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Inhibition of Enzyme Activities by 12-Keto Oleic Acid[1]

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The effect of the long chain keto acid, which is one of the secondary products from oxidative deteriolation of lipids, on the enzyme activity was investigated *in vitro*. Cholinesterase and succinate dehydrogenase were inhibited more effectively by 12-keto oleic acid than by linoleic acid, whereas the inhibition of trypsin by linoleic acid was greater than that caused by 12-keto oleic acid. This fact may show that the enzyme inhibition by 12-keto oleic acid is not merely due to nonspecific denaturation.

The inhibitory effect of 12-keto oleic acid on acetylcholinesterase was not protected by the presence of α-tocopherol, while the inhibition by linoleic acid was completely restored when α-tocopherol was added simultaneously. When both linoleic and 12-keto oleic acids were added at the same time, the synergistic inhibition was observed, which was partially protected by α-tocopherol. Thus the inhibition caused by 12-keto oleic acid is not only the reflection of the stimulation of lipid peroxidation induced by 12-keto oleic acid, but the specific irreversible interaction with enzyme protein.

It is well known that oxidized fats cause various damages to the physiological constituentes and functions. The essential toxic substance in oxidized fats has been considered to be lipohydroperoxides which will give damages to SH enzymes,[3] cytochrome C,[4] and lipoproteins.[5] Therefore, the toxicity of oxidized fats must be generally enhanced when peroxide value is increased as the time proceeds during oxidation.[6] However, it is reported that though peroxide value of oxidized fats decreased, the toxicity did not always decrease. Namely, the change of their peroxide value is not always correlate with that of their oral toxicity.[7] It has been generally accepted that lipohydroperoxide itself is hardly absorbed through intestinal wall. These facts reveal that the toxic substance other than lipohydroperoxide will be present in the oxidized fats and it may be absorbed through intestinal wall. The identification of absorbable oxidation products has been made, among which it is known that hydroxy- and epoxy-acids were not toxic.[8] As to long chain keto acid, many investigators reported the production of various keto acids in oxidized fats under different conditions.[9–11] Some of those are efficiently absorbed through intestinal wall.[12] In the preceding paper,[13] we reported the promotive activity of long chain keto acid on lipid peroxi-

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2) Location: *Aobayama, Sendai.*
3) E.D. Wills, *Biochem. Pharmc.*, **7**, 7 (1961).
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5) T. Nishida and F.A. Kummerow, *J. Lipid Res.*, **1**, 450 (1960).
6) N. Matsuo, "Lipids and Their Oxidation," eds by H.W. Schultz, *et al.*, Avi published, Westport, 1962, p. 321.
7) Y. Ito, K. Isobe, H. Sekita, M. Osawa, M. Takeda, H. Tanabe, S. Nagoya, and T. Kuwamura, *J. Food Hygienic Soc. Japan*, **11**, 268 (1970).
8) N.W. Hanson, *Chemistry and Industry*, **1964**, 1591.
9) G.W. Ellis, *Biochemistry*, **46**, 129 (1950).
10) N.R. Artman and J.C. Alexander, *J. Am. Oil Chemist's Soc.*, **45**, 643 (1968).
11) L.R. Wantland and E.G. Perkins, *Lipids*, **5**, 191 (1970).
12) M. Sato and M. Uchiyama, The 89th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, April, 1969.
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dation. In the present paper, the effect of such long chain keto acid on some enzymes, succinate dehydrogenase, trypsin and cholinesterase, which are all known to be inhibited by linoleic acid or lipid peroxide, was investigated *in vitro*.

Material and Method

Preparation of Fatty Acids——The methods of preparation of ricinoleic, 12-keto olcic and 12-keto stearic acids have been previously described.[13] Pure linoleic acid was prepared by hydrolysis of ethyl linoleate obtained from Ono Seiyaku Co., Ltd. (Osaka) before use. Oleic acid was prepared by the method of Rubin, *et al.*[14]

Materials——DL-α-Tocopherol was the gift from Eisai Co., Ltd. (Tokyo). Bovine erythrocyte acetylcholinesterase and neotetrazorium chloride were purchased from Sigma Chemical Company. Trypsin was obtained from Tokyo Kasei Co., Ltd. (40000 Fuld-Gross units/g). Glutathione was obtained from Wako Pure Chemical Industries Ltd. (Tokyo). Methyl parathione was obtained from Sumitomo Chemical Industry Ltd. (Osaka) and L-cysteine was obtained from SS Pharmaceutical Co., Ltd. (Tokyo).

Measurement of Enzyme Activity——Succinate-neotetrazolium chloride oxidoreductase (succinate dehydrogenase, E.C. 1.3.99.1) was determined by measuring the amount of formazan formed by reduction of neotetrazolium chloride[15] using mitochondria prepared according to the method of Schneider, *et al.*[16] Fatty acids were added as ethanol solutions giving a final concentration of 4.8% ethanol. Control experiments using buffer containing 4.8% ethanol were always carried out testing the effect of these emulsion on enzyme. Preincubation at 37° for 10 min under air and incubation for 10 min under N_2 were carried out.

Acetylcholinesterase (E.C.3.1.1.7) was determined by a modification of the method of Ellman, *et al.*[17] utlizing acetylthiocholine iodide as a substrate. Enzymes were previously incubated with lipids at 37° for 60 min before determining the activity. The reaction took place at 30° in a total volume of 2.9 ml consisting of 0.1M phosphate buffer (pH 7.4), 2 μmoles of acetylthiocholine iodide, 0.4 μmole of 5,5′-dithiobisnitrobenzoic acid (DTNB), varing amount of lipid compounds, 0.2% Tween 80 and 0.02 mg of crystalline bovine erythrocyte acetylcholinesterase. Progressive formation of yellow pigment by the reaction of released thiocholine with DTNB was followed at 412 mμ with a 1.0 cm light path in Shimazu multiconvertible spectrophotometer Double-40.

Cholinesterase (E.C.3.1.1.8) in human plasma was determined by a modification of the method of Ellman, *et al.*[17] A solution of 8 mg of lyophilized human plasma (Nippon Seiyaku Co., Tokyo) dissolved in 1.0 ml of saline was diluted to 2.0 ml with 0.1M phosphate buffer (pH 7.4). To this solution was added 0.04 ml of an ethanol solution of lipid and the mixture was placed in a bath of 25° and incubated for 30 min. Thereafter 25 μmoles of acetylthiocholine iodide dissolved in 0.5 ml of 0.1M phosphate buffer (pH 8.2) was added as a substrate and maintained at 25° for 3 min. The reaction was stopped by the addition of 10 μmoles of eserine and then 1 μmole of DTNB dissolved in 0.5 ml of 0.1M phosphate buffer (pH 7.4) was added. The solution was diluted with 5 ml of distilled water and the optical density was read at 412 mμ. A calibration curve was prepared by using reduced glutathione.

Trypsin (E.C.3.4.4.4) activity was measured according to Kunitz.[18] Fatty acids were added as ethanol solutions giving a final concentration of 2.4% ethanol. The amount of alcohol thus added to reaction mixture was found to be non-effect in control experiment. Preincubation at 37° for 30 min and incubation for 20 min were carried out with constant shaking. The activity was determined by measuring the optical density at 280 mμ.

Result

Effect of Various Fatty Acids on Some Enzymes

The effect of emulsions containing 1×10^{-3}M stearic, oleic, linoleic, 12-keto oleic (12-KOA), 12-keto stearic and ricinoleic acids in 0.1M phosphate buffer (pH 7.4) on bovine erythrocyte acetylcholinesterase (BE-AChE) and human plasma cholinesterase (HP-ChE) was examined. The results are shown in Table I. BE-AChE was inhibited to much greater extent by 12-KOA than by linoleic acid. 12-Keto stearic and oleic acids were found to be only

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16) G.H. Hogeboon and W.C. Shneider, *J. Biol. Chem.*, **196**, 111 (1952).
17) G.L. Ellman, K.D. Courtnery, V. Andres, Jr., and R.M. Featherstone, *Biochem. Pharmac.*, **7**, 88 (1961).
18) K. Kunitz, "Method in Enzymology," Vol. 2, Academic Press Inc., New York, 1955, p. 26.

Table I. Effect of Various Fatty Acids on Bovine Erythrocyte Acetylcholinesterase (BE-AChE) and Human Plasma Cholinesterase (HP-ChE)

Fatty acid	Relative activity (%)	
	BE-AChE	HP-ChE
None	100	100
Stearic acid	100	—
Oleic acid	92.7	—
Linoleic acid	82.9	88.6
12-Keto oleic acid	76.8	63.0
12-Keto stearic acid	98.4	92.1
Ricinoleic acid	88.7	89.5

Enzyme was preincubated with fatty acid in 0.1M phosphate buffer, pH 7.4, at 37° for 60 min (BE-AChE) or 30 min (HP-ChE). 1 mM of fatty acid was used in ethanolic solution (HP-ChE) or suspension (BE-AChE). Values are an average of duplicate determination in a representative experiment performed on two (BE-AChE) or three (HP-ChE) separate occasions.

slightly inhibitive. The similar result was obtained when HP-ChE was tested. This inhibition on cholinesterase may connect with the presence of carbonyl and double bond at the same time.

The influence of fatty acids *in vitro* on succinate dehydrogenase of rat liver mitochondria were almost identical at the lower concentration and these results will be explained by the detergent property of fatty acid and subsequent swelling of mitochondria. But, the higher concentration of 12-KOA produced the stronger inhibition that the other fatty acids in the same concentration (Table II).

Table II. Effect of Various Fatty Acids on Succinate-Neotetrazolium Chloride Oxidoreductase of Rat Liver Mitochondria

Fatty acid	Enzyme activity	
	1×10^{-3}M	2.5×10^{-3}M
None	1.00	1.03
Stearic acid	0.38	0.34
Oleic acid	0.41	0.37
Linoleic acid	0.39	0.32
12-Keto oleic acid	0.29	0.16
12-Keto stearic acid	0.46	0.44
Ricinoleic acid	0.36	0.33

The basal medium consisted of 100 μmoles of phosphate buffer (pH 7.4), 100 μmoles of sodium succinate and fatty acids in a total volume of 1.2 ml. Mitochondria in the reaction mixture were preincubated with fatty acid at 37° for 10 min under air. The reaction was started by the addition of succinate and neotetrazolium salt. Incubation was carried out for 10 min under N_2. Specific activity was expressed as mμmoles formazan formed/min/mg protein.

The susceptibility of trypsin against fatty acids was different from above three enzymes, namely linoleic or 12-keto stearic acid was more inhibitory than 12-KOA (Table III).

Changes of the activity of BE-AChE by increasing the concentration of 12-KOA are shown in Fig. 1. 12-KOA gave a powerful inhibition at the specified concentration at which the inhibition increased progressively with the increase of concentration of 12-KOA.

Effect of α-Tocopherol on 12-KOA-inactivation of Cholinesterase

It is reported that the inhibition of bovine red cell AChE and intact human erythrocyte is caused by lipid peroxide during ultraviolet radiation.[19] In the present paper, crystalline

19) B.W. O'Malley, C.E. Mengel, W.D. Meriwether, and L.G. Zilkle, Jr., *Biochemistry*, **5**, 40 (1966).

Table III. Effect of Various Fatty Acids on Trypsin Activity

Additive	Relative activity (%)	Additve	Relative activity (%)
None	100	12-Keto oleic acid	89.1
Stearic acid	90.7	12-Keto stearic acid	63.3
Oleic acid	81.7	Ricinoleic acid	91.3
Linoleic acid	72.6		

1 mM of each fatty acid in ethanolic solution was added. Preincubation was carried out at 37° for 30 min with constant shaking. Values are an average of duplicate determination in a representative experiment performed on four separate occasions.

Table IV. Effect of α-Tocopherol, 12-Keto oleic Acid and Linoleic Acid on Bovine Erythrocyte Acetylcholinesterase

Additive	Activity (mμmoles/min)
None	27.3 (100)
Linoleic acid	20.1 (74.0)
Linoleic acid+α-tocopherol	27.3 (100)
12-Keto oleic acid	18.6 (68.2)
12-Keto oleic acid+α-tocopherol	15.6 (57.0)
Linoleic acid+12-keto oleic acid	0.1 (0.4)
Linoleic acid+12-keto oleic acid+α-tocopherol	5.1 (18.7)

1×10^{-3}M of 12-keto oleic, linoleic acids and α-tocopherol was used. Preincubation was carried out at 37° for 60 min with constant shaking. Activity is expressed as mμmoles of acetylthiochloline iodide hydrolyzed/min. Figures in parenthesis indicate percent of corresponding control. Values are an average in representative experiment performed on three separate occasions.

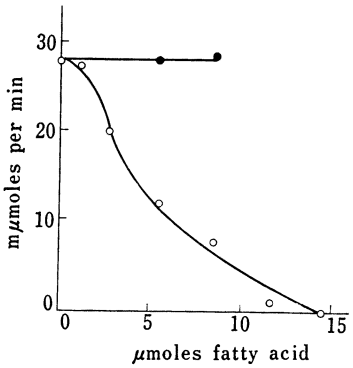

Fig. 1. Changes in the Activity of Bovine Erythrocyte Acetylcholinesterase by the addition of 12-Keto oleic Acid

The enzyme in 0.1M phosphate buffer, pH 7.4, was incubated for 60 min with fatty acid suspended with 0.2% Tween 80. Activity is expressed as mμmoles of acetylthiochloline iodide hydrolyzed per min.
○—○ : 12-keto oleic acid
●—● : stearic acid

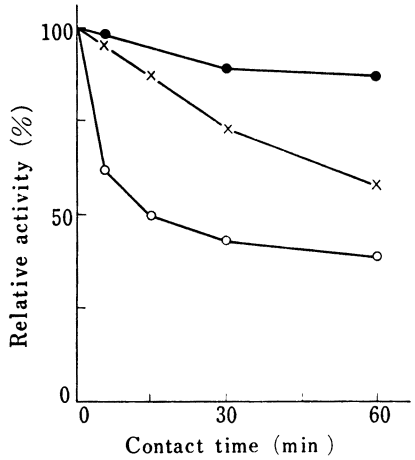

Fig. 2. Changes in the Activity of Bovine Erythrocyte Acetylcholinesterase by the Duration of Preincubation

BE-AChE was incubated with 12-keto oleic acid or methyl parathione in 0.1M phosphate buffer, pH 7.4 with constant shaking. 12-Keto oleic acid (1 mM) and methyl parathione (0.1 mM) were added in emulsion. ●—●: control, ×—×: 12-keto oleic acid, ○—○: methyl parathione

BE-AChE was weakly inhibited by linoleic acid. Moreover, because the inhibitory effect of linoleic acid was completely recovered by the addition of α-tocopherol, the inhibition must be caused by lipohydroperoxide produced from linoleic acid during incubation (Table IV). However, the inhibition by 12-KOA was not prevented by the addition of α-tocopherol. No inhibition by α-tocopherol itself was recognized. When both linoleic acid (1 mM) and 12-KOA (1 mM) were added at the same time, enzyme activity was completely inhibited, which was far stronger than the effect brought by 2 mM of sole fatty acid and only partially restored by α-tocopherol.

In human plasma cholinesterase, the similar result was observed (Table V). The synergistic effect by the presence of linoleic acid and 12-KOA, however, was not so remarkably. The inhibition by 12-KOA, in this case, was slightly recovered by the addition of higher concentration of α-tocopherol, which suggests the partial contribution of lipohydroperoxide formed from unsaturated fatty acids in human plasma.

TABLE V. Effect of Linoleic acid, 12-KOA and α-Tocopherol on Human Plasma Cholinesterase

Additive	Relative activity (%)		
	A	B	C
None	100	100	100
Linoleic acid	88.2	70.0	84.6
12-KOA	65.7	38.5	64.8
Linoleic acid + 12-KOA	48.5	28.6	—
Linoleic acid + α-tocopherol	100	—	100
Linoleic acid + 12-KOA + α-tocopherol	45.0	29.7	—
12-KOA + α-tocopherol	51.3	—	60.3 (5×10^{-3}M)
	—	—	66.4 (2.5×10^{-2}M)
	—	—	72.0 (5×10^{-2}M)
α-Tocopherol	—	100	—

A: 1×10^{-3}M of each fatty acid and 5×10^{-3}M of α-tocopherol were used. Values are an average of determination in a experiment performed on six separate occasions.
B: 2.7×10^{-3}M of each fatty acid and 5×10^{-3}M of α-tocopherol were used. Values are an average of determination in a experiment performed on two separate occasions.
C: 1×10^{-3}M of each fatty acid was used. Values in brackets indicate concentration of α-tocopherol.
The enzyme preparation was incubated with fatty acids and α-tocopherol for 30 min at 37° before determining the activity. As controls, the enzyme preparation was incubated for 30 min in the absence of lipid. The assay method was described in method section.

Properties of 12-KOA Inactivation

The effect of preincubation time on inhibition of BE-AChE was investigated at 37° (Fig. 2). The slight decrease of activity was found in the absence of inhibitors. The inhibition by 12-KOA increased proportionally with the increase of the period of preincubation. The inhibition by methyl parathione reached the maximum at 15 min of contact time.

To determine whether 12-KOA inhibition is reversible, an effort was made to overcome the inhibition by dilution. The system used was similar to that used by Christner, Nand and Mhatre to study the reversal of inhibition of β-glucuronidase by cummen hydroperoxide.[20] As shown in Table VI, it is evident that enzyme could not be released from enzyme-inhibitor complex by dilution, which reveals the inhibition is irreversible.

Since it is known that the sulfhydryl enzymes are most susceptible to inactivation by lipid peroxide and the inhibition is protected by SH compound,[3] effect of SH reagents for inhibition of 12-KOA was investigated. The preincubation of 12-KOA with glutathione was not effective to prevent the inhibition even if the molar ratio of glutathione: 12-KOA was increased

20) J.E. Christner, S. Nand, and N.S. Mhatre, *Biochem. Biophys. Res. Commun.*, **38**, 1098 (1970).

Table VI. Reversibility of 12-KOA Inhibition of Bovin Erythrocyte Acetylcholinesterase by Dilution

Experiment	Pre-assay mixture		Assay mixture		Activity	Inhibition (%)
	Enzyme (μg)	Inhibitor (mM)	Enzyme (μg)	Inhibitor (mM)		
1	20	0	18.6	0	33.2	—
2	4	0	3.7	0	5.49	—
3	20	2	18.6	1.86	9.25	72.2
4	4	2	3.7	1.86	1.99	63.8
5	20	0.4	18.6	0.37	34.1	0
6	4	0.4	3.7	0.37	6.66	0
7	20	2	3.7	0.37	16.6	68.7

The pre-assay mixture contained 0.1M phosphate buffer, pH 7.4, the indicated amount of acetylcholinesterase and the indicated amount of inhibitor (12-keto oleic acid) suspended with Tween 80 in a final volume of 2.7 ml. The mixture was allowed to incubate 30 min at 37° after addition of the inhibitor. In experiment 1 to 6, 0.1 ml of 20 mM acetylthiocholine iodide was added to the pre-assay mixture to yield the assay mixture. In last experiment, 2.7 ml of pre-assay mixture was diluted five fold in a solution containing the same concentration of buffer. Substrate was added and acetylthiocholine iodide hydrolyzed was measured in a cuvette as the absorbance change at 412 mμ with a 1.0 cm light path. Activity was expressed as mμmoles of acetylthiocholine iodide hydrolyzed per min.

(Table VI). 12-KOA was found to be more inhibitory on succinate dehydrogenase in rat liver mitochondria than other fatty acids (Table II). The inhibition was partially reversed by the addition of excess cysteine. If the concentration of cysteine was much greater, *e.g.* 10 mM, the recovery of enzyme activity reached to 40% (Table VIII).

Table VII. Effect of GSH and 12-KOA on Human Plasma Cholinesterase

Molecular ratio 12-Keto oleic acid : Glutathione		Relative activity (%)
—	—	100
1	—	78.0
1	3	78.2
1	5	78.5
1	8	76.2

Enzyme activity was determined by a modification of the method of Hestrin. Glutathione in contact with 6×10^{-4}M 12-keto oleic acid (12-KOA) for 30 min before addition of enzyme.

Table VIII. Effect of 12-KOA and L-Cysteine on Rat Liver Mitochondria Succinate-Neoterazolium Chloride Oxidoreductase

12-Keto oleic acid (M)	L-Cysteine (M)	Activity (%)
—	—	100
10^{-3}	—	17.8
10^{-3}	1×10^{-3}	21.8
10^{-3}	2.5×10^{-3}	20.6
10^{-3}	5×10^{-3}	34.5
10^{-3}	7.5×10^{-3}	45.0
10^{-3}	1×10^{-2}	59.3

The basal medium consisted of 100 μmoles of phosphate (pH 7.4), 100 μmoles of sodium succinate and other additions in a total volume of 1.72 ml. 12-KOA and L-cysteine were preincubated at 37° for 30 min afterthat with mitochondria for 10 min.

Discussion

In the present paper, effect of 12-KOA and the related fatty acids on enzyme activities was investigated. The inhibition pattern of enzyme by 12-KOA described in these results showed two cases: (a) inhibition by 12-KOA was much greater than linoleic acid (b) inhibition by linoleic acid was much greater than 12-KOA. These results may show that the inhibitory effect by 12-KOA is not merely due to denaturation of protein.

When the same amount of 12-KOA and linoleic acid was incubated with enzyme at the same time, the synergistic inhibition was observed as compared with the inhibition brought by sole fatty acid (Table IV). It is considered that 12-KOA enhances the effect of linoleic acid, presumably through linoleic acid hydroperoxide formed during incubation. In fact, the inhibitory effect of linoleic acid was prevented in the presence of α-tocopherol. α-Tocopherol also partially restored the enzyme inhibition in the presence both linoleic acid and 12-KOA. But, the enzyme inhibition by 12-KOA was almost not prevented in the presence of α-tocopherol. These facts show that the inhibitory effect of 12-KOA described in this work clearly falls into two categories: (1) that is some modification on enzyme protein (direct action) and (2) that is through the promotive effect on peroxidation (indirect action). These are a common observation that the inhibition by oxygenated fats was not completely protected by the addition of α-tocopherol in human erythrocyte acetylcholinesterase.[19] Moreover, these results also agree with Shauenstein's report[21] that glyceraldehyde dehydrogenase and lactate dehydrogenase were largely inhibited by linoleic acid hydroperoxide and at the same time non-peroxidic compounds have also apparent inhibitory activity.

It is considered that the inhibitory effect of fatty acid or fatty acyl CoA, except the effect through peroxide, on enzymes are due to detergent properties.[22,23] The inhibitory effect by anionic detergents is strong when pH of the solution is acidic.[24] It is also known that soaps of long chain unsaturated fatty acids are better detergents than those of corresponding saturated fatty acids.[25] In the present paper, since the reaction described in Table I was carried out at neutral pH, the inhibitory effect of 12-KOA could not be applicable to the detergent effect.

Since the inhibitory effect by 12-KOA on succinate dehydrogenase was protected by the addition of excess of cysteine, the inactivation is probably due to modification of SH group of enzyme protein. Linoleic acid hydroperoxide causes a rapid destruction of SH group of proteins and amino acid and inhibits the activity of SH enzymes.[26,27] In fact, succinate dehydrogenase in rat liver and muscle was strongly inhibited when the rats were fed a basal diet containing 5% of autoxidized ethyl ester of highly unsaturated fatty acids for few days.[6] And, the inhibition of succinate oxidase by lipid peroxide was partially retired by the addition of glutathione.[26] We observed that 12-KOA reacts with SH group of protein and amino acid.[28]

On the other hand, because the protective effect by SH compound was not recognized in the case of the inhibition of cholinesterase, it is likely that the inhibition was due to interaction with other moiety of enzyme protein than SH group. The protective effect was not recognized when used BE-AChE as enzyme and various SH compound other than glutathione as protec-

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23) J.A. Dorsey and J.W. Porter, *J. Biol. Chem.*, **243**, 3512 (1968).
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25) T.O. Henderson and J.J. McNeil, *Biochem. Biophy. Res. Commun.*, **25**, 662 (1966).
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27) S.E. Lewis and E.D. Wills, *Biochem. Pharmac.*, **11**, 901 (1962).
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tive agents. Mounter, *et al.*[29] proved that cholinesterase are not SH enzyme in all probability. Moreover, Castro[30] regarded the possibility that the alkylating agents on cholinesterase can be expected to undergo four reactions with protein such as lysine, histidine, tyrosine and methionine residues and considered that a SH group is not involved during the inhibition by alkylating agents and the possibility of reaction on histidine or methionine groups of enzyme has been discussed.

Tappel, *et al.*[4] reported that when linolenic acid peroxide was reacted with cytochrome C, the insoluble polymeric materials were formed and amino acids such as serine, proline and arginine were also destroyed as much as cysteine. Malonaldehyde, end product of lipid peroxide, is probably the reagent responsible for cross-linking of lysine in enzyme protein.[31] As mentioned above, the toxicity of oxygenated fats is not merely due to destruction of SH group but the destructive effect on various constituents. We preliminarily recognized[28] that 12-KOA decolorized hemoglobin through the destruction of porphyrin when incubated with aqueous solution of hemoglobin at 37° (pH 7.4). Generally, straight chain aldehydes combine with protein.[32] 12-KOA, as mentioned, reacts with SH compounds as cysteine and glutathione to decrease free SH.[28] The structure of binding products is now under investigation.

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