by Barber (1966) in which ascorbate and Fe^{2+} are the normal catalysts.

The formation of $^{14}CO_2$ was completely inhibited by flushing the system with N_2 or by heating

horseradish peroxidase suspended in phosphate buffer in a boiling-water bath for 2 h. Flushing the boiled peroxidase with air or O_2 did not restore decarboxylase activity. Activity was completely lost by lowering the pH to 1 and immediately restoring it to 6.4. Optimum pH was 6.4 in phosphate buffer (0.1 M) and no decarboxylation occurred when non-phosphate buffers were used.

Maximum decarboxylation (40%) was obtained with 0.1 ml (1 mg) of the resuspended liver powder, 1 mM-ferrous chloride, 10 μM -[15- ^{14}C]retinoic acid and 0.1 M-phosphate buffer, pH 6.4, in a final volume of 2 ml. Decarboxylation was proportional to protein concentration up to 1.25 mg/ml. As with the intact microsomal fraction, 1 mM-NADPH and 1 mM-ascorbate were each effective as cofactor and were nearly equivalent to 1 mM- Fe^{2+} . Activities of the liver powder in the presence of 1 mM-NADPH, 1 mM-ascorbate or 1 mM- Fe^{2+} usually ranged from 20 to 30% decarboxylation. Heating of the resuspended liver powder in a boiling-water bath completely inhibited both the NADPH- and the ascorbate-catalysed reactions, but only lowered the Fe^{2+} -catalysed decarboxylation to 75% of the value for the unheated control. In addition, the liver powder catalysed the decarboxylation of retinoic acid in the absence of added Fe^{2+} to the extent of 10–15%. The Fe^{2+} -catalysed degradation of retinoic acid by heated liver powder has some similarity to the haematin-catalysed lipid peroxidation reviewed by Tappel (1962) and the observation by Eriksson, Olsson & Svensson (1970) that after being heated for 1–2 min the haemoproteins catalase and horseradish peroxidase became more efficient haem catalysts of fatty acid oxidation.

It is of additional interest that in both the peroxidase- and the liver-powder-catalysed reactions decarboxylation never exceeded 50%. This observation is in agreement with the earlier reports by Roberts & DeLuca (1968) and Lin (1969). An explanation for this and some insight into the mechanism may be forthcoming with the isolation and identification of the intermediates and products of both the peroxidase- and the liver-powder-catalysed decarboxylations.

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