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The Enzymic and Non-Enzymic Degradation of Colneleic Acid, an Unsaturated Fatty Acid Ether Intermediate in the Lipoxygenase Pathway of Linoleic Acid Oxidation in Potato (*Solanum tuberosum*) Tubers

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Colneleic acid is an unsaturated ether fatty acid derived from linoleic acid via a lipoxygenase-mediated enzyme pathway. It is degraded (a) by an enzyme in potato tubers which is heat-labile and non-dialysable and (b) by a model system containing catalytic amounts of Fe^{2+} ions. Both enzyme- and Fe^{2+}-catalysed systems have similar properties with respect to pH optima (pH 5.0–5.5), oxygen requirement (0.6–0.7 mol of O_2 consumed/mol of ether degraded), inhibitors and reaction products. An unstable product breaks down to C_8 and C_9 carbonyl fragments. Both systems are inhibited by low concentrations of antioxidants (e.g. 5μM-butylated hydroxytoluene) and some chelating agents (e.g. 5μM-diethyldithiocarbamate). The model system is strongly inhibited by metal ions, particularly Cu^{2+} and Fe^{3+}, at 20μM. Hydrogen peroxide and haemoproteins do not substitute for the enzyme or Fe^{2+} ions but the non-haem iron protein, ferredoxin, does catalyse the degradation.

Disruption of many plant tissues results in rapid autolytic degradation processes. Previous work from this laboratory has established a sequence of enzyme reactions in extracts of potato tuber, catalysing the breakdown of the predominant classes of endogenous lipids (phospholipids and galactosyl diglycerides).

The sequence so far established (see Scheme 1) involves: (*a*) a lipolytic acyl hydrolase, which releases free fatty acids from membrane-bound lipids (Galliard, 1970, 1971*a*,*b*); (*b*) lipoxygenase (EC 1.13.11.12), which converts the major fatty acids (linoleic acid and linolenic acid) into their 9-D-hydroperoxy derivatives (Galliard & Phillips, 1971); (*c*) the enzymic conversion of these hydroperoxides into novel butadienyl vinyl ether derivatives (containing the structure –CH=CH–CH=CH–O–CH=CH–) by a process involving insertion of an atom of oxygen into the fatty acid carbon chain (Galliard & Phillips, 1972; Galliard *et al.*, 1973).

The present paper describes two systems that catalyse the breakdown of the butadienyl vinyl ether derivatives. One is enzymic and is catalysed by an enzyme fraction isolated from potato tubers; the other is non-enzymic and is catalysed by Fe^{2+} ions.

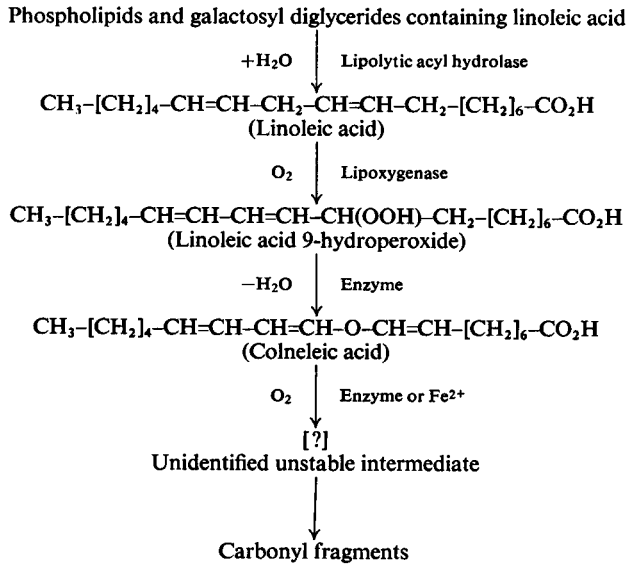
Systematic nomenclature of the polyunsaturated fatty acid ether derivatives is complex and does not readily relate the structures to their biochemical origin in fatty acids. Trivial nomenclature has been introduced as follows (see Galliard *et al.*, 1973): colneleic acid (derived from linoleic acid) is 9-(nona-*trans*-1′,*cis*-3′-dienyloxy)-*trans*-8-nonenoic acid; colnelenic acid (derived from linolenic acid) is 9-(nona-*trans*-1′,*cis*-3′,*cis*-6′-trienyloxy)-*trans*-8-nonenoic acid.

Materials and Methods

Materials

Potatoes (*Solanum tuberosum*) of 'Desiree' and 'Majestic' varieties were grown locally and mature tubers were stored at 5°C until required. Horseradish peroxidase, EC 1.11.1.7 [300 purpurogallin (20-s) units/mg], catalase, EC 1.11.1.6 (20000 Sigma units/mg) and linoleic acid (grade III, approx. 99% pure) were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., and glucose oxidase, EC 1.1.3.4 (grade 1, 210 units/mg), was obtained from Boehringer Corporation (London) Ltd., London W5 2TZ, U.K. [1-^{14}C]Linoleic acid (58mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Ferredoxin (ferric form), prepared from parsley leaves, was a gift from Dr. H. E. Davenport of this Institute.

Colneleic acid was isolated from potato tuber extracts as described previously (Galliard & Phillips, 1972). The method was slightly modified (Galliard *et al.*, 1973) in that linoleic acid (10mg/100g of tissue) was added to homogenates, citric acid (0.1M) replaced acetic acid in the biphasic extraction system and silicic acid for column chromatography was pretreated with EDTA. [1-^{14}C]Colneleic acid was prepared essentially in the same way as the unlabelled compound except that [1-^{14}C]linoleic acid (40μCi) was added to a small-scale homogenate preparation (10g fresh wt. of tuber in 15ml of the buffer). The [^{14}C]colneleic acid isolated by silicic acid column chromatography was checked for radiopurity by radioscanning of thin-layer chromatograms (Galliard & Phillips, 1972).



Scheme 1. *Sequential reactions catalysing the enzymic degradation of lipid-bound linoleic acid in extracts from potato tuber*

Enzyme preparation

Tubers of the 'Desiree' variety are used in this laboratory for preparation of most enzymes and subcellular fractions from potato because of their relatively low content of the lipolytic acyl hydrolase, which causes hydrolysis of membrane lipids (Galliard & Matthew, 1973). Diced tuber tissue (54g) was homogenized at 0°C in 100ml of 0.05M-Tris–HCl, pH8.0, containing 0.2mM-mercaptobenzothiazole, in a domestic juice extractor (Moulinex Type CFIA) lined with Miracloth (Evans Adlard and Co., Cheltenham, U.K.). The time taken to dice the tissue is not critical with this homogenization medium. The homogenate was centrifuged at 15000$g_{av.}$ for 30min. A portion (2ml) of the supernatant was loaded on a column (10cm×1cm internal diam.) of Sephadex G-25, previously equilibrated with 0.05M-Tris–HCl, pH7.5. The column was eluted with the same buffer and 10ml fractions were collected. The bulk of the protein and all the colneleic acid-degrading enzyme activity were recovered in the third 10ml fraction and as a routine this was used as the source of the enzyme.

Substrate preparation

From stock solutions in benzene, portions containing 1.5μmol of colneleic acid and 0.2mg of Triton X-100 were combined. The solvent was evaporated with a stream of N_2 and 2 drops of 2M-NH_3 and 2ml of water were added. Excess of NH_3 was removed under a stream of N_2 at 40°C until a pH of approx. 8 was obtained, the volume was made up to 2ml with water and this substrate preparation was used immediately. For routine enzyme assays, 0.2ml of this solution (containing 0.15μmol of substrate) was added to a 0.5ml incubation system.

Assays of colneleic acid degradation

The routine assay, as used for experiments described in this paper, unless otherwise stated, takes advantage of the u.v. absorption of colneleic acid ($\lambda_{max.} = 250$nm; $\epsilon_{250} = 2.2\times10^4$; Galliard *et al.*, 1973). Duplicate incubation mixtures (total vol. 0.5ml) contained 0.3mM-colneleic acid, 0.1M-sodium acetate buffer (pH5.0), either enzyme or other catalyst and further additions as indicated in the text. The mixtures were prepared at 0°C and incubated by shaking aerobically at 25°C. Reactions were terminated (as a routine after 10min) by the addition of ethanol (4ml) and water (0.5ml). The mixtures were centrifuged to remove any turbidity and u.v. absorption at 250nm was determined spectrophotometrically. Enzymic reactions gave approximately linear progress curves with time up to 10min of incubation, although Fe^{2+}-catalysed reactions were non-linear with time (see Fig. 3).

The extent of degradation of colneleic acid was determined by the fall in E_{250} compared with duplicate samples containing an equivalent amount of substrate to that used in the incubations, but taken directly from the stock solution into 80% (v/v) ethanol. The extent of non-enzymic breakdown of

substrate was obtained from duplicate control incubation mixtures containing no added catalyst. All experimental results presented in this paper (with the exception of Table 5) are corrected for breakdown in the absence of added catalyst.

A second assay used an oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) and air-saturated incubation mixtures (total volume 2ml) were stirred at 25°C under the electrode. Oxygen uptake was recorded continuously and a dissolved $[O_2]$ of 20.4mmol·l^{-1} was assumed at air saturation. In some experiments 1ml of the incubation mixture was rapidly removed from the electrode cell into ethanol (4ml) for u.v. spectrophotometric determination of colneleic acid.

A third assay involved radioscanning of thin-layer chromatograms of reaction products. Chloroform-soluble incubation products were applied to 0.25mm layers of silica gel G; t.l.c. plates were developed in light petroleum (b.p. 60–80°C)–diethyl ether–acetic acid (60:40:1, by vol.). Developed plates were scanned with a Radioscanner (Panax Equipment Ltd., Redhill, Surrey, U.K.) and areas under the peaks were determined. I_2 vapour was used to detect lipid spots on chromatograms.

Analytical methods

Quantitative determination of the iron content of ferredoxin and peroxidase was done by the method of Doeg & Ziegler (1962). Protein was determined by the biuret method (Gornall *et al.*, 1949). ^{14}C-labelled oxo acids were tentatively identified by radio-g.l.c. of the methyl ester derivatives (Galliard & Phillips, 1972).

Results and Discussion

Degradation products from colneleic acid

In preliminary experiments [1-^{14}C]colneleic acid was incubated with aqueous extracts of potato tubers and reaction products were analysed by t.l.c. Fig. 1 shows a typical radiochromatogram of the ^{14}C-labelled products. Almost all the added radioactivity was retained in the chloroform-soluble products, the major reaction product (peak A) being much more polar than colneleic acid (peak C). This product (peak A), which is as yet unidentified, is unstable. Attempts to isolate the material (see Fig. 1*d*) have resulted in further breakdown to ^{14}C-labelled fragments (peak B) identified as a mixture containing mainly 9-oxononanoic acid with a smaller amount of 8-oxo-octanoic acid. Use of U-^{14}C-labelled colneleic acid as substrate resulted in an additional ^{14}C-labelled product (peak D, Fig. 1*e*). Degradation of colneleic acid is accompanied by a complete loss of the u.v. chromophore ($\lambda_{max.}$ 250nm) and this has provided a simple assay procedure.

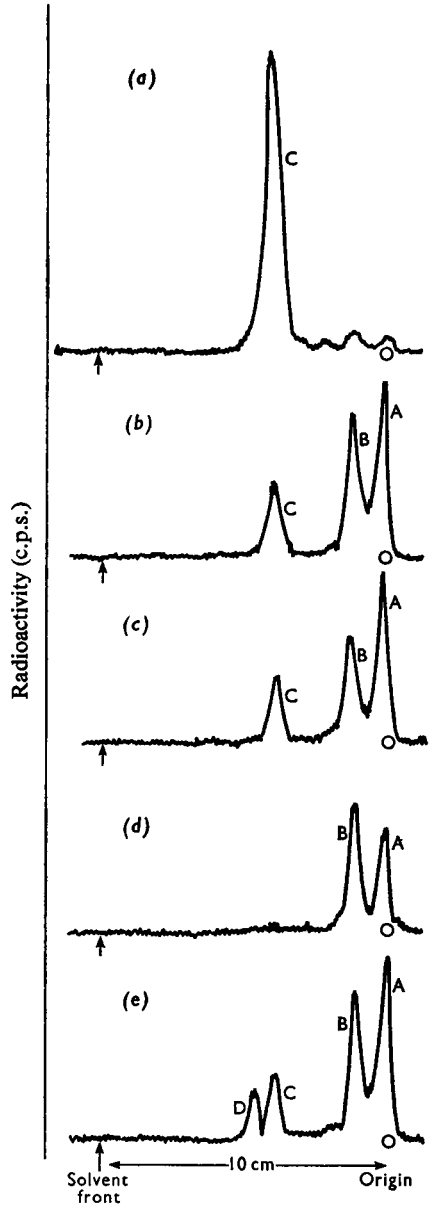


Fig. 1. *Radioactive traces of t.l.c. separations of reaction products formed during the degradation of ^{14}C-labelled colneleic acid*

[1-^{14}C]Colneleic acid (10^6 d.p.m.; 0.3mM) was added to 0.1M-sodium acetate buffer, pH5.0, and incubated for 10min at 25°C in a total volume of 0.5ml with additions as follows: (*a*) no additions (substrate control); (*b*) $FeSO_4$ (20μM); (*c*) 15000*g* supernatant from potato tuber (20μg of protein). Trace (*d*) shows the result of re-chromatography of material in peak A of trace (*c*). Trace (*e*) represents the products obtained with the 15000*g* supernatant enzyme when [U-^{14}C]colneleic acid was used as substrate. See the text for identification of peaks A–D.

Isolation of enzyme activity

Differential centrifugation of aqueous homogenates of potato tuber showed that most of the activity was recovered in the 100000 *g*/60min supernatant fraction (Table 1). As a routine a 15000 *g*/30min supernatant was passed through a small column of Sephadex G-25 to obtain an active protein fraction for use as source of the enzyme in these studies. Partial activity was recovered in precipitates obtained by acetone or $(NH_4)_2SO_4$ treatment of the extracts (Table 1).

Non-enzymic breakdown of colneleic acid

It was observed that colneleic acid and colnelenic acid were degraded to a significant and variable degree either during their preparation and purification (Galliard *et al.*, 1973) or during incubation in the absence of enzyme. The extent of non-enzymic breakdown was determined from control incubations (see the Materials and Methods section). This breakdown was probably due to the presence of trace amounts of metal ions. The non-enzymic control values ranged from 1 or 2% to 20% breakdown of substrate. Values of 10–12% were considered to be the maximum limits for this non-enzymic breakdown in critical experiments. Preliminary experiments had indicated that the non-enzymic breakdown occurred mainly during the incubations (at pH 5.0) rather than in the preparation (at alkaline pH) of the aqueous form of the substrate. Subsequent work showed that Fe^{2+} ions specifically catalysed the degradation of colneleic acid. The degradation products from the Fe^{2+}-catalysed reaction were identical on t.l.c. analysis (Fig. 1*b*) with those obtained from the enzyme-catalysed breakdown of colneleic acid (Fig. 1*c*). Yavin & Gatt (1972*a*,*b*) have demonstrated the catalysis of vinyl ether cleavage in plasmalogens by a Fe^{2+}–ascorbate complex. It was essential therefore to discriminate between the enzymic process and possible metal-catalysed non-enzymic processes.

Comparison between enzymic and Fe^{2+}-catalysed degradation of colneleic acid

The enzymic and non-enzymic processes were distinguished by dialysis and by heat treatment. Table 2 summarizes the results of several experiments which showed that the enzymic process was completely inhibited by pre-heating the enzyme at 80°C for 10min. Boiling in the absence of reducing agent actually decreased the catalytic activity of the Fe^{2+}-catalysed system, but this was probably due to partial oxidation to the ferric form, which is inactive (see Table 6). By Sephadex G-150 chromatography, the enzymic activity was eluted in fractions corresponding to a molecular weight around 10^5.

Table 1. *Colneleic acid-degrading activities of enzyme preparations from potato tubers*

Incubation mixtures contained colneleic acid (0.3mM), sodium acetate buffer, pH 5.0 (0.1M), and enzyme (equivalent to 5 μl of crude homogenate except where indicated) in a total volume of 0.5ml. After incubation for 10min with shaking at 25°C, ethanol (4.0ml) and water (0.5ml) were added. Degradation of substrate was measured spectrophotometrically. $(NH_4)_2SO_4$ precipitate, acetone-dried powder and Sephadex G-25 preparations were made from 15000*g* supernatant fractions. Boiled preparations were heated at 100°C for 10min. Results of three separate experiments are presented.

	Colneleic acid degraded (% of added substrate)	Enzyme activity (μmol converted/10min per mg of protein)
Expt. 1		
Crude homogenate	71	3.6
Crude homogenate (boiled)	0	0
15000*g* supernatant	67	3.8
15000*g* sediment	6	0.8
100000*g* supernatant	67	5.0
100000*g* sediment	6	—
Expt. 2		
Crude homogenate	82	5.0
15000*g* supernatant	82	6.7
15000*g* supernatant (boiled)	0	0
80%-satd.-$(NH_4)_2SO_4$ precipitate	61	5.0
Acetone-dried powder	46	2.2
Expt. 3		
15000*g* supernatant (3 μl)	61	7.2
Protein fractions from Sephadex G-25 (equivalent to 3 μl of supernatant)	49	7.3

Table 2. *Comparisons between enzyme- and Fe^{2+}-catalysed degradation of colneleic acid*

Mixtures (total vol. 0.5ml) contained 0.1M-sodium acetate buffer, pH5.0, and either the Sephadex G-25 enzyme fraction (approx. 16μg of protein) or $FeSO_4$ (0.2mM). Assays were started by addition of colneleic acid (0.3mM) and after 10min incubation at 25°C, substrate degradation was determined spectrophotometrically.

Conditions	Colneleic acid degraded (% of control reactions)	
	Enzyme system	Fe^{2+}-catalysed system
Control	100	100
Dialysis against 0.05M-Tris–HCl buffer for 18h	94	—
Dialysis against 0.05M-Tris–HCl buffer containing 2mM-EDTA	0	—
Preincubation for 10min at:		
0°C	98	—
40°C	93	—
50°C	68	—
70°C	12	—
80°C	0	103
Boiling at 100°C for 10min	0	55
Boiling at 100°C for 10min with cysteine (1mM)	0	100

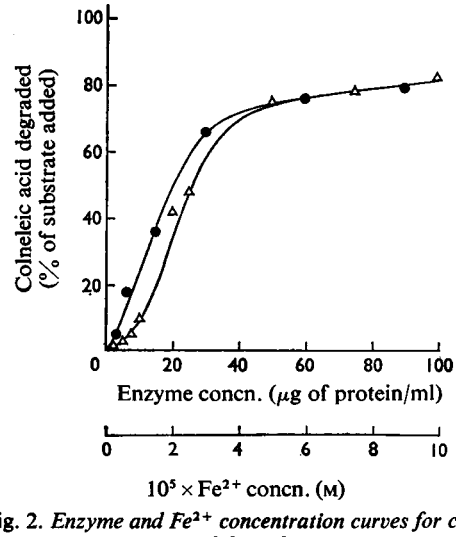


Fig. 2. *Enzyme and Fe^{2+} concentration curves for colneleic acid degradation*

Incubation mixtures contained colneleic acid (0.3mM), enzyme (Sephadex G-25 fraction) or $FeSO_4$ as indicated and 0.1M-sodium acetate buffer, pH5.0, in a total volume of 0.5ml. After 10min incubation at 25°C colneleic acid degradation was determined spectrophotometrically. ● and △ represent curves for enzyme and $FeSO_4$ respectively.

Properties of enzymic and Fe^{2+}-catalysed degradation of colneleic acid

(a) *Catalyst concentration.* The enzymic reaction was proportional to enzyme concentration up to approx. 50–60% conversion of substrate (Fig. 2). The Fe^{2+}-concentration curve shows a similar shape (Fig. 2) except for some sigmoidicity at low Fe^{2+} concentration, possibly owing to partial oxidation to inactive Fe^{3+} ions. The catalytic nature of the Fe^{2+}-catalysed process is illustrated; at 50% conversion of colneleic acid, 0.012μmol of Fe^{2+} was required to degrade 0.05μmol of colneleic acid in the experiments presented in Fig. 2. The extent of colneleic acid breakdown catalysed by a given amount of enzyme (on a protein basis) was reproducible in all experiments except when tubers stored longer than 6 months were used; a decrease in activity with sprouting tubers was observed. The Fe^{2+} concentration giving approx. 50% degradation of colneleic acid varied over several experiments from 10 to 25μM. The Fe^{2+}-concentration curve in Fig. 2 represents a typical result.

(b) (i) *Progress curve (Fig. 3).* The enzymic reaction was linear up to about 10min. The Fe^{2+}-catalysed reaction showed a [Fe^{2+}]-dependent lag phase followed by a rapid reaction. This supports the possibility of a free-radical mechanism in the non-enzymic system.

(ii) *pH (Fig. 4).* Both enzymic and non-enzymic systems were optimum at about pH5; below this pH activity always fell sharply in both systems.

(c) *Kinetics of enzyme-catalysed reaction.* With optimum incubation conditions approx. 0.5μmol of colneleic acid was typically degraded/min per mg of protein in the 15000*g* supernatant. This is equivalent to approx. 5μmol converted/min per g fresh wt. of tuber tissue and is of the same order of magnitude as the lipoxygenase enzyme from the same tissue (Galliard, 1970). Lineweaver–Burk reciprocal plots from substrate concentration curves gave an apparent K_m (colneleic acid) of approx. 0.13mM.

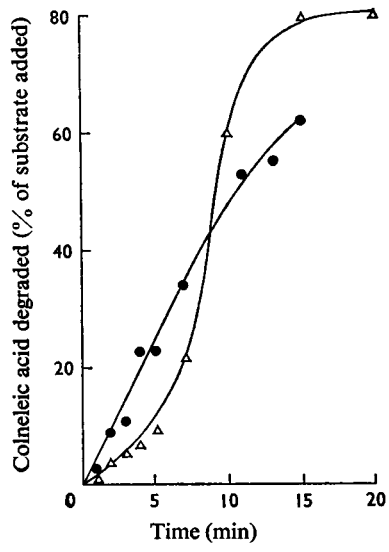


Fig. 3. *Progress curves for enzymic and Fe^{2+}-catalysed degradation of colneleic acid*

The mixture (5ml total vol.) contained 0.1M-sodium acetate buffer, pH5.0, 0.3mM-colneleic acid and either the Sephadex G-25 enzyme fraction (approx. 80μg of protein) or 25μM-$FeSO_4$. Samples (0.5ml), removed at intervals during incubation at 25°C, were analysed spectrophotometrically to determine colneleic acid degradation. ● and △ represent curves for enzyme and $FeSO_4$ respectively.

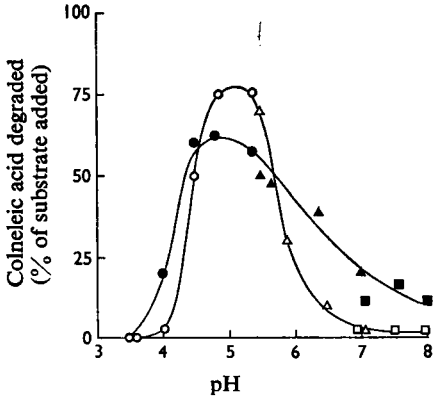


Fig. 4. *pH curves for enzymic and Fe^{2+}-catalysed degradation of colneleic acid*

Incubation mixtures contained colneleic acid (0.3mM), enzyme (approx. 16μg of protein) or $FeSO_4$ (20μM) and 0.1M buffer in 0.5ml total volume. After 10min incubation at 25°C colneleic acid degradation was determined spectrophotometrically. Solid and open symbols represent enzyme and $FeSO_4$ systems respectively. Buffers used were sodium acetate (●, ○), 2-(*N*-morpholino)ethanesulphonic acid (Mes) (▲, △) and Tris–HCl (■, □).

Table 3. *Oxygen requirement for enzymic and Fe^{2+}-catalysed degradation of colneleic acid*

Mixtures (total vol. 5ml) in Thunberg tubes contained 0.1M-sodium acetate buffer, pH5.0, [1-^{14}C]colneleic acid (0.3mM; 0.1μCi) and either the Sephadex G-25 enzyme fraction (approx. 80μg of protein) or $FeSO_4$ (50μM). After 10min shaking at 25°C incubations were stopped by rapid freezing to −30°C. Ethanol (40ml) and water (5ml) were added to the frozen mixtures, which were then allowed to attain room temperature before u.v. measurements. Samples were also taken for analysis by radioscanning.

Incubation conditions	Colneleic acid degraded (% of added substrate)		
	Enzyme system		
	Expt. 1	Expt. 2	Fe^{2+}-catalysed system
Air	64	68	77
Vacuum	6	—	—
N_2	—	4	0

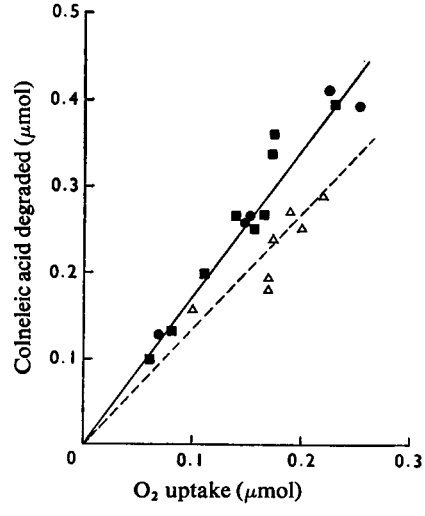


Fig. 5. *Relationship between colneleic acid degradation and oxygen uptake for enzyme and Fe^{2+}-catalysed systems*

Incubation mixtures in oxygen-electrode cells contained colneleic acid (0.3mM), enzyme (Sephadex G-25 fraction, 560μg of protein; or 800μg of 15000*g* supernatant protein) or $FeSO_4$ (1–0.1mM) and 0.1M-sodium acetate, pH5.0, in a total volume of 2.0ml. Assays were started by addition of enzyme or $FeSO_4$ at 25°C and oxygen consumption was monitored continuously. At oxygen-consumption values indicated, 1ml portions were rapidly transferred to ethanol (4ml) for spectrophotometric determination. Points on the figure represent results from several experiments. Symbols used are: ●, enzyme (Sephadex G-25 fraction); ■, 15000*g*/30min supernatant; △, $FeSO_4$.

(d) *Dependence on oxygen.* Breakdown of colneleic acid, determined either by examination of reaction products on t.l.c. or by determination of the u.v. chromophore (Table 3), was repeatedly and completely inhibited in the absence of oxygen, in both enzyme and Fe^{2+}-catalysed systems. Polarographic measurements with an oxygen electrode illustrated the dependence of reaction on oxygen concentration. Results of several studies on the stoicheiometry of the reaction are combined in Fig. 5, which shows proportionality of O_2 uptake and colneleic acid breakdown, with values of 0.60 μmol of O_2 consumed/μmol of colneleic acid changed in the enzyme system. The corresponding value for the Fe^{2+}-catalysed reaction was 0.74 μmol of O_2 consumed/μmol of colneleic acid.

Until the immediate product of the reaction is characterized, it is difficult to interpret these data. If it is assumed that the reaction involves uptake of one mol of O_2 per mol of unsaturated ether, loss of the u.v. chromophore without fragmentation of the molecule suggests an oxygenation of the butadienylvinyl ether structure; the polarity of the reaction product is also indicative of an oxygenated product. The aerobic nature of the reaction would then suggest a hydroxylase reaction rather than a hydration. Yavin & Gatt (1972*b*) have proposed such a hydroxylation reaction catalysed by 'active oxygen' in the catalysis of plasmalogen vinyl ether cleavage by the Fe^{2+}–ascorbate complex.

(e) *Inhibitors.* Table 4 summarizes the results of several experiments designed to study the effects of various reagents on both enzyme- and Fe^{2+}-catalysed reactions. A marked similarity was observed between the effect on both systems of the inhibitors except for $Na_2S_2O_5$, which at 5mM markedly inhibited the enzyme but had no effect on the metal-catalysed reaction. EDTA and CN^- were relatively poor inhibitors. A range of iron- and copper-chelating agents were inhibitory in the range 5–30 μM; diethyldithiocarbamate and Dithizone (diphenylthiocarbazone) were particularly effective on both systems. Marked inhibition was obtained by low concentrations (1 μM) of the antioxidants butylated hydroxytoluene and butylated hydroxyanisole; these are one-electron-transfer reagents and their inhibitory effect suggests the involvement of free-radical mechanisms in both enzymic and non-enzymic breakdown of colneleic acid. These results suggest the involvement of a metalloprotein in the enzymic process.

(f) *Effect of metal ions.* Table 5 summarizes the typical results of experiments in which metal ions either replaced enzyme and Fe^{2+} ions in the standard assays or were added to enzymic and Fe^{2+}-catalysed reaction systems. In these results no corrections are made for colneleic acid breakdown in control assays (see the Materials and Methods section) and the 11% breakdown observed with no additions (Table 5) represents this control value.

Table 4. *Effect of inhibitors on enzymic and Fe^{2+}-catalysed degradation of colneleic acid*

Mixtures (total vol. 0.5ml) contained 0.1M-sodium acetate buffer, pH5.0, 0.3mM-colneleic acid and either the Sephadex G-25 enzyme fraction (approx. 16 μg of protein) or $FeSO_4$ (20 μM). Inhibitors, as indicated, were added to assay mixtures immediately before incubation at 25°C for 10min. Colneleic acid degradation was determined spectrophotometrically.

Inhibitor	Concn.	Percentage inhibition of colneleic acid degradation: Enzyme system	Percentage inhibition of colneleic acid degradation: Fe^{2+} system
EDTA	1mM	24	100
KCN	1mM	20	10
$Na_2S_2O_5$	5mM	64	0
	0.5mM	5	0
Diethyldithiocarbamate	5 μM	91	100
	1 μM	22	6
Diphenylthiocarbazone (Dithizone)	10 μM	100	100
	5 μM	33	24
α,α'-Bipyridyl	30 μM	0	4
8-Hydroxyquinoline	30 μM	16	10
1,10-Phenanthroline	30 μM	16	10
2,9-Dimethyl-1,10-phenanthroline (neocuproine)	30 μM	13	100
2,2'-Biquinoline (cuproine)	30 μM	22	25
Butylated hydroxytoluene*	5 μM	95	79
	1 μM	63	24
Butylated hydroxyanisole*	5 μM	94	90
	1 μM	88	8

* Incubations contained 0.5 μg of Tween-20, which was used to disperse these compounds.

Table 5. *Effect of metal ions on enzymic and non-enzymic degradation of colneleic acid*

Incubation mixtures contained 0.1 M-sodium acetate buffer (pH 5.0), 0.3 mM-colneleic acid and, as indicated, enzyme (approx. 16 μg of protein) and metal salts (20 μM) in a volume of 0.5 ml. After 10 min aerobic incubation at 25°C colneleic acid degradation was assayed spectrophotometrically.

Metal ion added	Catalyst added	Colneleic acid degraded (% of added substrate) ... None	Fe^{2+}	Enzyme
None		11	83	81
Ca^{2+}		0	24	77
Cu^{+}		8	4	70
Cu^{2+}		0	0	55
Co^{2+}		4	36	75
Fe^{2+}		83	—	90
Fe^{3+}		9	2	77
Mg^{2+}		8	26	75
Mn^{2+}		11	53	73

Table 6. *Effect of various catalysts on the degradation of colneleic acid*

Mixtures (total vol. 0.5 ml) contained 0.1 M-sodium acetate buffer, pH 5.0, and 0.3 mM-colneleic acid and additions as indicated. After incubation at 25°C for 10 min, colneleic acid degradation was determined spectrophotometrically. Heated catalysts had been subjected to 80°C for 10 min.

Additions	Colneleic acid degraded (% of added substrate)
H_2O_2 (1 mM)	10
Glucose (1 mM) + glucose oxidase (50 μg)	0
Catalase (200 units)	6
Catalase (200 units)—heated	0
Peroxidase (100 μg)	3
Peroxidase (100 μg)—heated	13
Ferredoxin (0.25 μg of iron)	65
Ferredoxin (0.25 μg of iron)—heated	70
$FeSO_4$ (10 μM)	73

Table 5 shows that no metal ions could effectively replace Fe^{2+}, and some (particularly Ca^{2+} and Cu^{2+}) even inhibited the non-enzymic breakdown in control assays. Similarly, Cu^{+}, Cu^{2+} or Fe^{3+} ions always strongly inhibited the Fe^{2+}-catalysed reactions and Ca^{2+}, Co^{2+} and Mg^{2+} ions showed some inhibitory effects. The enzyme-catalysed reaction was less susceptible to inhibition by metal ions, although Cu^{2+} ions showed some effect. The increased breakdown in the presence of Fe^{2+} ions and enzyme presumably represents an additive effect. Although Fe^{3+} did not substitute for Fe^{2+}, mixtures containing Fe^{3+} and a reducing agent, e.g. ascorbic acid and cysteine, were catalytically active.

Attempts to replace enzyme and Fe^{2+} ions with other catalysts

These results suggested that the enzymic breakdown of colneleic acid was an oxygen-requiring, possibly free-radical process. Thus model systems for oxygenase reactions, peroxide-generating systems and haemoproteins were tested as possible analogues for the enzyme system. Table 6 shows that neither H_2O_2 nor H_2O_2-generating systems could replace the enzyme. $SnCl_2$, which acts in an oxygenase model system, was also ineffective. The haemoproteins catalase and peroxidase were also inactive, although heat treatment of peroxidase did cause some slight activity. (Partially denatured haemoproteins are more active than natural haemoprotein in catalysing the autoxidation of unsaturated fatty acids; Eriksson *et al.*, 1970.) High concentrations of catalase were in fact inhibitory when added to either enzyme- or Fe^{2+}-catalysed colneleic acid degradation systems.

However, ferredoxin, a non-haem ferriprotein, did replace the enzyme and Fe^{2+} in colneleic acid degradation. Fig. 6 shows that, based on equivalent iron content, ferredoxin was as effective as Fe^{2+}, whereas the haemoprotein peroxidase was virtually inactive. It is interesting that ferredoxin, in which iron is present in the ferric state, catalysed the breakdown of colneleic acid (even after heating) whereas free ferric ions did not. Moreover, Fe^{3+} ions inhibited the catalytic activity of Fe^{2+} ions (Table 5). Ferredoxin has a very low redox potential (−0.4 V); Methyl Viologen, a non-metallic redox reagent with a similar low potential, was completely inactive in the system. The nature and amount of any metal in the enzyme from potato will only be determined when the enzyme has been purified. In the oxidative cleavage of plasmalogen vinyl ethers, ascorbic acid, free or protein-bound, was required in addition to Fe^{2+} ions for maximum activity (Yavin & Gatt, 1972*b*). In the present work, Fe^{2+} ions alone were effective and the

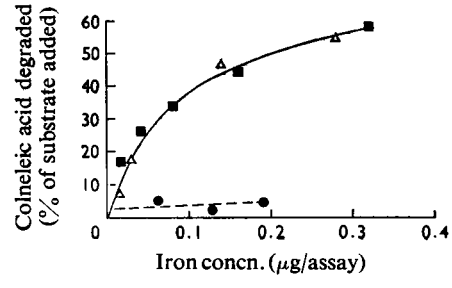


Fig. 6. *Effect of iron-containing catalysts on degradation of colneleic acid*

Incubation and assay conditions as in Table 6. The symbols refer to $FeSO_4$ (△), ferredoxin (■) and peroxidase (●).

addition of 0.1mM-ascorbate gave no increased activity. Also Cu^{2+}–ascorbate mixtures, which catalyse autoxidation of unsaturated fatty acids, were inactive in catalysing colneleic acid breakdown.

Conclusions

Previous studies on the enzymic cleavage of vinyl ether bonds have been limited to plasmalogens. These include a liver microsomal system (Warner & Lands, 1961), a Mg^{2+}-requiring system in brain mitochondria (Ansell & Spanner, 1965) and an anaerobic, non-enzymic system inhibited by chelating agents in acetone extracts of several tissues (Thiele, 1959*a*,*b*). The system in rat brain described by Yavin & Gatt (1972*a*,*b*) required oxygen and a heat-stable, low-molecular-weight component similar to, or identical with, ascorbic acid.

Colneleic acid contains a novel butadienyl vinyl ether structure (–CH=CH–CH=CH–O–CH=CH–). Only oxygen and catalytic amounts of Fe^{2+} ions were required for the non-enzymic breakdown of this compound. Since Fe^{3+} ions were inactive and Fe^{2+} ions were required in catalytic amounts, a stoicheiometric Fe^{2+}/Fe^{3+} redox couple was not involved. A likely mechanism would involve a free-radical process initiated by Fe^{2+}–O_2, a known peroxide-radical-generating system. Inhibition by butylated hydroxytoluene and butylated hydroxyanisole also suggests a free-radical mechanism.

The enzymic mechanism appears to have a similar mechanism although the metal involved is not identified. The fact that the non-haem ferriprotein, ferredoxin, can substitute for the enzyme may be significant. We have recently found that a commercial preparation of soya-bean lipoxygenase (which is also a non-haem-iron-containing enzyme; Chan, 1973; Rosa & Francke, 1973) also catalysed the breakdown of colneleic acid. Further, we have so far failed to separate the colneleic acid-degrading enzyme from lipoxygenase in potato tuber extracts. The possible involvement of lipoxygenase or a lipoxygenase-like enzyme is intriguing in view of the sequential nature of the enzyme reactions so far elucidated (see the introduction) in the oxidative degradation of linoleic acid in extracts of potato tuber (Scheme 1).

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