Discussion

From the study of the characteristics of the oil presented in Table I it can be noted that iodine value, free fatty acids, and unsaponifiable increase during germination. The increase in I.V. may result from the synthesis of unsaturated acids. However this may arise from the disproportionate metabolism of saturated and unsaturated acids. Therefore a study of only I.V. does not help to form any conclusion. To draw a reliable conclusion it is necessary that the composition of component acids in 100 g. of seeds be considered. The increase in free fatty acids may be caused by the hydrolysis of glycerides by water, hence it will not be correct to say that free fatty acids are first accumulated before conversion of oil into glucides as concluded by Johnston and Sell (10).

The study of the composition of the component fatty acids in 100 g. of seeds has revealed that none of the acid has been synthesized during germination. All acids are metabolized at different rates during germination. Myristic acid has been metabolized completely. Stearic acid is metabolized slowly while lignoceric acid is not metabolized in the initial stages of germination. The metabolism of lignoceric acid during later stages of germination was found to be very slow. Other saturated acids are metabolized more rapidly. Among unsaturated acids, oleic acid is metabolized more rapidly than linoleic acid. The rate of the metabolism of unsaturated acids is slower than that of saturated acids. Thus a disproportionate metabolism of saturated and unsaturated acids is observed in the present investigation. Hence increase in I.V. is due to this disproportionate metabolism of component acids and cannot be attributed to the synthesis of unsaturated acids.

The study of the composition of seeds has revealed the significant change in crude fiber, oil, and glucide contents of seeds. Reducing sugar, which was not originally present, has been synthesized in the first stage and grows during subsequent stages. Sucrose and starch contents also increase during germination. It is therefore concluded that the oil is metabolized during germination and that reducing sugar, sucrose, and starch are synthesized at the expense of oil. This observation is in close agreement with those reported by previous workers (10,11,12). The nitrogen content of seeds was found to be constant. Ash content and moisture content have been increased to a small extent.

From the present investigation it has been concluded that saturated acids are metabolized at a greater rate than that of unsaturated acids during germination; some acids are not metabolized during the initial stages of germination; and fatty acids are converted into soluble and insoluble glucide during the course of germination.

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Unsaturated Lipid Peroxidation Catalyzed by Hematin Compounds and Its Inhibition by Vitamin E[1]

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Evidence favoring hematin catalysis over autoxidation as the dominant mechanism of lipid peroxidation in animal tissues is presented. Lipid peroxidation in Erlich ascites tumor cells and isolated electron transport particles has been studied. Random destruction of the cytochromes and a loss of catalytic activity correlate with peroxidation of the electron transport particle.

Mixtures of α-, β-, and γ-tocopherols show no synergistic effect. Synergism with ascorbate and citrate greatly enhance the antioxidant activity of α-tocopherol. A tocopherol-ascorbate-glutathione-triphosphopyridine nucleotide couple could act synergistically to inhibit lipid peroxidation in animal tissues.

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LIPID PEROXIDATION catalyzed by hematin compounds is a basic pathological reaction *in vivo* and a deteriorative reaction *in vitro*. In vitamin E-deficient animals hematin-catalyzed lipid peroxidation appears to be widespread but is particularly damaging in the mitochondria and microsomes, where the free radical intermediates react with enzymes and lead to metabolic derangements (28–30,33). Lipid peroxidation products have been found in human atherosclerosis (6,11); this pathological reaction may involve catalysis by hemoglobin (9). Stored whole blood appears to deteriorate by hemolysis involving hemoglobin-catalyzed oxidation of the unsaturated

lipids of the membrane (4). Hematin-catalyzed oxidative rancidity of fish, poultry, and meats is the reaction which usually limits their frozen storage life (31).

This reaction has been strongly implicated *in vivo* and *in situ* because hematin compounds are the most powerful lipid peroxidation catalysts found in animal tissues (24–27), and they are often in close molecular proximity to the unsaturated lipid undergoing peroxidation (28). However since the products of hematin-catalysis and autoxidation are similar (16), it is difficult to determine the relative amount of hematin catalysis occurring in tissues. This paper deals with a number of important problems in hematin-catalyzed lipid peroxidation and its inhibition by vitamin E. First, two approaches for defining the amount of hematin-catalysis are used. One is selective inhibition, and the other is a comparison of relative rates of oxidation of highly unsaturated lipids where unstable peroxides make autocatalysis very rapid. Secondly, further studies have been made of lipid peroxidation at the cellular and subcellular levels. Thirdly, synergism involving tocopherols, ascorbate, and citrate was studied. Although the tocopherols are the major lipid antioxidants found in nature, by standards of synthetic antioxidants they appear weak (13,27). Their effectiveness and capacity may be greatly increased by the synergistic action of other tissue components.

Experimental

Hematin-Catalyzed Lipid Peroxidation in Situ. A disk of adipose tissue weighing 7.7 g. with a surface area of 13.4 cm.^{2} was allowed to oxidize in a 125-ml. Warburg vessel containing oxygen at 20°C. for 2 days, then at 38°C. for 1 day. Lard was prepared from a paired sample by low-temperature rendering. For comparison the same size lard sample, 7.7 g. and 13.4 cm.^{2} surface area, was held in a Warburg vessel under the same conditions as the adipose tissue. Peroxide values were determined iodometrically. Initial values were 1.8 meq./kg. for adipose tissue and 1.5 meq./kg. for lard.

For inhibition studies the pork tissue was made into a slurry in order to achieve good inhibitor-incorporation. This was obtained by blending 1 part by weight of ground pork containing 70% lipid with 2 parts of water. To serve as a bacteriostat, 35 p.p.m. aureomycin were added. Butylated hydroxyanisole and nordihydroguaiaretic acid were added in a minimum amount of alcohol. Inhibition was determined by manometric measurement of oxygen absorption at 30°C. and peroxide titration. Initial peroxide concentration was 4.3 meq./kg.

Lipid Peroxidation of Ascites Cells and Electron Transport Particles. Erlich ascites tumor cells freshly drawn from mice were washed three times in 0.15 M NaCl. Two ml. of packed cells were diluted to 4 ml. in 0.15 M NaCl, containing 10 p.p.m. aureomycin, and added to each Warburg vessel. Oxidative metabolism of the cells was inhibited by 1×10^{-2} M iodoacetate. They were allowed to oxidize in oxygen at 37°C. For tocopherol inhibition 0.13 mg. α-tocopherol/flask was added. Thiobarbituric acid reactants were measured as previously described (28), using 0.5 ml. of suspended cells per assay.

Electron transport particles from beef heart mitochondria (5,7), containing approximately 3 mg. of protein per ml., were suspended in 50 ml. of aerated .01 M phosphate buffer containing 10 p.p.m. aureomycin. Lipid peroxidation at 25°C. was initiated by adding 20 μ moles ascorbic acid at 0 and 2 hrs. As a function of time, thiobarbituric acid reactants, succinoxidase activity, and cytochrome content were measured. Thiobarbituric acid reactants in 5 ml. of the reaction mixture were measured as previously described (28). Succinoxidase activity was determined by polarographically measuring the rate of oxygen consumed. Cytochrome concentrations were measured by difference spectra, using the techniques described by Chance and Williams (3).

Comparison of Hemoglobin Catalysis and Autoxidation. Pure linoleic, linolenic, and arachidonic acids and methyl esters were obtained from the Hormel Foundation. Fish oils and the methyl ester of sardine oil were obtained from the Fishery Technological Laboratories, Seattle, Wash. Fish oils were purified by passing a 1:1 oil-petroleum ether mixture through activated alumina columns. Purified hemoglobin was prepared from cattle blood. Oxidation was measured manometrically at 37°C. in oxygen with 0.50 g. of lipid per Warburg vessel. For hemoglobin catalysis 0.5 ml. of 1×10^{-4} M hemoglobin was added from the side arm. Rapid shaking at 180 strokes/min. provided thorough mixing. For autocatalysis there was no aqueous phase.

Inhibition by Vitamin E and Synergists. Reaction systems were similar to those previously described (13). Ten ml. of linoleic acid [4] were emulsified with 20 ml. of 0.1 M phosphate buffer, pH 7.0, with the aid of 0.25 ml. of emulsifier.[5] Three ml. of emulsion were added to each flask. Hemoglobin at a final concentration of 5×10^{-5} M was in a side-arm. Antioxidants and synergists were added to the emulsion in the Warburg flasks, and the final volume was brought to 4 ml. Rates of oxidation at 37°C. in oxygen were measured manometrically. The results are conveniently defined as "protective indices," which are the ratios of the oxidation rate of the control sample divided by the oxidation rate of the sample with added inhibitors.

Results and Discussion

Hematin-Catalyzed Lipid Peroxidation in Situ. To compare the rate of hematin-catalyzed lipid peroxidation *in situ* with autocatalysis, the oxidation of pork adipose tissue was compared to extracted lard. Table I shows the correlation between the rate of oxygen absorption and the increase in peroxide values. The increase in peroxide value shows that lipid peroxidation occurred in the pork adipose tissue. Because the adipose tissue was not sterile, it is possible that a small fraction of the oxygen absorption was caused by microbial growth. The evidence that a large fraction of the oxygen absorption is not microbial is that there were no indications of microbial

TABLE I

Rate of Peroxidation of *in Situ* and Rendered Lard

Sample	Full rate of oxygen absorption μl. O_2/hr.	Increase in peroxide value meq./kg.
Pork adipose tissue	640	7.1
Lard	0	0.6

[4] Nutritional Biochemicals Company.
[5] Tween 40 or Myrj 53.

growth, few organisms will grow readily on adipose tissue which is actively peroxidizing, and the oxygen-absorption rate is much too large to ascribe to microbial oxidation. This comparison shows that *in situ* lipid is much more labile to peroxidation than the lipid which is no longer in contact with other tissue constituents. Since one major difference between adipose tissue and lard is the presence of the hematin compounds (in this case, hemoglobin, myoglobin, and the cytochromes) this study implicates them as dominant catalysts in *in situ* lipid peroxidation. These results are reinforced by studies on fish tissues, which varied in total hematin content from 5×10^{-5} M to 1×10^{-6} M (2). In these fish the extractable lipid peroxidation catalysts were found to be hematin compounds by selective inhibition with cyanide. These fish muscles underwent lipid peroxidation at a rate proportional to their content of total hematin compounds.

A more critical test to differentiate between hematin catalysis and autoxidation is that of selective inhibition. The only mechanism of inhibition which is selective for hematin catalysis is that of blocking the reaction of hydroperoxide with the iron in the heme nucleus. Cyanide has been found as the most effective inhibitor of this type (1,28). Some comparative tests of the phenothiazine type of dyes, methylene blue and new methylene blue, suggest that they could be used as selective inhibitors (26). However, since the phenothiazine dyes appear to inhibit both as free-radical acceptors and by direct decomposition of peroxides, they do not allow a clear differentiation (15). In Table II is shown the inhibition of lipid peroxidation in pork adipose tissue. Comparison of new methylene blue, butylated hydroxyanisole, and nordihydroguaiaretic acid is interesting because these are all good inhibitors of hematin catalysis in model reaction systems. Furthermore new methylene blue and similar compounds, under certain conditions, replace vitamin E *in vivo* (17). Butylated hydroxyanisole and nordihydroguaiaretic acid are also good inhibitors of autoxidation. Butylated hydroxyanisole and nordihydroguaiaretic acid would inhibit lipid peroxidation by either mechanism while cyanide appears to inhibit hematin catalysis selectively. Then, from the similar inhibition given by these three, it may be concluded that hematin catalysis is a dominant mechanism of lipid peroxidation in this study.

TABLE II

Inhibition of Lipid Peroxidation in Pork Tissue

Inhibitor	Concentration of inhibitor, M	Oxygen absorbed, μl.		Change in peroxide value meq./kg.
		In 30 hr.	In 43 hr.	
Control	....	737	>>5000	+5.9
Cyanide	2.0×10^{-2}	177	310	−4.3
New methylene blue	2.8×10^{-3}	40	50	−4.3
Butylated hydroxyanisole	2.1×10^{-3}	53	1060	−3.0
Nordihydroguaiaretic acid	1.7×10^{-3}	88	74	−4.3

Lipid Peroxidation at the Cellular and Subcellular Level. It would be desirable to have simpler systems than experimental animals or isolated tissues for study of cellular lipid peroxidation. Figure 1 shows the course of lipid peroxidation of ascites cells as measured both by oxygen absorption and the thiobarbituric acid reaction. Since these cells were washed and oxidative metabolism was inhibited, the oxygen absorption should be a good measure of lipid peroxidation. The correlation of the thiobarbituric acid reactants with oxygen absorption shows that these cells are undergoing lipid peroxidation. The oxidized cells developed a painty odor characteristic of peroxidized lipid. Further it was found that the oxidative reaction as measured by oxygen absorption and the thiobarbituric acid reaction is strongly inhibited by added α-tocopherol. The use of free-floating animal cells, like the ascites tumor cell, may prove useful for studies of the mechanism of hematin catalysis and vitamin E inhibition *in vivo*.

Studies of *in vivo* lipid peroxidation in vitamin E-deficient animals and *in vitro* lability of isolated subcellular fractions have shown that the mitochondria are very labile and are the site of much lipid peroxidation damage within the cell (28,29,33). These studies of the mechanism of mitochondrial lipid peroxidation and damage have been extended to the electron transport systems as shown in Table III. Isolated electron transport particles are labile to lipid peroxidation because the unsaturated lipids are in close molecular proximity to the cytochromes and molecular oxygen. Table III shows that there was a close correlation between the increase in lipid peroxidation, as measured by thiobarbituric acid reactants, and damage to the catalytic activity of the electron transport system, as measured by the decrease in the rate of oxidation of succinate. Measurements of the cytochrome components by difference spectra showed increasing destruction as a function of lipid peroxidation. In a number of similar experiments the same pattern prevailed, namely, random destruction of the cytochromes *a*, *b*, and *c* as a function of lipid peroxidation. Random destruction of cytochromes is the most probable reaction in a free-radical, chain-reaction lipid peroxidation. Free-radical intermediates can damage the cytochromes and other enzymes of mitochondrial electron transport by hydrogen abstraction and by addition reactions.

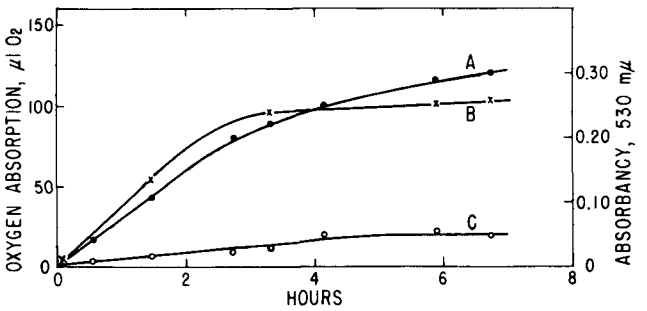

FIG. 1. Lipid peroxidation of ascites cells. A. Oxygen absorption. B. Thiobarbituric acid reaction, absorbancy at 530 mμ. C. Oxygen absorption of tocopherol-inhibited reaction.

Comparison of Hematin Catalysis and Autoxidation. In Table IV are comparisons of the induction

TABLE III

Lipid Peroxidation of the Electron Transport System

Time, hr.	Thiobarbituric acid reactants, abs. 530 mμ	Rate of oxidation of succinate, % initial rate	Cytochromes, % remaining		
			a_3	$c+c_1$	b
0	0	100	100	100	100
2.2	.42	80	....	....	....
3.2	.56	76	....	....	....
3.9	.74	20	95	83	95
5.5	.88	20	92	86	68
6.7	.92	0	73	80	61

TABLE IV

Comparison of Hemoglobin Catalysis and Autoxidation

Lipid	Iodine number	Hemoglobin catalysis		Autoxidation		Ratio of hemoglobin catalysis rate / autoxidation rate
		Induction period, hr.	Rate ml. O_2/hr./g.	Induction period, hr.	Rate ml. O_2/hr./g.	
Linoleic acid	181	2.3	2.8	7.5	0.45	6.2
Linolenic acid	273	0	9.0	2.5	6.1	1.5
Arachidonic acid	315	0.1	4.5	2.5	2.7	1.7
Methyl linoleate	173	0.3	0.96	10.0	0.08	12.0
Methyl linolenate	260	0.4	1.3	2.2	0.68	1.9
Methyl ester of sardine oil	352	0.3	10.6	0.5	9.6	1.1
Sardine oil	191	1.2	2.6	2.0	2.9	0.87
Menhaden oil	167	1.8	0.88	23.0	0.3	2.9
Cod liver oil	164	7.4	1.6	11.3	0.4	4.0

periods and rates for hemoglobin-catalyzed oxidations and autoxidations of highly unsaturated fatty acids, esters, and triglycerides. These comparisons of the rates show that the hemoglobin-catalyzed linoleic acid and methyl linoleate oxidations are much greater than the corresponding autoxidations. For linolenic acid, its ester, and arachidonic acid, the hemoglobin-catalyzed reaction is only 50–100% faster than autoxidation. Among the highly unsaturated oils tested, sardine oil had a rate of autoxidation slightly greater than hematin-catalyzed oxidation. For sardine oil methyl esters the rates were approximately equal. For all of these unsaturated lipids the induction period of the hemoglobin-catalyzed reaction was shorter than that for the corresponding autoxidation. Rapid initiation and propagation reactions are characteristic of hematin catalysis. Fatty acids, methyl esters, and triglycerides of less unsaturation than these shown always have higher rates of hematin catalysis than corresponding autoxidation (13,26). Collectively these results suggest that hematin catalysis would be of greater physiological importance as a mechanism of lipid peroxidation where the bulk of the lipid present is not highly unsaturated, such as in animal fats. Where highly unsaturated lipids occur in localized concentrations in the animal body, autoxidation may also be an important pathological and deteriorative reaction.

Inhibition by Vitamin E and Synergists. Table V shows some results of studies of α-, β-, and γ-tocopherol as inhibitors of hemoglobin-catalyzed linoleic acid oxidation. Four concentrations of each tocopherol were tested; 3,6,12, and 30×10^{-4} M. Greater antioxidant activity was always obtained at the higher concentrations. Table V shows results for 3 and 30 x 10^{-4} M; the values obtained for other concentrations were intermediate to these. Both the β- and γ-tocopherol act more effectively than α-tocopherol as antioxidants in this system. Because of their different redox potentials and stabilities of intermediate oxidation products, it is possible that the various tocopherols might act synergistically in inhibiting hemoglobin-catalyzed linoleic acid oxidation. An experimental test of this possibility shows, in Table V, that mixtures of α, β, and γ give no apparent synergistic effect.

TABLE V

Tocopherols as Inhibitors of Hemoglobin-Catalyzed Linoleic Acid Oxidation

Tocopherol	Concentrations M x 10^4	Protective index control μl. O_2/1 hr. / + tocopherol, μl. O_2/1 hr.
α	3	1.3
α	30	1.4
β	3	1.8
β	30	2.7
γ	3	2.1
γ	30	2.4
$\alpha + \beta$	3 each	2.1
$\alpha + \gamma$	3 each	2.1
$\beta + \gamma$	3 each	2.0
$\alpha + \beta + \gamma$	3 each	2.0

The tocopherol in animal tissues would be in contact with many constituents which could act synergistically in inhibiting lipid peroxidation. For the experiment described, ascorbate, citrate, and histidine were chosen as examples from classes of probable synergists. Previous work on inhibition of hematin-catalyzed reactions indicated that ascorbate and some of the amino acids function synergistically with a variety of phenolic antioxidants (13,25). Table VI shows some of the results of this study where a great effect of ascorbate and citrate synergism was obtained. The concentrations of tocopherol, ascorbate, and citrate were chosen to approximate the ratios that are found in animal tissues. As can be seen from the data in Table VI, ascorbate was a powerful synergist with tocopherol. Histidine at 5×10^{-3} M was not an outstanding synergist in any combination. In fact, when added to tocopherol plus ascorbate plus citrate, it greatly decreased their effectiveness as antioxidants. Of all the possible synergistic reactions of tocopherol with other tissue constituents, that with ascorbic acid appears most useful to the cell. Present knowledge indicates that the series of couples shown in Figure 2 could act synergistically to inhibit hematin-catalyzed lipid peroxidation or autoxidation in tissues. The synergistic effect of ascorbate and tocopherol was also noted in previous research (25) and by other investigators (10,14,32). The reduction of a transitory tocopherol semi-quinone radical by ascorbate is a likely possibility (8), but further studies of the mechanism of ascorbate synergism are needed. The reduction of

TABLE VI

Synergism of Ascorbate and Citrate with Tocopherol

Inhibitors	Protective indices control μl. O_2 / + inhibitors, μl. O_2	
	0.5 hr.	1.0 hr.
Tocopherol, 3.0×10^{-3} M	1.3	1.6
Ascorbate, 1.5×10^{-3} M	1.7	1.2
Citrate, 6.5×10^{-3} M	0.9	0.9
Ascorbate + citrate	1.2	1.0
Tocopherol + ascorbate	110	46
Tocopherol + citrate	1.7	2.0
Tocopherol + ascorbate + citrate	179	72

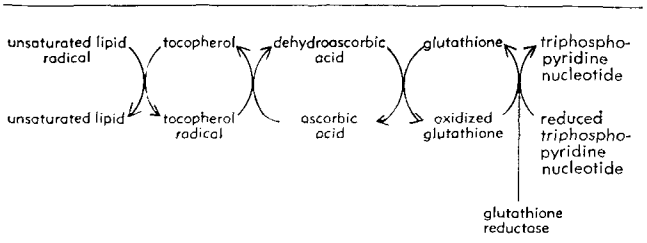

FIG. 2. Redox couples which could increase the antioxygenic effectiveness of tocopherol.

dehydroascorbic acid by glutathione and the coupling of glutathione reduction to reduced di- and triphosphopyridine nucleotides by the enzyme glutathione reductase (20) are well known. Through such coupled systems the capacity of tocopherol would be greatly increased.

The normal range for vitamin E in human blood plasma is 0.9–1.9 mg. per 100 ml.; similar values for ascorbic acid and glutathione in whole blood are 0.1–1.3 and 25–41 mg. per 100 ml., respectively. Thus glutathione coupled to tocopherol would increase its lipid antioxygenic capacity. Further, since glutathione is coupled to tissue respiration, its oxidation-reduction capacity is very great.

Prooxidants Other than Hematin Compounds. The lipid prooxidant-antioxidant balance found in nature is often more complicated than that of hematin compounds and tocopherol. White muscle disease in lambs and calves appears to be a good example of a complicated vitamin E deficiency (12,18,19). Lipoxidase, the only known lipid peroxidation catalyst more active than hematin compounds, appears to play an important role in white muscle disease which has not been noted in previous research. Feeds characteristically associated with white muscle disease (12,18,19), red beans, pea vines, and alfalfa, are known to be good sources of lipoxidase (21–23). Lipoxidase-catalyzed lipid peroxidation in the rumen would be an important peroxidative stress.

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Solubility of Linoleic Acid in Aqueous Solutions and Its Reaction with Water[1,2]

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In a study of stable emulsions of linoleic acid in 0.1M-KH_2PO_4/Na_2HPO_4 buffer solutions prepared by sonic vibrations, the influence of linoleic acid on pH was manifested in buffer solutions of pH 8.00 and decreased gradually till it became negligible in pH 4.50. This change in pH values was due to differences in solubility of linoleic acid in the buffer solutions.

Ultraviolet spectra of soluble linoleic acid in buffer solutions indicated the presence of conjugated dienes, which increased with the increasing of the pH of the system.

Unbuffered aqueous emulsions of linoleic acid had a pH value which ranged between 4.69 and 5.10. Saturated aqueous solutions, obtained by high-speed centrifugation, had concentrations of 15.80 to 16.00 mg. linoleic acid per 100 ml. of D.I. water.

From the solubility data and conductivity values of linoleic acid the apparent classic and thermodynamic ionization constants were calculated to be $6.974 \pm 0.023 \times 10^{-6}$ and $6.905 \pm 0.017 \times 10^{-6}$ at 0.7°C. and $1.730 \pm 0.009 \times 10^{-5}$ and $1.689 \pm 0.007 \times 10^{-5}$ at 25°C., respectively.

The result of the chemical interaction of linoleic acid and water is a saturated hydroxy fatty acid. This acid gave a positive test for glycol groups with periodic acid oxidation test and appeared to be a tetrahydroxy compound with the exact structure unknown.

THE SOLUBILITIES of the members of the normal saturated fatty acid series and of oleic, linoleic, and linolenic acid have long been known with great accuracy in a wide variety of organic solvents. In 1955 Kolb and Brown (13) provided further data on the solubility of fatty acids as a guide to their separation by low-temperature crystallization from organic solvents.

The solubilities in water of the normal saturated fatty acids from caproic to stearic at various temperatures between 0° and 60°C. are reported by Ralston

[1] Presented at the 51st Annual Meeting, American Oil Chemists' Society at Dallas, Tex., April 4–6, 1960.
[2] American Meat Institute Foundation Journal Paper No. *204*.