STUDIES ON THE 'LIPOXIDASE' IN THE FLESH OF BRITISH COLUMBIA HERRING

By

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THE LIPOXIDASE SYSTEM IN THE FLESH OF BRITISH COLUMBIA HERRING

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THESIS

THE LIPOXIDASE SYSTEM IN THE FLESH OF BRITISH COLUMBIA HERRING

(Summary)

From the dark muscle of British Columbia herring *(Clupea pallasii* Valenciennes) a highly active enzyme capable of peroxidising unconjugated unsaturated fatty acids was isolated. This lipoxidase, which was shown to be a nitrogenous complex possessing no heavy metals or sulphydryl group as the active centre, is heat-labile and can act only in presence of activators such as certain iron-containing organic nitrogenous compounds. Two such compounds, namely haemoglobin and cytochrome 'C' were isolated. The enzyme exhibits optimum activity at 15C°. and pH. 6.9. There is also an optimum concentration of enzyme, substrate, and of the activators for maximum enzyme activity. The presence of the activiators appears to change the kinetics of the reactions. The inhibition of the enzyme reaction brought about by cyanides and azides is possibly due to the inactivation of the iron-containing activators rather than of the enzyme itself.

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ABSTRACT

From the dark muscle of British Columbia herring a highly active enzyme capable of peroxidising unconjugated unsaturated fatty acids was isolated. This 'lipoxidase', which was shown to be a nitrogenous complex possessing no heavy metals or sulphydryl group as the active centre, is heat-labile and can act only in presence of activators such as certain iron-containing organic nitrogenous compounds. Two such compounds, namely haemoglobin and cytochrome 'C' were isolated. The enzyme exhibits optimum activity at 15°C. and pH 6.9. There is also an optimum concentration of enzyme, substrate, and of the activators for maximum enzyme activity. The presence of the activators appears to change the kinetics of the reactions. The inhibition of the enzymic reaction brought about by cyanide and azide is possibly due to the inactivation of the iron-containing activators rather than of the enzyme itself.

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INTRODUCTION

The deterioration of quality due to rancidity that develops in fatty foods during storage causes considerable loss to packers, operators of frozen food lockerplants, and their patrons, and all those concerned in general with production, processing, storage, sale, or consumption of the products. The problem is particularly important to the fisheries industries because of the high fat content of many fishes and more particularly because of the composition of fish fats. These fats are especially rich in unsaturated fatty acids which are markedly prone to deterioration due to the development of rancidity during storage.

In frozen fish the oxidation of the indigenous fats is accompanied by such undesirable changes as a yellow or brown discolouration of exposed fats (rusting), bleaching of the natural red astacin pigments (in salmon), and production of unpleasant flavours variously described as rancid, bitter, 'salt-fish', and by similar descriptive terms. The off-flavours are known to be caused by the compounds formed as a result of oxidative break-down of fats. Various products have been identified in autoxidised lipides. Among them the most common are peroxides, peracids, ketones, aldehydes, substituted ethylene oxides, carboxyl compounds, hydroxyl compounds, water, and carbon dioxide (Black 1945, Mattil 1941). In general, however, these studies have been concerned with

fats that were oxidised far beyond the stage where rancidity first appeared.

Conditions affecting the onset of rancidity in fats have occupied the attention of many investigators during the past few years. The development of rancidity in fatty foods may be attributed to both external and internal factors. Among the former are micro-organisms, temperature, moisture, atmospheric oxygen, and light; while among the latter are proteins, enzymes, co-enzymes, minerals of the tissue, and chemicals which may be added during processing as well as materials (e.g. metal foils) composing the package. The effects and control of micro-organisms have been reviewed in detail (Lea 1939) and the effects of curing, different curing mixtures, and impurities in the curing salts extensively studied (Lea 1931, 1933, 1939; Hills and Conochie 1945, 1946; Greenwood, Striter and Kraybill 1945; Oser 1946.)

A factor comparatively little investigated but probably of the first order of importance is the effect of the enzymes or enzyme systems in fatty tissue. Banks (1937, 1944) demonstrated the presence of a heat-labile system in herring muscle which stimulated rancidity in extracted herring oil. Lea (1931, 1933) suggested that an enzyme system might be responsible for the rapid deterioration of bacon and later Lea (1937) presented evidence for its presence. Watts and Pang (1947) have compared the catalytic effects of pork muscle extracts and haemoglobin on peroxidation of lard. Other enzymes capable of oxidizing fatty acids and fats have been

reported. Munoz and Leloir (1943) prepared an enzyme from liver that catalyses the oxidation of low-molecular-weight fatty acids, work which has been considerably extended by Lehninger (1945, 1948) and Lang (1939). Hove (1943) found that rat liver and gastric mucosa contained an oxidase capable of oxidising unsaturated fats but the intestine and muscle were inactive. Extract of gastric mucosa retained its activity at pH 3. The capacity of this extract to catalyse reactions at such a high hydrogen ion concentration is possibly due to the fact that the enzyme involved is produced in an environment, namely the gastric mucosa, characterised by high acid production. Süllmann(1947) has demonstrated the presence in the striated muscle of the rabbit of an enzyme that strongly peroxidises unsaturated fatty acids and fats. Brocklesby and Rogers (1941) reported that an extract of salmon liver destroyed 200 units of vitamin A in 45 minutes at room temperature, indicating the probable presence of a similar fat-oxidising peroxidase. Reiser (1949) gave evidence of the presence of two heat-labile factors in bacon which may be responsible for the development of rancidity in bacon.

A 'lipoxidase' present in legumes was investigated and use made of it to study the mechanism of the oxidation of isolated unsaturated fatty acids (Bergström 1945, Holman 1947). The literature on this enzyme has recently been reviewed (Holman 1948).

However, the effects of the tissue enzymes in fatty foods on the development of rancidity have not been adequately

studied. The investigations of Lea (1937), Watts and Pang (1947), and Banks (1944) were not carried to a stage where the nature of the enzyme or enzyme systems could be conclusively ascertained. The present author's work (Khan 1948) with rancidity in frozen stored fish resulted in some interesting findings and it was thought a further study of the enzyme involved was desirable, resulting in the present more detailed investigation of the 'lipoxidase' of herring flesh.

EXPERIMENTAL

Material

Herring, Clupea pallasii, were chosen as material because of their relatively rich fat content and the nature and extent of the unsaturation of the fats. High unsaturation of herring oil has been known to provide suitable conditions for rancidity to progress with a fair degree of rapidity. As a result, in all likelihood, the proneness of herring flesh to undergo oxidative rancidity is most likely due to the activity of the lipoxidase in the flesh of this fish. Available information regarding lipoxidase activity in fish flesh record the presence of such an enzyme only in herring flesh. Banks (1937), studying the enzyme system in herring influencing rancidity, reported that the enzyme is mostly concentrated in the dark streak of muscle along the lateral lines of the fish and the flesh exhibits either very little or no lipoxidase activity at all. These findings were checked and the results agree fairly well (Table I). Hence, the dark streak of herring flesh was used for extracting the enzyme.

Substrate

In order to find a workable method for standardizing the lipoxidase of herring muscle it was necessary at first to ascertain the essential substrate for the enzyme. A discussion of this part of the studies will, therefore, precede the subsection on "Standardization".

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TABLE I

Lipoxidase activity of herring flesh and dark muscle along the lateral lines. Substrate: 2 mg. of linoleic acid.

Time of reaction in minutes	Amount of Fe^{+++} as 10^{-6} gm. produced per 100 gm. tissue	
	Flesh	Dark strip
0	0	0
1	10	81
2	13	223
3	21	240
5	30	245
10	32	250
15	32	250
20	32	250
30	33	252
60	33	254

It was observed by Sumner and Sumner (1940) that the lipoxidase in legumes oxidised carotene only in presence of unsaturated fats. This observation was later confirmed by Tauber (1940). Sumner (1942b) submitted evidence that carotene is not oxidised either directly or indirectly by the peroxides formed from unsaturated fats. It was also shown that the oxidation of carotene requires that the peroxidation of unsaturated fat be actually in progress. Sumner (1942a), investigating the effect of lipoxidase on various unsaturated compounds, reported that the enzyme is most effective on the unsaturated part of the structural group:

$$- CH_2 - CH = CH - CH_2 - CH = CH - (CH_2)_7 - COOH$$

the configuration of the groups about the double bond nearest to the carboxyl group being probably of the cis type. Similar results were reported by Holman and Burr (1945) and Holman and Elmer (1947). These authors, using purified fatty acids and their esters, found (1) that lipoxidase attacked linoleic acid, linolenic acid, their esters and methyl arachidonate; (2) that linoleate, linolenate, and arachidonate esters were oxidised at the same rate; (3) that only the cis isomers of linoleic and linolenic acids were attacked. From these results it is suggested that the essential substrate structure for attack by legume lipoxidase seems to be the methylene-interrupted, doubly unsaturated system

$$- CH_2 - CH = CH - CH_2 - CH = CH - CH_2 -$$

with a cis configuration about both double bonds. A repetition

of the above studies with herring lipoxidase and using oleic, linoleic, and linolenic acids as substrates produced results which indicate that the essential substrate structure for herring lipoxidase is similar to that for legume lipoxidase.

Oleic acid was peroxidised by herring flesh extract very slowly and the maximum effect, obtained in 20 minutes, was less than 20% of the theoretical. Linoleic acid with both double bonds having cis configuration was peroxidised with great rapidity, the maximum being over 80% of the theoretical total peroxidation of one double bond. Linoleic acid with one double bond having trans configuration (obtained from Hormel Foundation, Austin, Minn., U.S.A.) was peroxidised at a rate about a third of that observed in case of linoleic acid with both double bonds having cis configuration, the final amount of peroxide formed also being about one third that from the cis-cis linoleic acid. Linolenic acid (cis) was peroxidised at a rate appreciably higher than that for cis-cis linoleic acid and the maximum was about 110% of the theoretical total peroxidation of one double bond (Table II, Fig. 1 and 2).

Reaction mechanism

It is known that the oxidation products of unsaturated fatty acids and their esters in reactions catalysed by soybean lipoxidase give a positive ferric thiocyanate test (Sumner 1943) and it is also known the primary oxidation products of linoleic, linolenic, and arachidonic acids and their esters in reactions catalysed by soybean lipoxidase show increased absorption near 2300 and 2700Å (Holman and Burr 1945).

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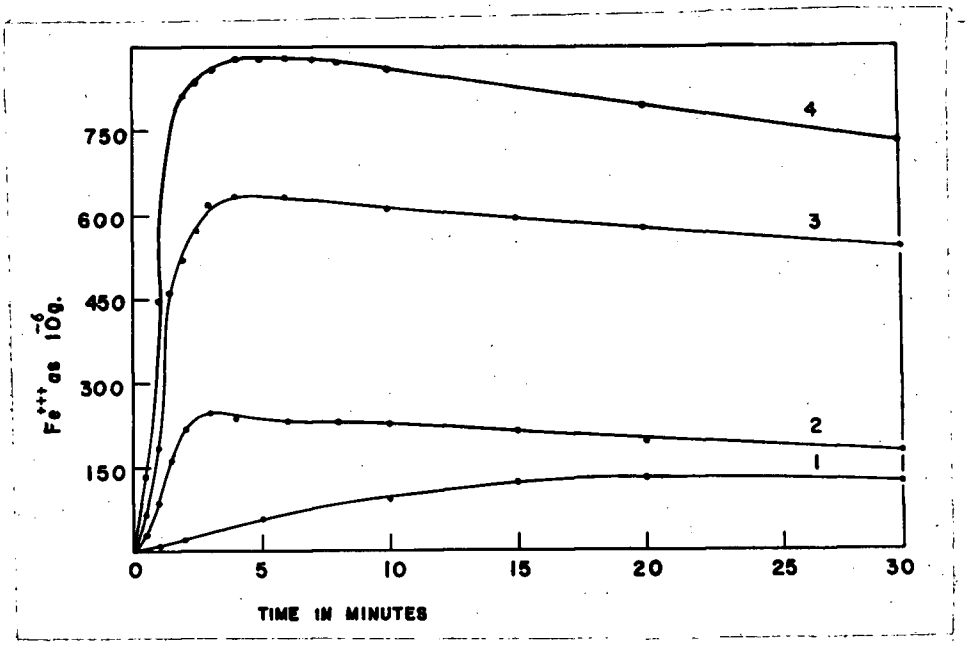

Fig. 1 - Effect of herring lipoxidase on different substrates. In presence of 2 mg. of substrate and 0.1 mg. of crude enzyme. (1) Oleic acid. (2) Linoleic acid cis-trans. (3) Linoleic acid cis-cis. (4) Linolenic acid.

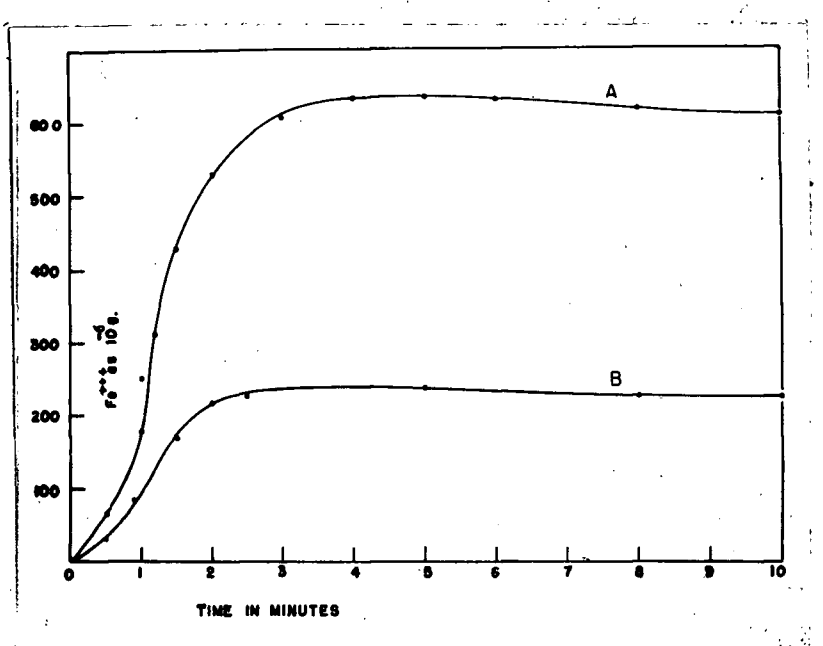

Fig. 2- Effect of herring lipoxidase on linoleic acid.
A. Linoleic (cis-cis). B. Linoleic (cis-trans).
These are same graphs from Fig. 1 drawn on a larger scale to show the nature of the reaction in the initial stages.

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TABLE II

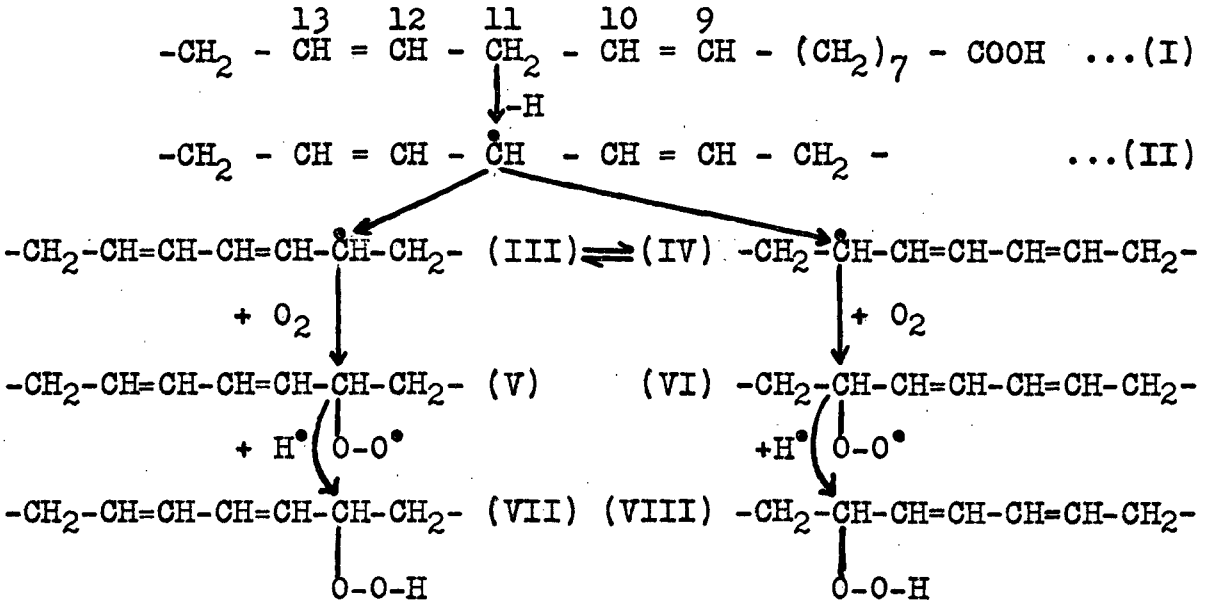
Effect of herring lipoxidase on different substrates

Time in minutes	Activity as 10^{-6} gm. of Fe^{+++}															
	0.0	0.5	1.0	1.5	2.0	2.5	3	4	5	6	8	10	15	20	30	60
Oleic (cis)	0	-	8	-	18	-	-	-	60	-	-	112	124	130	124	110
Linoleic (cis-trans)	0	29	85	178	218	234	246	236	236	230	230	228	213	192	176	
Linoleic (cis-cis)	0	65	182	462	518	575	618	632	630	624	618	610	596	574	542	
Linolenic cis	0	135	420	763	812	843	856	878	874	868	861	853	826	790	730	

NOTE: Amount of substrate was 2 mg. Enzyme added was 0.01 mg.

Experiments were carried out to ascertain whether or not similar facts would hold good in case of herring lipoxidase. Oxidation products of oleic, linoleic, and linolenic acids gave a positive ferric thiocyanate test (Table II). And these also showed pronounced absorption near 2300 and 2700 Å (Table III and Fig. 3). On the basis of the above results it seems quite possible that the reaction mechanism involved in the oxidation of unsaturated fatty acids and their esters catalysed by herring flesh extract is the same as that where the oxidation is catalysed by soybean lipoxidase. As such, a brief review of the present knowledge regarding the reactions involved in the oxidation of unsaturated fatty acids and their esters, taking the case of linoleic acid as a typical example, will be presented here.

In accordance with the evidence now at hand, as a result of investigations by Bergström (1945), Holman (1946) and other workers in the field, particularly Bergström and Holman (1948), and Holman (1947), the mechanism of reaction involved in the oxidation of linoleic acid catalysed by lipoxidase might be as follows:



(• indicates activation)

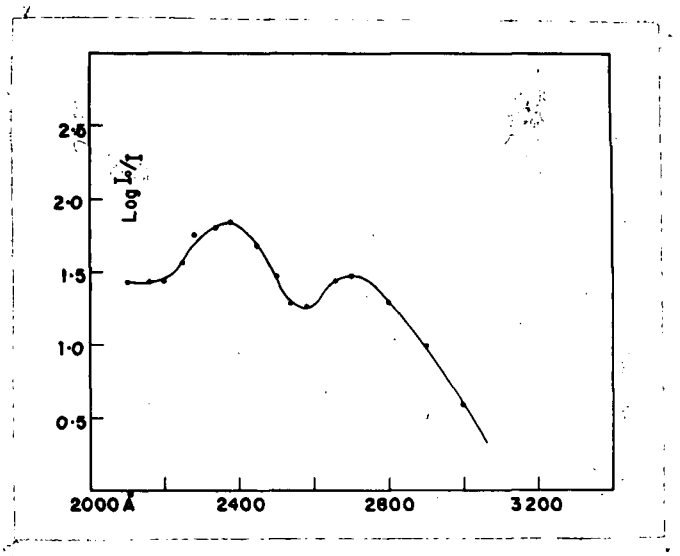


Fig. 3 - Absorption spectrum of the primary oxidation products of linoleic acid in herring lipoxidase catalysed reaction. Two mg. of linoleic acid and 0.01 mg. of active enzyme solution.

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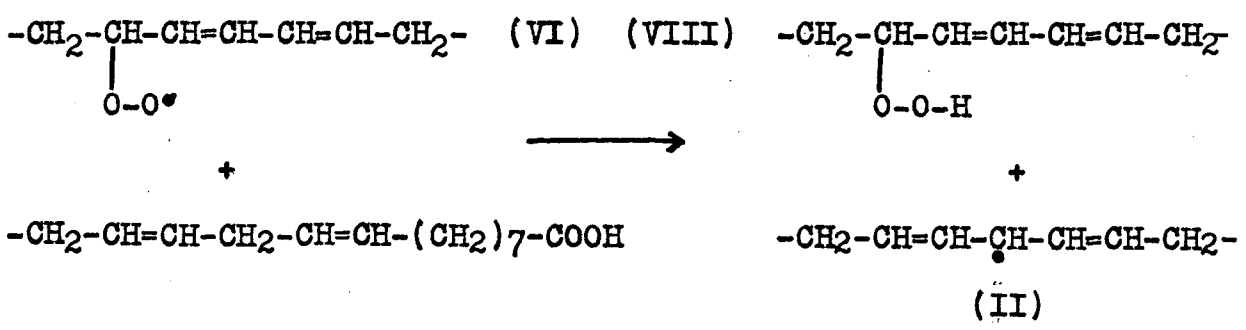
TABLE III

Absorption spectrum of the primary oxidation products of linoleic acid in herring lipoxidase catalysed reaction. (2 mg. substrate).

Wavelength	$\log I_o/I$
2100 Å	1.425
2150	1.430
2200	1.440
2250	1.553
2300	1.760
2320	1.751
2340	1.820
2360	1.846
2380	1.854
2400	1.827
2420	1.783
2450	1.678
2500	1.483
2550	1.580
2600	1.262
2650	1.442
2700	1.480
2750	1.445
2800	1.296
2900	0.954
3000	0.596

The oxidation of linoleic acid, once started by lipoxidase, could continue by means of a chain reaction. For example, the radicals V or VI could accept a hydrogen atom from linoleic acid:



This would give rise to another radical, II, which would go through the same cycle. Lipoxidase may thus have its function in initiating the chain reactions by removal of hydrogen from the linoleic acid methylene group.

Other authorities (Swern, Scanlan and Knight, 1948) have suggested that the oxygen addition may occur at stage II, followed by re-arrangement of the unsaturated double bond, instead of the double-bond re-arrangement preceding the addition of oxygen at stage III in the scheme indicated above. The end result is the same in either case.

Standardization

Various methods of assay of lipoxidase, involving its different properties, have been suggested. These methods can be divided into the following classes: (1) those which employ the Warburg apparatus and measure the absorption of oxygen during the oxidation of the unsaturated substrates (Craig 1936, Hummel and Mattil 1944, Süllmann 1941, Tauber 1940); (2) methods in which the peroxides formed are estimated (Balls, Axelrod

and Kies 1943, Banks 1937, Kokatnur and Jelling 1941, Sumner and Dounce 1939, Sumner and Somers 1944, Sumner 1943, Süllmann 1941, Young, Vogt and Nieuwland 1936); (3) methods based on decolorization of a suspension of carotenoid compounds containing unsaturated fat (Balls, Axelrod and Kies 1943, Reiser and Fraps 1943, Sumner and Sumner 1940, Sumner 1942). Sumner (1943) employed the ferric thiocyanate method for determination of peroxide products. More recently Sumner and Smith (1947) used an assay based on the destruction of bixin, a carotenoid dicarboxylic acid.

All these methods of assay are subject to considerable error. In most of comparatively old methods of measuring peroxide products, the substrates used were not homogeneous. The recent methods employing linoleic acid or its esters as substrate ensure a homogenity and a favourable degree of dispersion of the substrate. And it is known that the rate of oxidation of the unsaturated compounds is influenced by the degree of dispersion of the substrate. When one follows lipoxidase activity with the Warburg apparatus, it is found that there is no simple relation between enzyme action and the consumption of oxygen, at least over a range where convenient enzyme concentrations are employed. Those methods using carotenoid destruction are subject to the disadvantages that the rate of carotenoid destruction is proportional to the enzyme concentration only over a narrow range, and that a side reaction, rather than the primary phenomenon, is being measured.

At the commencement of the present investigation the

method of Sumner (1943), using linoleic acid suspension as substrate and the ferric thiocyanate method for determination of peroxide products, was employed for estimating lipoxidase activity of herring flesh. Although positive results with this method established the lipoxidase activity of herring flesh, it had to be discarded for the disadvantage that a side reaction, namely oxidation of Fe^{++} to Fe^{+++} by the hydroperoxides rather than the primary phenomenon, namely the hydroperoxides formed from the unsaturated fatty acids, is being measured. Next, the bixin decolorization method of Sumner and Smith (1947) was tried. Herring flesh extract failed to decolorize bixin at pH 4-9. This failure will be explicable on an examination of the chemical changes involved in the peroxidation of unsaturated fatty acids which are discussed under a separate heading. In coupled oxidation of carotenoid compounds and linoleic acid, the decolorization of the pigment occurs as a consequence of an interruption of the chain oxidation of linoleic acid by the carotenoid compounds. The radicals V and VI (see under 'Reaction mechanism') may accept hydrogen atoms from carotene, leading to its destructive oxidation and the termination of the chain reaction. It is quite likely that in case of herring lipoxidase there is a substrate competition as a result of which compounds V and VI or both receive their hydrogen atoms from linoleic acid in preference to carotenoids. Consequently, the chain reaction is not terminated and carotenoids are not bleached.

In the course of investigations of the spectra of

autoxidized fatty acids Holman and Burr (1945) studied the spectra of lipoxidase-oxidized fatty acids. These authors noted that linoleic acid, ethyl linoleate, linolenic acid, ethyl linolinate and methyl arachidonate showed increased absorption near 2300 and 2700 Å, indicating that these substances were changed by lipoxidase. The products which gave the increased absorption were considered to be the conjugated unsaturated carbonyl compounds. A method of assay was developed by Theorell, Bergström and Åkeson (1946), making use of the absorption of the primary products of oxidation of unsaturated fatty acids. This method overcomes the disadvantages of the previously mentioned ones and has the added advantage of being a very quick and reproducible method. A homogeneous substrate solution is used, and the products of the reaction are measured spectrophotometrically. Under these conditions, peroxide production is proportional to time and to enzyme concentration over wide ranges.

This spectrophotometric method was adapted in the present case with certain modifications in order to standardize the lipoxidase of herring flesh. The wavelength of light for maximum absorption by the primary products of oxidation of linoleic acid in presence of herring lipoxidase was found to be 2380 Å (Fig. 3) instead of 2340 Å as employed by Theorell et al (1946) in standardization of soybean lipoxidase. The optimum temperature and pH employed were those for herring lipoxidase. Furthermore, a larger quantity of ethyl alcohol (5.0 ml.) was necessary to stop the enzyme reaction as against

2.0 ml. used by Theorell et al (1946). The details of the modified method are as follows.

One milliliter of 0.200% cis-cis linoleic acid in 0.2 M acetate buffer of pH 6.9 is placed in a Warburg respiration bottle; into the side arm is put 0.2 ml. of enzyme solution, the bottle is filled with oxygen and placed in a water bath at 15°C. After temperature equilibrium is reached, the contents of the bottle are mixed and the mixture shaken well. At the end of two minutes 5.0 ml. of 95.0% ethyl alcohol (free from ammonia and oxides of nitrogen) is added to stop the reaction. The mixture (6.2 ml.) is diluted with 75% ethyl alcohol (NH_3- and N-oxide-free) to a strength measurable with a Beckman spectrophotometer. The absorption of the diluted mixture at 2380 Å is measured with a Beckman spectrophotometer. The blank was prepared in the same manner except that the enzyme was inactivated by boiling prior to its incorporation into the reaction mixture.

A unit of lipoxidase will be defined as that amount of the enzyme which under the above experimental conditions produces in the final mixture (6.2 ml.) an optical density of two at the end of two minutes of reaction.

Extraction and purification

The conditions for the extraction and purification of the enzyme were ascertained by a series of exploratory experiments carried out with a lot of Point Grey herring caught in May, 1948 off Burrard Inlet close to Vancouver, B.C. To avoid undue increase in the bulk of the thesis, the results of these

exploratory experiments are placed in the appendix. Final results recorded here were obtained with another lot of herring caught in August of the same year at a different locality about 100 miles north of Vancouver along the coast of British Columbia. Both lots were frozen and stored before extraction was performed. It was observed that there was a noticeable difference between the lipoxidase activity of the two lots of herring (Table IV). This difference might be considered to have been caused by variable factors such as locality of catch (e.g. water temperature in relation to activity), season of catch (e.g. maturity in relation to spawning), type of food consumed and age. A future extension of this study might lead to interesting observations on possible relations of lipoxidase activity to certain of these factors when taken into account more completely than the present work allowed.

Fifteen kilograms of the dark streak of muscle were finely ground, lyophilized and extracted in the cold with low-boiling "Skellysolve" petroleum spirits. The defatted tissue was extracted in a Waring blendor at 0°C. with acetate buffer of pH 5.6. The supernatent slightly yellowish fluid obtained by centrifuging the mixture was treated with ammonium sulphate. At 35% concentration of ammonium sulphate an inactive fraction precipitated out. The concentration of ammonium sulphate in the clear filtrate was now raised to 60% whereupon an active fraction precipitated out. The precipitate was filtered and washed with 60% ammonium sulphate; dissolved in a minimum amount of water and dialysed overnight in running water until

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TABLE IV

Lipoxidase activity of the dark muscle of two different lots of herring

	Sample no.	Amount of Fe^{+++} as 10^{-6} gm. produced per 100 gm. tissue	
Lot no. I (1000 herring)	1	146	140.8 (Av.)
	2	131	
	3	157	
	4	127	
	5	143	
Lot no. II (2500 herring)	1	231	245.6 (Av.)
	2	249	
	3	261	
	4	245	
	5	242	

NOTE: Each sample represents ten herring taken at random from the lot concerned. Storage conditions for Lot no. I and II were similar within narrow limits of temperature and elapsed storage time.

free from sulphate. The dialysed solution was then adjusted to pH 6.8 with dilute ammonium hydroxide. The inactive precipitate was filtered off. The filtrate was neutralized with 0.1 N NaOH to pH 7, and treated with ethyl alcohol in the cold (0°C.). At an ethyl alcohol concentration between 16 and 18% an inactive precipitate was obtained. The filtrate from this was dialysed and dried in vacuum and the residue dissolved in a minimum amount of water. This solution was now treated with 20% basic lead acetate. With 3 volumes of lead acetate to 2 volumes of the solution a precipitate was obtained. At this stage an interesting point was noted. The precipitate with basic lead acetate did not exhibit any activity. At the same time the activity of the soluble fraction was lowered to less than 30% of its expected value. That this lowered activity was not due to oxidation or denaturation of the enzyme, or its distribution between the precipitate and the soluble fraction was evident from the following facts:

(I) The precipitate did not show any activity.

(II) Treatment of the soluble fraction with mild reducing agents (Zn dust and acetic acid; sodium hyposulphite $Na_2S_2O_4$) did not increase the activity of the soluble fraction.

(III) Addition of the fraction precipitated with basic lead acetate to the soluble fraction, raised the enzyme activity of the latter to its expected level.

That the enzyme proper was entirely in the soluble fraction was further evidenced by the fact that boiling the soluble

fraction for five minutes completely destroyed its enzyme activity, whereas boiling had no effect whatsoever on the activating capacity of the precipitate. From here on the soluble fraction will be referred to as the enzyme proper and the fraction precipitable with basic lead acetate as the activator.

In later experiments the non-dialysable activator was broken into dialysable and non-dialysable fractions. However, the activity was entirely in the dialysable fraction. The dialysable portion gave negative biuret, Millon's, Molisch and ninhydrin reactions, but positive tests for organic nitrogen. Tests for sulphur, halogens and phosphorus were negative. The material contained less than 0.45% iron. Tests for copper were negative. These results indicate that the dialysable fraction is a non-protein iron-containing nitrogenous compound. The absorption spectra of the dialysate showed two maxima, one near 4480 Å and one at 5610 Å (Table V and Fig. 4).

Experiments were repeated to obtain the activator without the use of lead acetate since attempts to dissolve the activator from the lead acetate precipitate resulted in a breakdown of the activator into a dialysable non-protein and a non-dialysable protein components. The dispensing of the use of lead acetate was effected according to the following scheme.

After separation of the precipitate obtained by the use of 16% ethyl alcohol, the concentration of alcohol was raised to 60% (at 0°C.). A reddish yellow precipitate was obtained. This showed all the properties of the activator.

Accordingly in the final purification the fractionation

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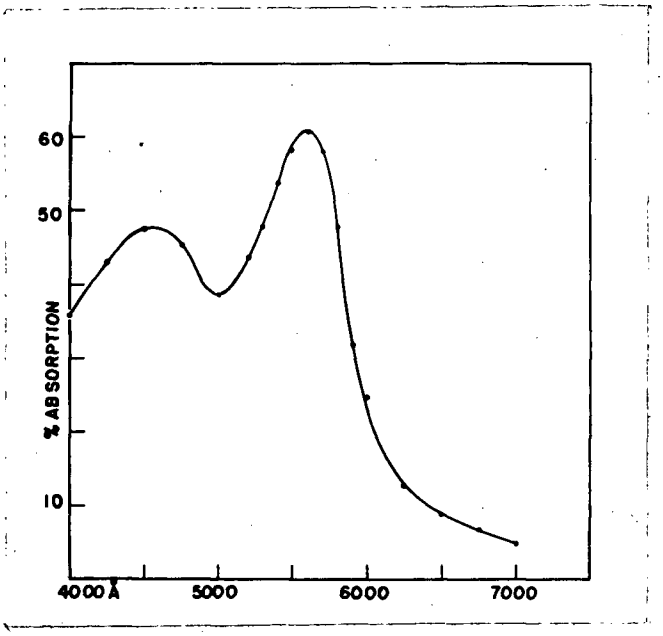

Fig. 4 - Absorption spectrum of the dialysable breakdown product of activators. Concentration of substrate was 0.2%.

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TABLE V

Absorption spectrum of the dialysable breakdown product of activator

Wavelength in Å units	% absorption in 0.2% solution	Wavelength in Å units	% absorption in 0.2% solution
4000	36.0	5400	53.8
4100	39.0	5500	58.2
4200	41.5	5520	59.4
4300	44.2	5540	60.4
4400	46.2	5560	60.6
4420	46.4	5580	60.8
4440	46.6	5600	61.0
4460	47.2	5610	61.5
4480	47.9	5620	61.3
4500	47.3	5630	61.0
4520	47.0	5640	60.8
4540	46.7	5650	60.7
4560	46.3	5660	60.4
4580	46.0	5680	59.2
4600	45.7	5700	58.0
4700	45.2	5800	47.6
4800	44.5	5900	32.0
4900	39.7	6000	24.8
5000	38.6	6250	12.8
5100	40.6	6500	9.0
5200	43.8	6750	7.0
5300	47.6	7000	5.0

with basic lead acetate was discarded and the activator fraction was separated from the enzyme by precipitation at 60% concentration of ethyl alcohol.

Enzyme Proper

After separation of the activator fraction, the alcohol was removed by vacuum distillation at room temperature. The pH of the aqueous solution was adjusted to 6.8 with 0.2 M acetate buffer, sodium chloride was added to 50% concentration, the inactive precipitate was removed and the filtrate dialysed and lyophilized. This lyophilization was necessary from three considerations: (1) to minimize bulk, (2) to get an idea of the amount of material and (3) to preserve the enzyme until needed. The lyophilized tissue was dissolved in the minimum amount of water and 1 volume of 50% trichloroacetic added to 5 volumes of the solution. The inactive precipitate was removed, the solution lyophilized, re-dissolved and subjected to electrophoresis. Progressive concentration of the enzyme at various stages of fractionation are shown in Table VI.

Electrophoresis

A simple apparatus was designed (Plate I) for the purpose of electrophoresis. This proved quite satisfactory for the electrophoresis of relatively large volumes of materials, especially when the components were coloured.

In a phosphate buffer (0.008 M NaH_2PO_4-0.064 M Na_2HPO_4) of pH 7.7, the active fraction migrated to the cathode when the applied potential was 130 volts and the current flowing through the solution was 10 milliamperes. The active fraction

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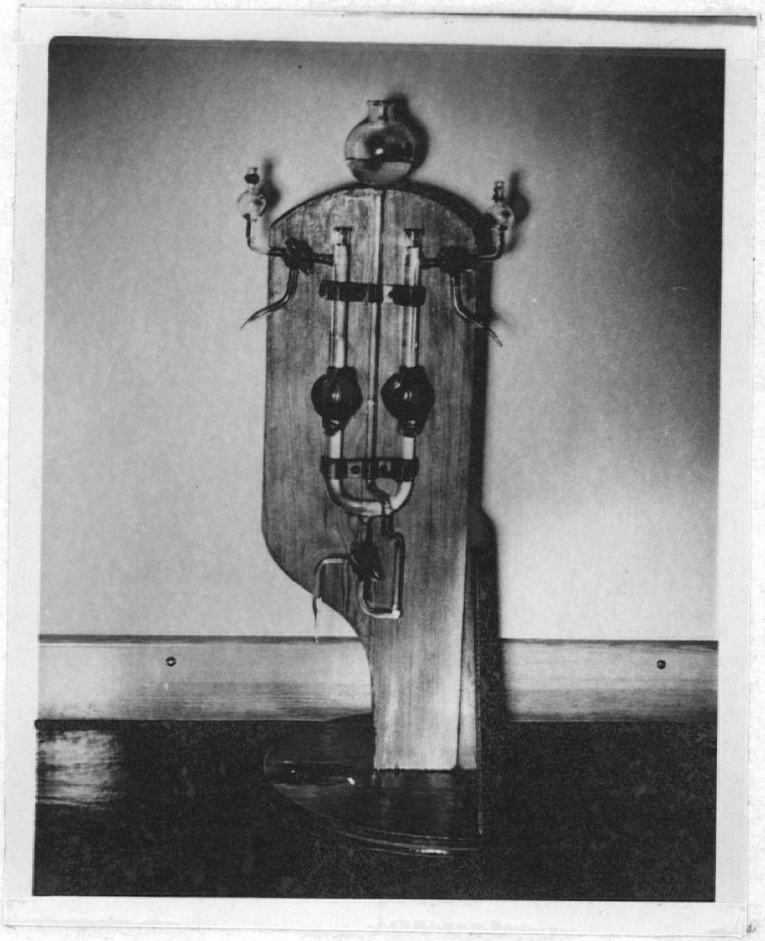

Plate 1 - Electrophoretic apparatus designed in the laboratory.

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TABLE VI

Lipoxidase activity at different stages of fractionation of the extract.

Fractionation	Total N_2	Total units	Units/mg.N_2
1. 60% Ammonium sulphate	40,000 mg.	2,040,000	51
2. pH 6.8	13,160 "	1,250,000	95
3. 16% Ethyl alcohol	4,835 "	870,000	180
4. precipitation with basic lead acetate	2,250 "	540,000	56)* 63) 240
5. precipitation with 60% ethyl alcohol	1,160 "	370,000	320
6. 50% sodium chloride	734 "	290,000	395
7. Trichloroacetic acid	512 "	230,000	450
8. Electrophoresis	80 "	96,000	1200

* Separation of activator from enzyme took place at this stage.

was pipetted out, dialysed, and dried under vacuum. This was later subjected to electrophoresis in a Tiselius electrophoretic apparatus at the Biophysics Laboratories of the University of British Columbia. The results of these experiments are shown in Table VII, and Fig. 5, 6 and 7. The three peaks were given by the enzyme concentrate obtained after fractionation with trichloroacetic acid. The other three patterns showing only one peak each were obtained with the active fraction separated by electrophoresis in the apparatus designed in the laboratory. From the electrophoretic patterns it appears that the isolated enzyme is a single substance.

Attempts were made to crystallize the enzyme from the electrophoretically separated highly active fraction. The enzyme failed to crystallize from alcohol and acetone at 0°C. Similar attempts to crystallize the enzyme by dialysing its concentrated solution against saturated ammonium sulphate solution also failed.

Purification of activator

The activator precipitate obtained at 60% concentration of alcohol was dissolved in a minimum amount of water, an equal volume of 50% ammonium sulphate was added, and the mixture held at 60°C. for one half hour; a precipitate formed. The mixture was refrigerated overnight and centrifuged. Both the precipitate 'a' and the filtrate 'b' exhibited activating properties. The precipitate 'a' was dissolved in water, brought to pH 6.8-7.0 with dilute ammonia, and sodium chloride added up to a concentration of 50%. The activator precipitated

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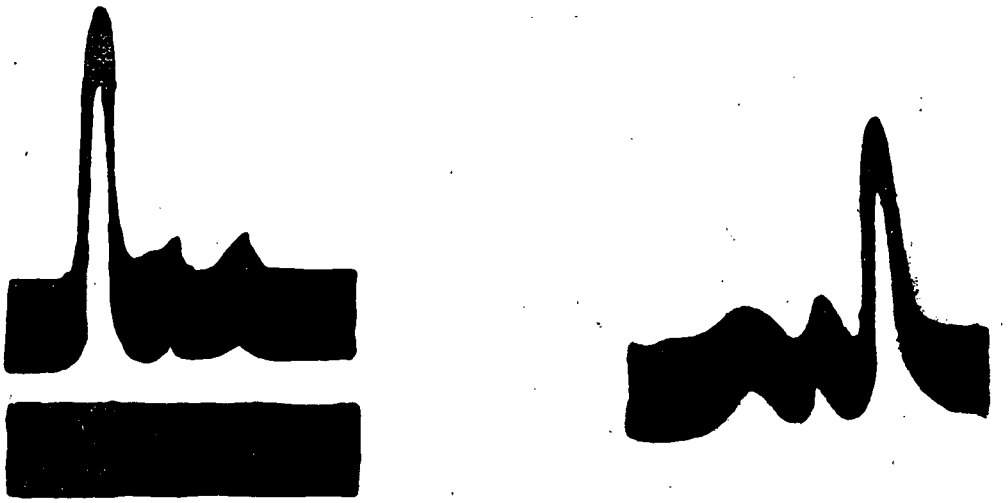
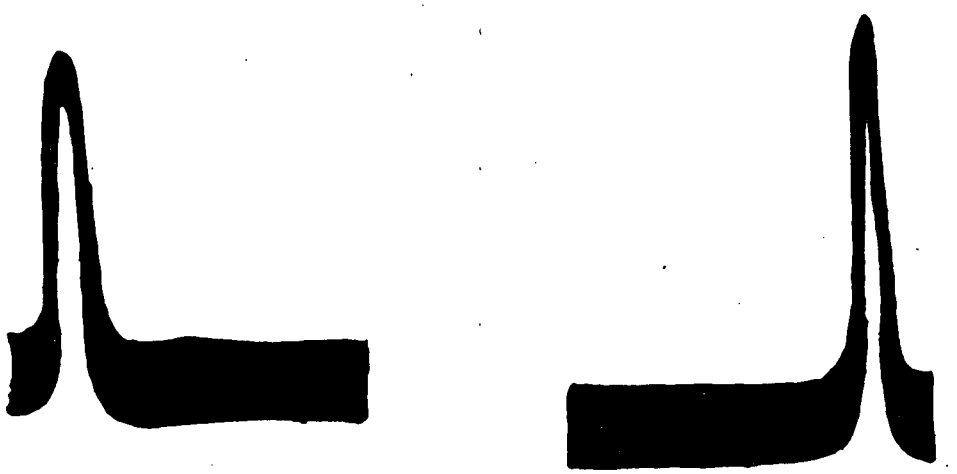


(A) (B)

Fig. 5 - Electrophoretic pattern of enzyme concentrate obtained after fractionation with trichloracetic acid. A. Ascending limb. B. Descending limb. (The lower Figure is the empty cell and the upper is the cell filled with enzyme solution).



(A) (B)

Fig. 6 - Electrophoretic pattern of herring lipoxidase in diethylbarbiturate buffer. A. Ascending limb. B. Descending limb.

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TABLE VII

Electrophoretic analysis of herring lipoxidase

Buffer	pH at 25°C.	Concentration of material	Time of electrophoresis	Number of peaks
0.008 M NaH_2PO_4 - 0.064 M Na_2HPO_4	7.7	1.0%	90 mins.	3
0.008 M NaH_2PO_4 - 0.064 M Na_2HPO_4	7.7	0.2%	"	1
0.1 N NaBar* - 0.02 N HBar	8.6	0.2%	"	1
0.1 N NaCac+ - 0.02 N HCac	6.8	0.2%	"	1

*Bar = diethylbarbiturate +Cac = Cacodylate

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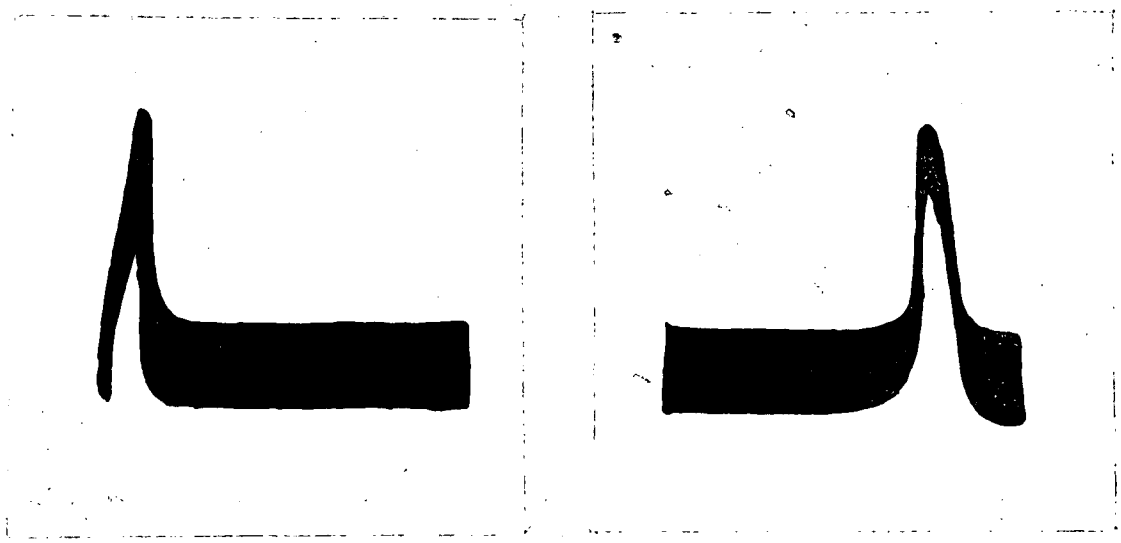

(A) (B)

Fig. 7 - Electrophoretic pattern of herring lipoxidase in cacodylate buffer. A. Ascending limb. B. Descending limb.

out. This was centrifuged, dissolved in water and then dialysed. Biuret, Millon's, xanthoproteic, ninhydrin and thiocyanate tests were positive. Tests for sulphur, halogens, phosphorus and copper were negative. The material contained less than 0.30% iron. This compound showed absorption maxima at 5450 Å and at 5630 Å, following reduction with $Na_2S_2O_4$. The filtrate 'b' was treated with one tenth of its volume of 90% trichloroacetic acid and the solution was refrigerated for 2 hours, then centrifuged. The active sediment was brought to a solution by stirring with 0.1 M NaOH and the solution diluted to a suitable volume with distilled water. This solution showed positive biuret, Millon's, xanthoproteic, ninhydrin and thiocyanate tests. Sulphur, halogens, phosphorus and copper were absent. The iron content was less than 0.35%. The absorption maximum was near 5560 Å, following reduction with $Na_2S_2O_4$. The results of absorption experiments appear in Tables VIII and IX, and Fig. 8 and 9.

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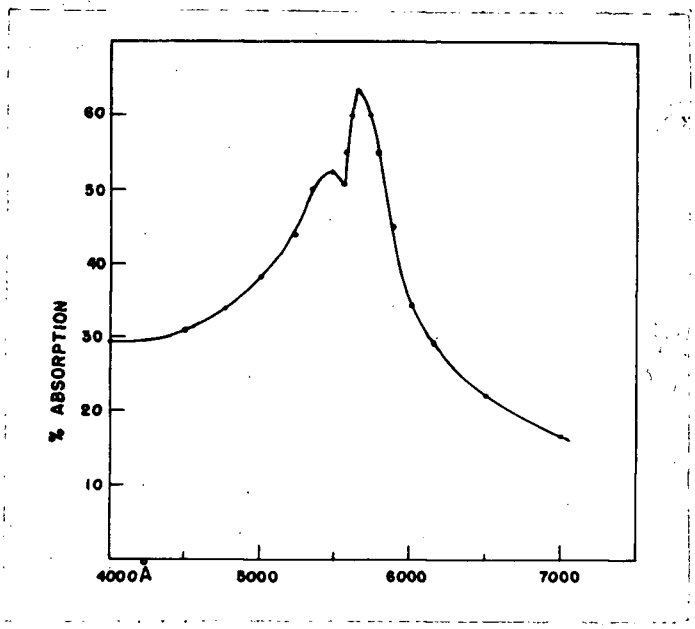

Fig. 8 - Absorption spectrum of activator 'a'. (0.2% solution).

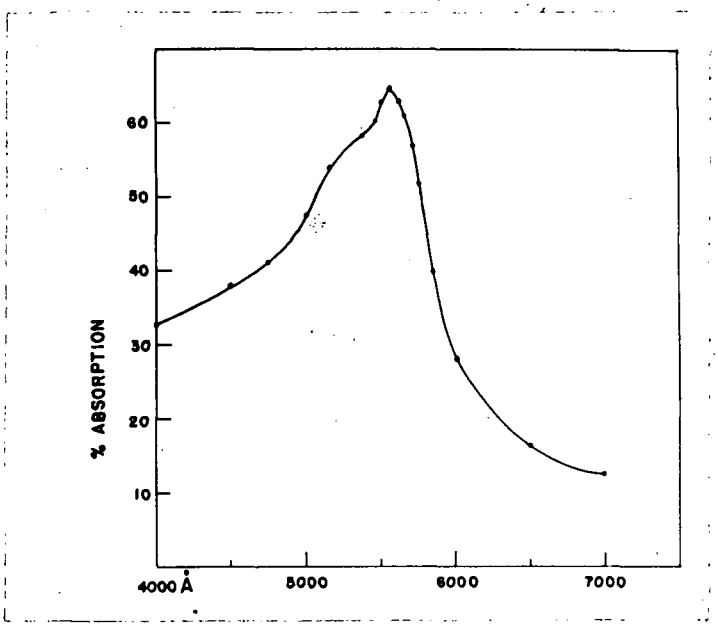

Fig. 9 - Absorption spectrum of activator 'b'. (0.2% solution).

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TABLE VIII

Absorption Spectrum of activator 'a'

Wavelength in Å units	% absorption in 0.2% solution	Wavelength in Å units	% absorption in 0.2% solution
4000	29.0	5580	58.1
4250	29.5	5600	60.3
4500	30.6	5610	61.2
4750	33.4	5620	62.3
5000	38.1	5630	63.4
5100	40.4	5640	63.0
5200	43.2	5650	62.6
5300	48.0	5660	62.2
5400	51.9	5680	62.0
5420	52.2	5700	59.0
5440	52.4	5800	50.5
5450	52.5	5900	42.0
5460	52.3	6000	36.0
5480	52.2	6250	27.5
5500	52.0	6500	22.5
5520	51.8	6750	18.8
5540	50.8	7000	17.0
5560	50.8		

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TABLE IX

Absorption Spectrum of activator 'b'

Wavelength in Å units	% absorption in 0.2% solution	Wavelength in Å units	% absorption in 0.2% solution
4000	32.6	5550	65.0
4250	35.0	5560	65.4
4500	37.6	5570	65.0
4750	41.0	5580	64.5
5000	47.2	5590	64.1
5100	51.3	5600	64.0
5200	54.4	5700	58.0
5300	56.8	5800	45.0
5400	58.2	5900	33.5
5500	61.8	6000	28.4
5510	62.2	6250	20.8
5520	62.6	6500	16.7
5530	63.3	6750	14.0
5540	64.2	7000	12.0

OBSERVATIONS

The slight activity exhibited by the enzyme fraction after removal of the activators might have been due to possibly incomplete removal of the activators, since with further fractionation it was evidenced that the enzyme alone failed to peroxidise linoleic acid, while addition of a small amount of the activator fraction restored the activity of the enzyme. It was evident, therefore, that the lipoxidase concerned acts only in conjunction with an activator or activators. The character of the activators is discussed elsewhere.

Tests performed in the course of the present investigation showed that this enzyme gave positive biuret, Millon's xanthoproteic and ninhydrin tests while tests for sulphur, halogens, phosphorus, iron and copper were negative. The protein nature of the enzyme is further evidenced by its inactivation with pepsin and trypsin. The enzyme is also inactivated by boiling. As such, the lipoxidase in herring flesh is a heat-labile enzyme exhibiting protein characters, which requires an activator for its activity. As shown elsewhere (Table XIX), several compounds can act as activator although their efficiency varies from compound to compound. Two such activators have been isolated from herring flesh.

Accordingly, in studying the characteristics of the enzyme, an optimum amount of the activator was added to the enzyme.

The effect of pH upon the enzyme activity

Sodium linoleate was used as substrate and the pH was adjusted to different values from 4-9 by the addition of NaOH or HCl. The activities were measured by the method employed for the standardization of the enzyme. The relative activities of the enzyme at various pH values are shown in Table X and Fig. 10.

It will be observed that the enzyme is most active around pH 6.9, but the activity falls much more quickly on the acid side of the pH than on the alkaline side. It is likely that this effect is due to the relative inaccessibility of substrate in the emulsion systems existing on the acid side of the pH, while high alkalinity possibly inactivates the enzyme.

The effect of temperature on the enzyme activity

To determine the effect of temperature upon the rate of reaction, a series of trials were made at each of several temperatures, using equal quantities of enzyme together with an optimum amount of the activator. The relative activities expressed as the optical density at 2380 Å ($\log I_o/I$) developed in two minutes reaction time are shown in Table XI and Fig. 11. It must be pointed out that the rapid decrease in reaction rate above 20°C. does not represent a thermal destruction of the enzyme, for the enzyme endured a much higher temperature for a longer period of time in its preparation without much loss in activity. The decrease in activity at higher temperatures might possibly be due to an inactivation of the enzyme in contact with substrate or reaction products.

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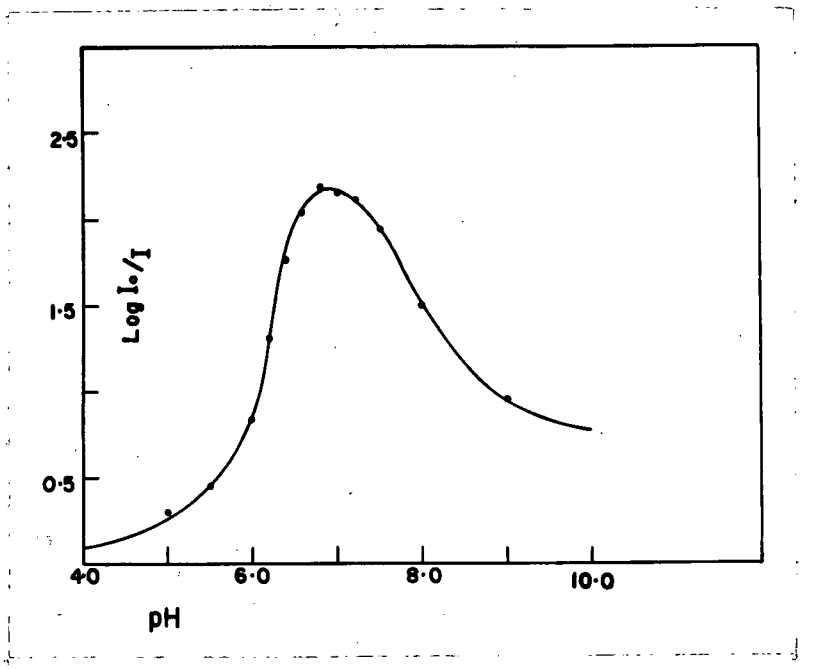

Fig.10 - Effect of pH on the activity of herring lipoxidase. (2 mg. linoleic acid and 0.01 mg. of enzyme).

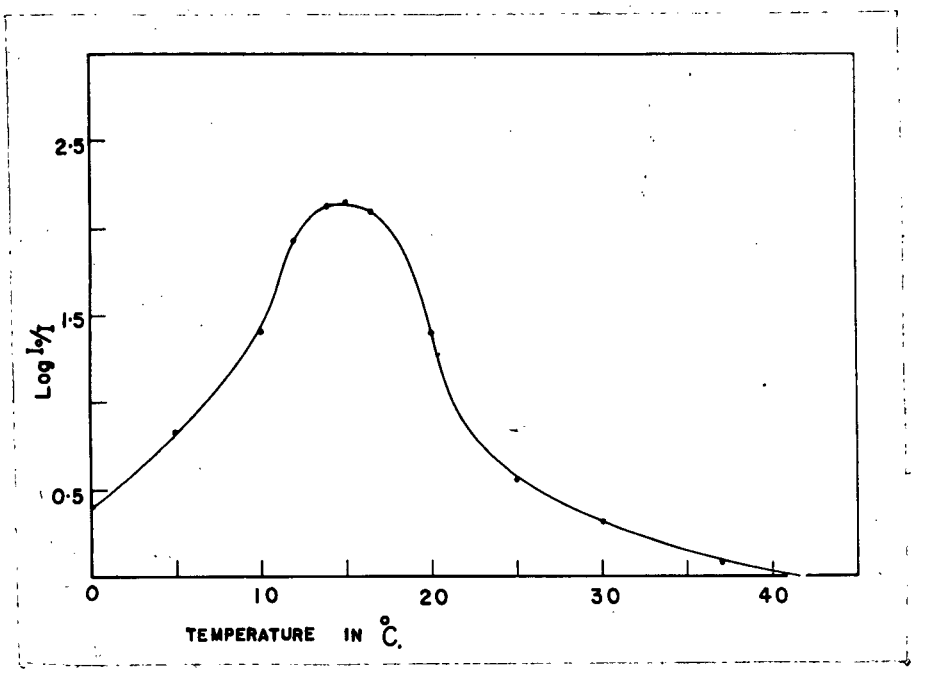

Fig. 11 - Effect of temperature on herring lipoxidase activity. (2 mg. linoleic acid and 0.01 mg. of enzyme.

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TABLE X

Effect of pH on the activity of herring lipoxidase (2 mg. linoleic acid and 0.01 mg. enzyme).

pH	log I_o/I at 2380 Å
4	0.1
5	0.3
5.5	0.45
6.0	0.83
6.2	1.30
6.4	1.78
6.6	2.08
6.8	2.17
7.0	2.14
7.5	1.95
8.0	1.54
8.5	1.16
9.0	0.95

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TABLE XI

Effect of temperature on herring lipoxidase activity. (2 mg. linoleic acid and 0.01 mg. enzyme).

Temperature ° C.	log I_o/I at 2380 Å
0	0.4
5	0.84
10	1.40
12	1.96
14	2.14
15	2.18
16	2.12
18	1.95
20	1.42
25	0.56
30	0.32
37	0.10
40	0.02
45	0.00

Effect of substrate and enzyme concentration on the enzyme activity.

In the presence of an optimum amount of enzyme and activator, the activity is a function of the substrate concentration. Thus in the presence of 0.01 mg. enzyme and 8 x 10^{-4} g. of activator 'a' the reaction is monomolecular up to a linoleic acid concentration of 2 mg. per ml., but in solutions between about 4-7 mg. per ml. of linoleic acid there is little or no influence of substrate concentration on enzyme activity. In other words, the reaction rate is of zero order. At higher concentrations of substrate the activity again decreases. These results are shown in Table XII and Fig. 12 .

Similar results are obtained when the concentrations of the substrate and the activator (in optimum amounts) are kept constant and that of the enzyme is varied. At concentrations below 0.01 mg. of enzyme per ml. of 0.200% linoleic acid the activity is directly proportional to the enzyme concentration. If the enzyme concentration is further increased, the reaction rate is not influenced; while with quantities of more than 0.10 mg. of enzyme the reaction rate decreases. These results are shown in Table XIII and Fig. 13.

Enzyme activators

The effects of various amounts of both activator 'a' and activator 'b' as well as of mammalian haematin, haemaglobin and cytochrome 'c' were studied. The effect was observed by measuring the total optical density produced at 2380 Å under the conditions similar to those employed in the

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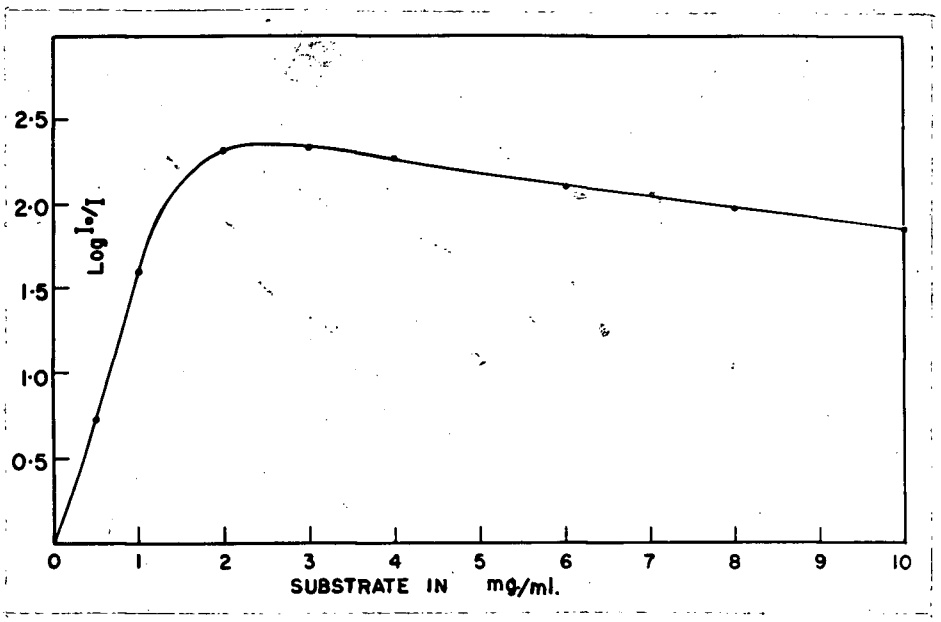

Fig. 12 - Effect of substrate concentration on enzyme activity. (0.01 mg. enzyme).

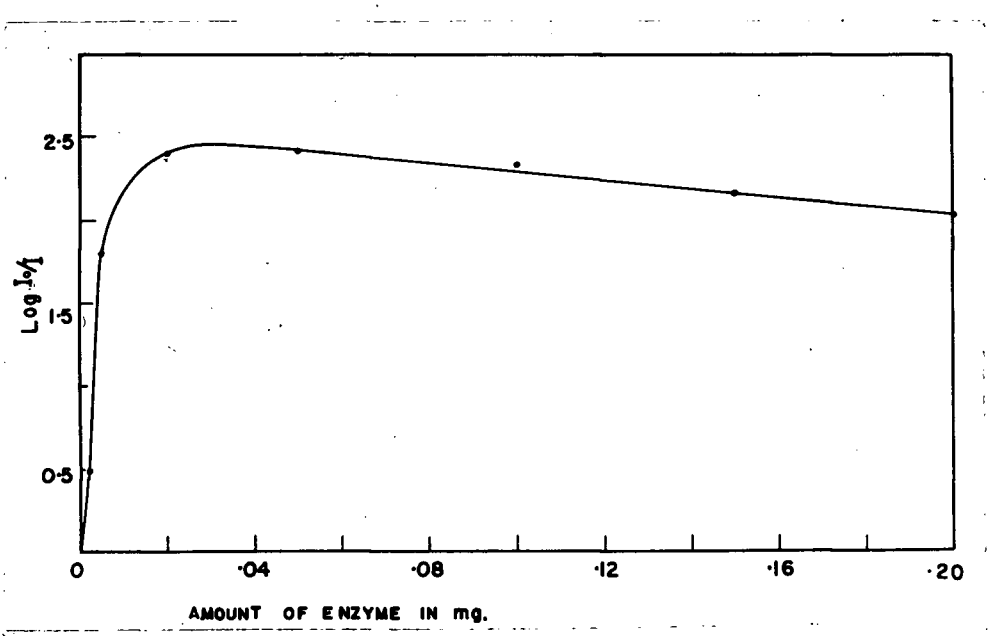

Fig. 13 - Effect of enzyme concentration on the activity of herring lipoxidase. (2 mg. linoleic acid).

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TABLE XII

Effect of substrate concentration on the activity of herring lipoxidase.

Amount of enzyme = 0.01 mg. Activator = 8 x10^{-4} g.

Linoleic acid per ml.	log I_o/I at 2380 Å
0.5	0.73
1.0	1.60
2.0	2.32
3.0	2.30
4.0	2.26
6.0	2.10
8.0	1.95
10.0	1.85
15.0	1.6
20.0	1.2

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TABLE XIII

Effect of enzyme concentration on the activity of herring lipoxidase. (2 mg. linoleic acid).

Amount of enzyme	log I_o/I at 2380 Å
0.002 mg.	0.50
0.005	1.80
0.010	2.32
0.020	2.40
0.050	2.42
0.100	2.34
0.150	2.16
0.200	2.02
0.500	2.00
0.725	1.90
1.000	1.80

standardization of the enzyme. Activator 'b' is roughly 1.6 times as active as activator 'a' (Table XIX, XV and XVI). The amount of activator needed to produce maximum peroxide formation in a given time depends upon the quantity of fat present. If activator and the enzyme are both held constant, the maximum peroxide formation occurs in the presence of a definite quantity of fat (Table XVII and Fig. 14). Either increase or decrease in the amount of fat results in a decrease in the peroxide formed. However, when the amount of activator was increased together with fat, the rate of oxidation increased with substrate concentration. The proportion of activator may, therefore, be increased to compensate for an increase in fat. Large proportions of activator are apparently inhibitory (Table XVII and Fig. 15), giving in effect a broad optimum of activator concentration. Furthermore, it is shown that the optimum amount of activator required to produce maximum peroxide formation at a given time is independent of the amount of enzyme present (Table XVIII and Fig. 16). Relative activities of various activators are shown in Table XIX.

Enzyme inhibitors

The activities in presence of inhibitors were determined in the manner described but using 0.5 ml. of substrate containing 4 mg. linoleic acid per ml. and 0.5 ml. of the appropriate inhibitor solution. Use of thiocyanate determination was necessary in cases where ultra-violet absorption of the inhibitor was too high in the blanks.

Effects of some commonly employed enzyme inhibitors

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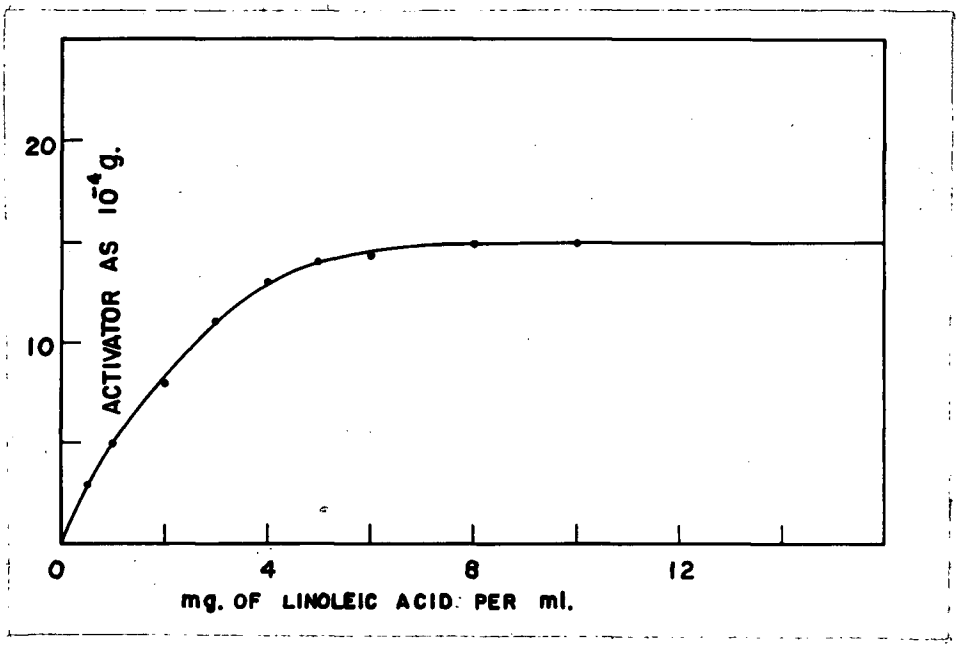

Fig. 15 - Optimum amount of activator at different substrate concentrations. Enzyme concentration is constant (0.01 mg.).

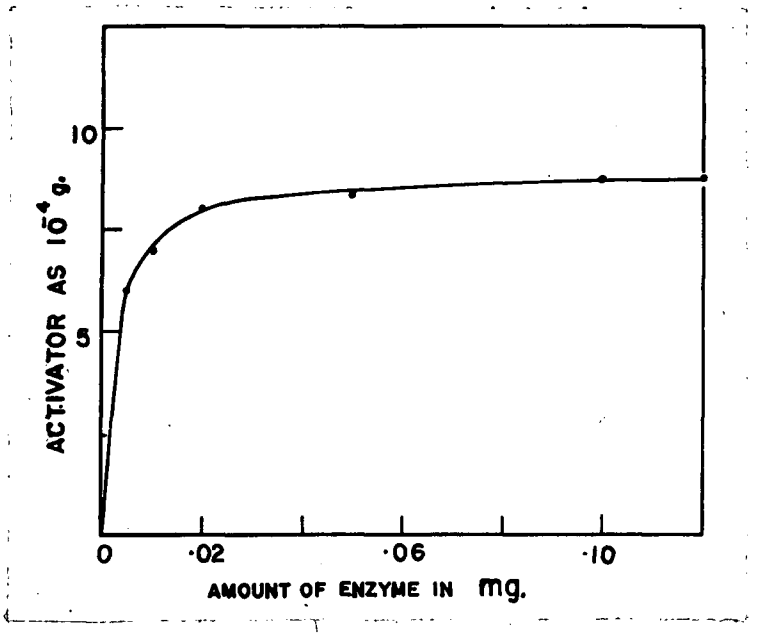

Fig. 16 - Optimum amount of activator at different enzyme concentrations. Substrate concentration constant (2mg.).

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TABLE XIV

Enzyme activity at different concentrations of activator 'a'. Reaction time was 2 minutes in presence of 2 mg. of linoleic acid and 0.01 mg. of herring lipoxidase.

Experiment	Amount of activator	log I_o/I at 2380 Å
A	2×10^{-4} gm.	1.65
(2 mg. substrate .010 mg. Enzyme)	$4 \times$ "	2.02
	$6 \times$ "	2.30
	$8 \times$ "	2.32
	$10 \times$ "	2.30
Reaction time =2 minutes	$15 \times$ "	2.00
	$20 \times$ "	1.60

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TABLE XV

Enzyme activity at different concentration of activator 'a'. Reaction time was 2 minutes in presence of 2 mg. of linoleic acid and 0.05 mg. of enzyme.

Experiment	Amount of activator	log I_o/I at 2380 Å
B (2 mg. subs .050 mg. enzyme Reaction time 2 minutes)	2×10^{-4} gm.	6.32
	4×10^{-4} "	8.25
	6×10^{-4} "	10.20
	8×10^{-4} "	10.78
	10×10^{-4} "	10.60
	15×10^{-4} "	9.30
	20×10^{-4} "	8.95

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TABLE XVI

Effect of the concentration of activator 'b' on enzyme activity. Reaction time was 2 minutes in presence of 2 mg. of linoleic acid and 0.01 mg. of enzyme.

Amount of 'b'	log I_o/I at 2380 Å
2×10^{-4} g.	1.2
3 x "	1.8
4 x "	2.12
5 x "	2.56
6 x "	2.60
8 x "	2.70
10 x "	2.5
15 x "	2.2
20 x "	1.9

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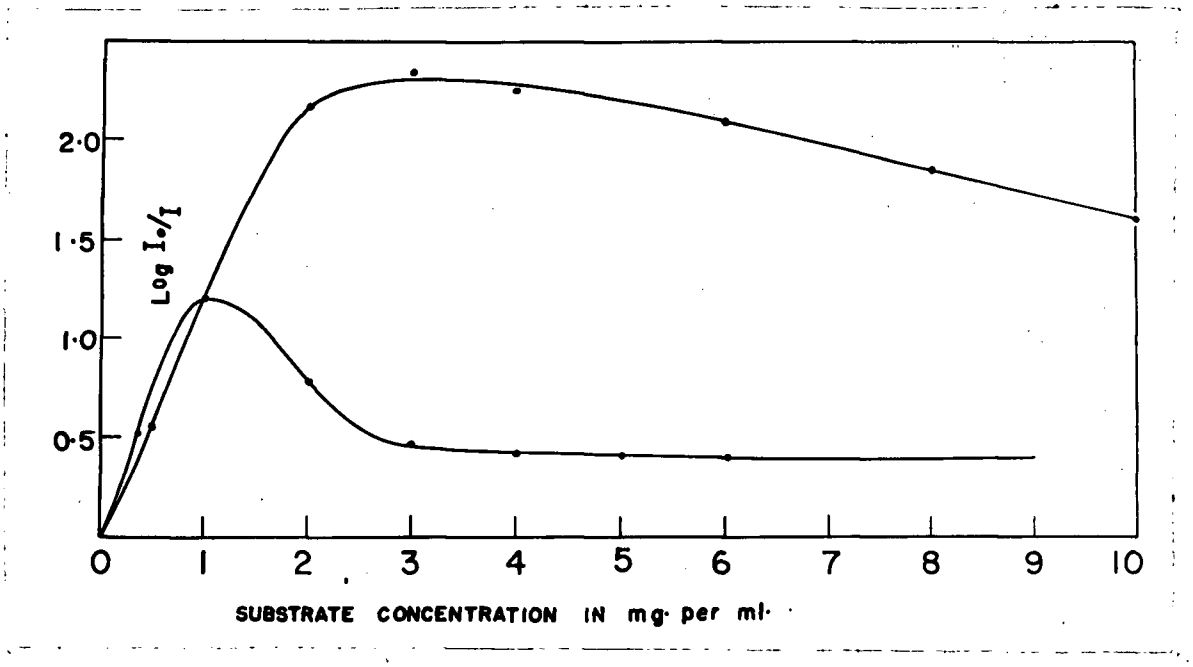

Fig. 14 - Effect of substrate concentration on the activators. The upper curve represents activity at different substrate concentrations in presence of their respective optimum quantity of enzyme and activator 'a'. The lower curve shows the effect of substrate concentrations in presence of an optimum amount of enzyme and activator 'a' for 1 mg. substrate.

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TABLE XVII

Effect of substrate concentration on the optimum amount of activator 'a' necessary for maximum enzyme activity. Amount of enzyme added was 0.01 mg.

Concentration of substrate/ml.	Optimum amount of activator 'a'	log I_o/I at 2380 Å
0.5 mg.	3 x 10^{-4} gm.	0.6
1.0 "	5 x "	1.40
2.0 "	8 x "	2.32
3.0 "	11 x "	2.12
5.0 "	13 x "	2.0
6.0 "	14 x "	1.96
8.0 "	14.6 x "	1.85
10.0 "	15.0 x "	1.75

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TABLE XVIII

Effect of enzyme concentration on the activity of activator 'a'

Linoleic acid/ml.	Amount of enzyme	Optimum amount of activator 'a'
2.0 mg.	0.005 mg.	6×10^{-4} gm.
"	0.01 "	7.0 x "
"	0.05 "	8.3 x "
"	0.10 "	8.8 x "
"	0.50 "	8.6 x "
"	1.00 "	9.0 x "

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TABLE XIX

Relative activity of various activators in presence of 0.01 mg. enzyme and 2 mg. linoleic acid.

Activator	Optimum amount
Dialysable breakdown product of activators	21×10^{-4} gm.
Activator 'a'	8 x "
Activator 'b'	5 x "
Haematin (mammalian)	23 x "
Haemoglobin "	10.3 x "
Cytochrome 'c' "	6.7 x "
Fe^{++}	inactive
Fe^{+++}	"

are shown in Table XX, XXI and Fig. 17. Cyanide inhibits the lipoxidase catalysed reaction to a pronounced degree at all concentrations of the inhibitor. Azide exhibits considerable inhibitory effects only at higher concentration, while fluoride and arsenite are slightly inhibitory at higher concentrations. Iodoacetate exhibits very little or no inhibitory effects at all concentrations. At lower concentrations, however, fluoride, arsenite and azide cause little or no inhibition.

Irrespective of whether activator 'a' or 'b' is present in the enzymic reaction these effects of the inhibitors are similar in character although they vary slightly in degree. As for example when activator 'a' is present with 0.5 M cyanide no activity of the enzyme is left while under similar conditions in presence of activator 'b' about 10% of the enzymic activity persists. At the time the experimental work had to be discontinued it is still uncertain whether the reduced activity is due to the inhibition of the enzyme proper or of its activator. But since the enzyme does not contain any heavy metal, while the activators are characterised by the presence of iron in organic combination, it would seem possible that the inhibition by cyanide and azide is due to the inactivation of the activators rather than of the enzyme proper. The failure of iodoacetic acid to inhibit the activity and also the fact that the nitroprusside and other tests for sulphur were negative, rule out the sulphydryl groups as active centres.

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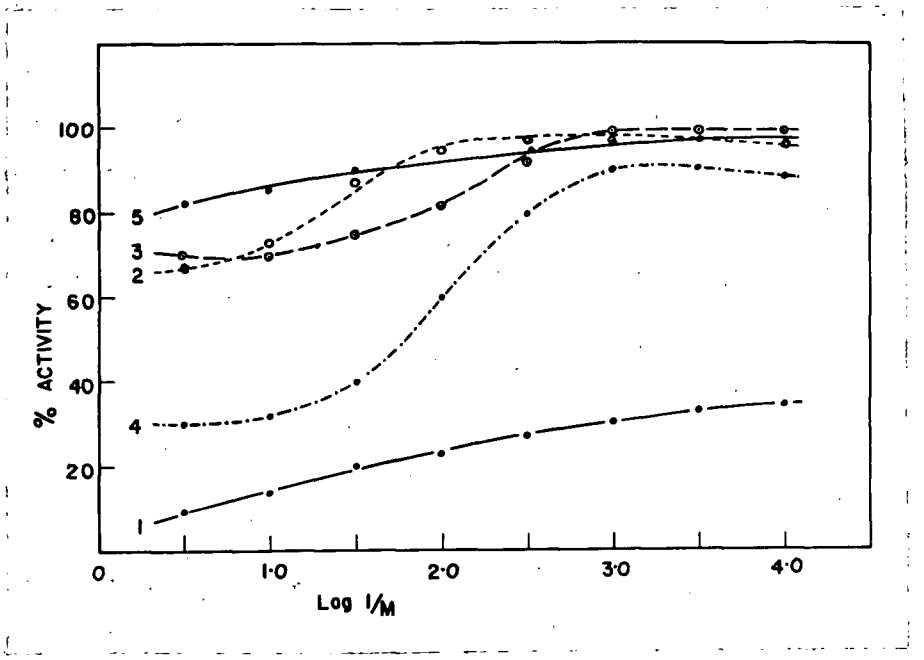

Fig. 17 - Effect of inhibitors on herring lipoxidase activity in presence of activator 'b', and 2 mg. linoleic acid and 0.01 mg. enzyme. 1. Cyanide. 2. Fluoride. 3. Arsenite 4. Azide. 5. Iodoacetic acid.

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TABLE XX

Effect of inhibitors on herring lipoxidase activity in presence of activator 'b' (2 mg. linoleic acid and 0.01 mg. enzyme).

Concentration as -log M	Percent activity				
	Cyanide	Fluoride	Arsenite	Azide	Iodoacetic acid
0.0					
0.5	9.0	67	70	30	83
1.0	14.0	73	70	32	85
1.5	20.0	87	75	40	90
2.0	23	90	83	60	91.0
2.5	27	95	92	81	93.0
3.0	30	95	98	89	96
3.5	33	96	98	90	96
4.0	34.2	98	99	92	96

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TABLE XXI

Effect of inhibitors on the enzyme activity in presence of activator 'a'. (2 mg. linoleic acid and 0.01 mg. enzyme).

Concentration as - log M	Percent activity				
	Cyanide	Fluoride	Arsenite	Azide	Iodoacetic acid
0.5	0.5	57	67	20	88
1.0	7.5	68	64	20.5	94
1.5	14.8	84	68	26	96
2.0	20.4	93	76	38	96
2.5	26.3	96	88	69	98
3.0	29.0	98	96	83	98
3.5	32.8	98	96	86	99
4.0	35.6	98	96	86	99

DISCUSSION

From the results it becomes evident that herring flesh contains a proteinaceous, heat-labile enzyme capable of peroxidizing naturally-occurring unsaturated fatty acids, particularly those possessing methylene-interrupted double bonds. This enzyme exhibits optimum activity at 15°C. and at pH 6.9 but fails to catalyse unless some iron-containing organic heat-stable activator is present along with it. The enzyme which is here termed 'lipoxidase' is characterised by an absence of heavy metals and sulphydryl group as the active centre. The inhibition of the enzymic reaction brought about by cyanide and azides is possibly due to the inactivation of the iron-containing proteinaceous activators rather than of the enzyme itself.

It must, however, be emphasized that the accessory substances here, termed 'activators', do not bring about the activation of the enzyme proper. In other words, the 'lipoxidase' is isolated in an active form, not as a zymogen; because if the enzyme were present as a zymogen and the compounds which are here called 'activators' were kinases, the alleged zymogen of lipoxidase would long have been converted to its active form since in the initial stages of purification of the enzyme the activators (or kinases) and the lipoxidase (or zymogen) were in contact with each other for a protracted period.

The activators appear to be conjugated proteins in combination with an iron-containing prosthetic group. This

group is rather firmly attached to the protein portion. However, the former can be removed from the latter by treatment with moderately strong alkalis and acids.

The absorption spectrum of the prosthetic group of the activators resembles very closely that of the mammalian haematin. From this evidence and also from the fact that the prosthetic group is an iron-containing non-protein nitrogenous compound, it is believed that the concerned component of the activators is haematin.

The absorption spectrum of activator 'a' resembles fairly closely that of mammalian haemoglobin. The slight deviations of the above spectrum from that of mammalian haemoglobin might possibly be due to differences in particle size, structure, and composition of the protein fraction of activator 'a'. In all probability, therefore, activator 'a' is herring haemoglobin. Further evidence of the haemoglobin character of activator 'a' is provided by the fact that the activity of herring lipoxidase is augmented by mammalian haemoglobin to a degree closely similar to that caused by activator 'a' (Table XIX).

Unlike the absorption spectrum of activator 'a', that of activator 'b' does not so closely resemble the absorption spectrum of any known haemochromogen. However, the maximum lies within the range for the cytochromes and is situated closer to the maximum for mammalian cytochrome 'c' than to that for cytochrome 'b'. This phenomenon could be explained on the assumption that the protein fraction of herring cyto-

chrome has a molecular size, composition and structure different from the protein fraction of mammalian cytochrome.

In inhibition experiments with cyanide it was found that even at 0.5 M concentration of the inhibitor about 8% of the activity persisted. It is known that cytochrome 'c' is completely inhibited at such a concentration while cytochrome 'b' is inhibited up to about 90%. Furthermore, the activity of mammalian cytochrome 'c' towards the herring lipoxidase-catalysed reaction is about 75% that of activator 'b' (Table XIX).

On the basis of the evidence obtained it is believed that activator 'b' is herring cytochrome 'c' possibly contaminated with herring cytochrome 'b'.

Although prosthetic group alone of the activators exhibits activity, its combination with a protein greatly modifies the degree of activity. At the necessitated termination of this study it is uncertain what specific role these activators play. But it would seem plausible to assume that due to the presence of the haematin fraction in their molecules, these compounds constitute an oxidation-reduction system. The activators possibly accept an atom of hydrogen from linoleic acid at stage I (see "Reaction mechanism") and donate the hydrogen atom back at stage V or VI.

In concluding the above discussion of the investigation accomplished, the writer wishes to point out possible extension of this work that might be of interest to future investigators who may at some time have an opportunity of

persuing further the ultimate solution of this complicated problem concerning the complete mechanism of the reactions involved in the rancidification of fats. Information regarding the molecular size, isoelectric point, and other physical properties as well as the chemical constitution of the enzyme and also of the activators, will make possible a better understanding of the enzymic reactions.

It would also be interesting here to consider the possible use of the information made available in this dissertation in controlling rancidity development in frozen fish foods. Any physical or chemical measures to inactivate the enzyme or its activators, or to exclude oxygen necessary for the enzymic reaction to progress, would serve the purpose. From the practical point of view, as well as from economic considerations, low storage temperatures to arrest the activity of the lipoxidase, or the employment of chemical antioxidants to make oxygen unavailable to the system, would seem logical. In employing chemical antioxidants the physiological toxicity of the added chemicals must be considered scrupulously. Among the commonly employed antioxidants, ascorbic acid, reductinic acid, α-tocopherol, the gallates and similar compounds seem effective and acceptable. Tarr (1947) reported the comparative values of a number of antioxidants in delaying the onset of rancidity in indigenous fats of fish. Similar reports were also made by Tarr and Cooke (1947) and Khan (1948), who studied the effects of certain cyclic and acyclic carbonyl enediols in retarding rancidity when fish fillets and steaks

are dipped in solutions of these compounds prior to freezing, as well as when these compounds are incorporated in the glaze. However, a further discussion of this subject goes beyond the intended scope of the present dissertation.

SUMMARY

1. Three different methods of estimating lipoxidase activity in herring flesh were investigated. The ultra-violet light absorption method was adopted with certain modifications.

2. Previous reports that lipoxidase in herring is concentrated in the dark muscle along the lateral lines and that the flesh exhibits little or no lipoxidase activity were confirmed. Different lots of herring were found to possess different lipoxidase activity.

3. From the dark muscle along the lateral lines in the body of the herring an enzyme was isolated by different physical and chemical methods of fractionation and finally by electrophoresis. Attempts to crystallise the enzyme failed. The isolated enzyme is over two thousand times as active as the raw material.

4. The herring 'lipoxidase' is a proteinaceous heat-labile enzyme capable of peroxidising naturally-occurring unsaturated fatty acids and fatty acid esters, particularly those possessing methylene-interrupted double bonds. The lipoxidase exhibits optimum activity at 15°C. and at pH 6.9 but fails to catalyse unless some iron-containing organic heat-stable activator is present. The enzyme is characterised by an absence of heavy metals and sulphydryl group as the active centre.

5. Among the activators for herring lipoxidase, two compounds were isolated from herring flesh. These compounds are conjugated proteins combined with an iron-containing nitrogenous prosthetic group. This group, which exhibits maximum absorption near 4480 Å and at 5610 Å, is believed to be haematin. The activator 'a', exhibits two absorption maxima, one at 5450 Å and the other at 5630 Å, while activator 'b', shows maximum absorption near 5560 Å. On the basis of evidence obtained activator 'a' is believed to be herring haemoglobin. Activator 'b' is considered to be herring cytochrome 'c' possibly contaminated with cytochrome 'b'.

6. There is an optimum concentration of enzyme, substrate, and also of the activators for maximum enzyme activity. The presence of activators appears to change the kinetics of the oxidation of the substrate.

7. The inhibition of the enzymic reaction brought about by cyanide and azide is possibly due to the inactivation of the iron-containing proteinaceous activators rather than of the enzyme itself.

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APPENDIX

Extraction Experiments

Extraction at pH	Amount of extract as gm. of N_2 per 100 gm. tissue	Activity as 10^{-6}gm. Fe^{+++} per gm. N_2 of extract	Total activity as 10^{-6} gm. of Fe^{+++}
4.0	0.9	0.0	0.
5.0	1.6	3.20	51.2
5.2	1.9	8.42	159.6
5.4	2.4	83.40	200.2
5.6	2.7	92.60	250.0
5.8	3.0	76.66	229.8
6.0	3.2	62.5	200.
7.0	3.6	50.0	180.
8.0	3.7	32.40	119.9
9.0	4.0	22.5	90.

FRACTIONATION EXPERIMENTS

Stage I: $(NH_4)_2 SO_4$

Concentration of $(NH_4)_2SO_4$ in extract	Amount of ppt. as gm. of N_2 per 100 gm. tissue	Activity in units/mg. N_2	
		ppt.	Filtrate
20%	0.4	Inactive	36.5
30%	0.9	"	46.6
35%	1.05	"	50.9
40%	1.4	20.8	42.2
45%	1.45	31.7	30.5
50%	1.80	41.	12.4
55%	2.3	34.8	9.6
60%	2.56	32.5	5.2
65%	2.62	31.9	2.1

Stage II: Fractionation with $NH_4(OH)$ at various pH.

pH	Amount of ppt. as gm. of N_2	Activity in units/gm. N_2	
		ppt.	Filtrate
5.6	0.00	-	31.1
6.0	0.20	Inactive	35.4
6.5	0.75	"	57.0
6.7	0.90	"	68.4
6.8	1.10	"	91.
7.0	0.90	5.6	61.73
7.5	0.82	4.8	64.5
8.0	0.50	5.6	42.2

Stage III: Ethyl Alcohol

Concentration of alcohol	Amount of ppt. as mg. N_2/kg. tissue	Activity in units/mg. N_2	
		ppt.	Filtrate
10%	60	Inactive	120.8
15%	170	"	156.8
16%	218	"	179.6
18%	227	50.2	132.1
20%	230	89.6	120.7
25%	243	108.2	103.2

Stage IV: Lead Acetate

Volume of		Activity in Units/mg. N_2		
Lead acetate	Enzyme solution	ppt.	Filtrate	ppt. + Filtrate
1	1	inactive	192	-
2	1	"	203	-
3	2	"	56	231.5
2	3	"	210.3	-
4	3	42	63	234.6

Stage V: 60% Ethyl Alcohol

Concentration of alcohol	Amount of ppt. as mg. of N_2	Activity in units/mg. N_2		
		ppt.	Filtrate	ppt. + Filtrate
20%	90	Inactive	93.5	106.5
30%	134	"	123.2	131.4
40%	190	"	157.1	187.2
50%	212	"	182.2	218.8
60%	245	"	Inactive	320.0
70%	263	26.8	"	320.0

Enzyme Proper: Fractionation with Nacl at various pH.

pH	Activity in units/mg. N_2 at Nacl concentration of							
	30%		40%		50%		55%	
	ppt.	Fil-trate	ppt.	Fil-trate	ppt.	Fil-trate	ppt.	Fil-trate
5.5	no ppt.	240	inactive	263	inactive	275	inactive	290
6.0	inactive	253	"	272	"	294	"	301
6.2	"	279	"	287	"	301	"	312
6.4	"	286	"	293	"	313	"	329
6.6	"	292	"	301	"	345	"	363
6.8	"	297	"	343	"	395	26	323
7.0	26	263	52	203	73	169	93	185
7.5	32	241	85	169	101	103	167	97

Trichloroacetic acid (50%)

Volume of		Activity in units/mg. N_2	
Trichloro-acetic acid	Enzyme solution	ppt.	Filtrate
1	7	inactive	450
1	6	"	450
1	5	"	450
1	4	26	412
1	3	53	360
1	2	134	286

Electrophoretic analysis of enzyme concentrate

Buffer	pH	Concentration of material	Time in minutes of electrophoresis	Number of peaks
0.008 M NaH_2PO_4 - 0.064 M Na_2HPO_4	7.7	1.0%	90	3
0.004 M NaH_2PO_4 - 0.032 M Na_2HPO_4	7.7	"	"	3
0.025 N $NaHCO_3$ - 0.1 N Nacl	8.2	"	"	2
0.1 N NaOH - 0.6 N Glycine	9.0	"	"	2
0.002 M Acetic acid - 0.2 M Na-acetate	6.5	"	"	3

Fractionation of activator 'b' with 90% trichloroacetic acid.

Ml. of 90% trichloroacetic acid per 100 ml. of activator solution.	Amount of activator as 10^{-4} gm. necessary for maximum activity in presence of 0.01 mg. enzyme.	
	ppt.	Filtrate
5.0	Inactive	12.0
10.0	5.0	Inactive
15.0	8.0	16.0
20.0	12.0	18.0