fed in a vitamin E and selenium-free diet containing torula yeast, but was innocuous when fed in a soybean protein type diet. Subsequent work revealed that the adverse effects of ethoxyquin in the torula yeast diet could be completely overcome by adding selenium, as selenite, in amounts which by themselves had very little effect in preventing exudates or mortality (Table VIII). Low dietary concentrations of α-tocopherol which were not completely active in preventing deficiency symptoms also eliminated the toxicity. It is probable that there is sufficient selenium in the soybean protein diet to prevent the toxicity of ethoxyquin. This protective action of selenium appears to be similar to that exerted by vitamin E under a variety of toxic conditions (23).

These various studies provide further information of metabolic effects of dietary selenium as related to vitamin E. The similarity of action of these two substances, as for example their relationship to coenzyme A and their prevention of certain toxicities, suggests a common biochemical mechanism. The hypothesis that vitamin E and selenium act *exclusively* as tissue antioxidants and thereby stabilize critical polyunsaturated fatty acids in the cell is weakened by the negative results with rat and guinea pig tissues; a general ground-theory should apply to a variety of species. On the other hand, there is insufficient evidence to support a hypothesis that α-tocopherol and selenium act specifically in one or more pathways of intermediary metabolism. Clearly, more information must be obtained to enable us to unravel the intriguing interrelationship between these substances.

REFERENCES

1. Schwarz, K., and C. M. Foltz, J. Am. Chem. Soc., *79*, 3292 (1957).
2. Patterson, E. L., R. Milstrey, and E. L. R. Stokstad, Proc. Soc. Exp. Biol. Med., *95*, 617 (1959).
3. Bieri, J. G., and A. A. Anderson, Arch. Biochem. Biophys., *90*, 105 (1960).
4. Folch, J., M. Lees, and G. H. Sloan-Stanley, J. Biol. Chem., *226*, 497 (1957).
5. Bragdon, J. H., J. Biol. Chem., *190*, 513 (1951).
6. Fiske, C. H., and Y. Subbarow, J. Biol. Chem., *66*, 375 (1925).
7. Pearson, S., S. Stern, and T. H. McGavack, Anal. Chem., *25*, 813 (1953).
8. Lester, R. L., Y. Hatefi, and F. L. Crane, Biochim, Biophys. Acta, *33*, 169 (1959).
9. Bieri, J. G., C. J. Pollard, I. Prange, and H. Dam, Acta Chem. Scand., *15*, 783 (1961).
10. Novelli, G. D., Methods in Enzymology, Vol. III, 917, Academic Press, Inc., New York, 1957.
11. Metcalf, L. D., and A. A. Schmitz, Anal. Chem., *33*, 363 (1961).
12. Bieri, J. G., Nature, *184*, 1148 (1959).
13. Bieri, J. G., H. Dam, I. Prange, and E. Søndergaard, Acta Physiol. Scand., *52*, 36 (1961).
14. Zalkin, H., A. L. Tappel, and J. P. Jordan, Arch. Biochem. Biophys., *91*, 117 (1960).
15. Corwin, L., Arch. Biochem. Biophys., *97*, 51 (1962).
16. Morin, R. J., S. Bernick, J. F. Mead, and R. B. Alfin-Slater, J. Lipid Res., *3*, 432 (1962).
17. Mead, J. F., G. Steinberg, and D. Howton, J. Biol. Chem., *205*, 683 (1953).
18. Diplock, A. T., J. Bunyan, E. E. Edwin, and J. Green, Brit. J. Nutrition, *16*, 109 (1962).
19. Yang, C., M. Riegl, and R. E. Olson, Fed. Proc., *17*, 498 (1958).
20. Yang, C., G. H. Dialameh, and R. E. Olson, Fed. Proc., *18*, 553 (1959).
21. Machlin, L. J., R. S. Gordon, and K. H. Meisky, J. Nutrition, *67*, 333 (1959).
22. Scott, M. L., Nutrition Abs. Rev., *32*, 1 (1962).
23. Hove, E. L., Am. J. Clin. Nutrition, *3*, 328 (1955).

[Received February 15, 1963—Accepted July 1, 1963]

The Biological Consequences of Feeding Polyunsaturated Fatty Acids to Antioxidant-Deficient Animals

L. J. MACHLIN, Monsanto Chemical Company, St. Louis, Missouri

Abstract

The addition of polyunsaturated fatty acids (PUFA) to diets deficient in vitamin E and other effective antioxidants results in a variety of symptoms in animals. For example, the feeding of such diets to rats results in muscular dystrophy, testis degeneration, dental depigmentation, brown discoloration of the fat and and uterus and creatinuria. Similar diets fed to rabbits and ruminants results in muscular dystrophy. In chickens the symptoms observed are encephalomalacia, lowered egg production, and poor hatchability. The addition of PUFA to diets is known to result in the destruction of vitamin E in the diet or in the tissues of animals as a result of free radicals produced during the autooxidation of the PUFA. However, in several studies, this possible explanation for the development of vitamin E deficiency symptoms has been made untenable. In such studies the more likely explanation for development of symptoms is the *in vivo* peroxidation of PUFA in the tissues of animals following incorporation of large amounts of PUFA in lipid structures and depletion from the tissues of vitamin E and other biologically effective antioxidants.

Introduction

THE TOPIC of antioxidant-unsaturated fatty acid relationships is not new and, in fact, began shortly after the initial discovery of our most important *in vivo* antioxidant vitamin E. In 1923, Evans recognized that vitamin E was necessary for the reproduction of the rat and, just a few years later, Agdur (1) and Evans (2) found that vitamin E deficiency symptoms were exaggerated by the addition of cod liver oil to the diet. Why the recent interest in this topic? One of the reasons is the popularization of the concept that unsaturated fatty acids will decrease blood cholesterol in humans, with the inference that this lowered cholesterol would reduce atherosclerosis. The increased consumption of PUFA has caused some concern, that excessively high intakes of unsaturated fatty acids may precipitate vitamin E deficiencies in humans (3). Another reason for renewed interest is the accumulation of evidence in the last few years that many synthetic antioxidants can prevent the deleterious effects of unsaturated fatty acids in animals. Thirdly, we now have available elegant procedures for the analysis of fatty acids in tissues, and also more refined nutritional techniques. This has led to experiments which help clarify and quantitate the initial observations on the relationships between polyunsaturated fatty acids (PUFA) and vitamin E.

Recent works which have helped establish some of the following points are briefly reviewed below:

1) PUFA are necessary for the development of most vitamin E deficiency symptoms. Where they are not absolutely necessary, PUFA almost invariably will exaggerate the requirement (3,4,5).

2) PUFA-induced symptoms can be prevented by a number of synthetic antioxidants of quite varied chemical structures (5,6,7,8,9).

3) The nutritional requirement for antioxidants is proportional to the polyunsaturated fatty acid content of the diet, i.e., as the unsaturated fatty acid content increases, the need for antioxidants will also increase (10,11).

4) The effect of PUFA in increasing the requirement cannot be explained solely on the basis of destruction of vitamin E either in the diet or in the animal.

5) We now know that the fatty acid composition of structural lipids is affected by the diets. We have long been aware that the adipose tissue can be altered by diet and now it is apparent that the lipid in all tissues and subcellular units, such as the mitochondria, can also be altered by diet.

Experimental

Observations on Vitamin E Deficiency

PUFA are necessary for the development of many vitamin E deficiency symptoms in the rat, viz., muscular dystrophy, brown adipose fat, brown coloring of the uterus, accumulation of ceroid, dental depigmentation, and degeneration of kidney.

Brown discoloration of the adipose tissue, brown discoloration in the uterus, and an accumulation of ceroid, evidently are all a result of peroxidation of the fatty acids in these tissues which leads to a lipid protein complex which is colored. Three other symptoms: testis degeneration, fetal resorption, and liver necrosis, apparently can be produced without PUFA in the diet, but the addition of PUFA will exaggerate all of these symptoms rather markedly.

In the chicken, the nutritional variables affecting the vitamin E deficiency symptoms are better defined (12). Encephalomalacia, a degeneration of the cerebellum resulting in nervous symptoms and death, is caused by the feeding of diets which are high in linoleic acid family fatty acids and low in antioxidants. Neither selenium nor sulfur amino acids have any effect on this symptom, which will be discussed in greater detail later. Exudative diathesis is caused by feeding diets which are extremely low in selenium and low in antioxidants. PUFA will increase the severity of exudative diathesis. Finally, muscular dystrophy in the chick is caused by feeding of diets low in sulfur amino acids and antioxidants; selenium seems to have some slight effect. This symptom can be produced on a fat-free diet. E-deficiency symptoms in the adult bird have not been examined as thoroughly. In the hen, when diets high in unsaturated fatty acids are fed, no encephalomalacia ensues but egg production decreases and also the eggs fail to hatch (7).

In one experiment (Fig. 1), hens were fed a purified diet containing peroxidized safflower oil as a source of linoleic acid for 2,8-wk periods. Unless Santoquin (1,2-dihydro-6-ethoxy,2,2,4,-trimethlylquinoline), or vitamin E, was added to the diet, egg production decreased markedly. Production could be restored by replacing the safflower oil with tallow, a saturated fat. This illustrates the profound effect of unsaturation on deficiency symptoms.

In another experiment (Fig. 2), eggs from hens fed the high unsaturated fatty acid diet failed to hatch unless vitamin E or an extremely high level of Santoquin were also in the diet. When low-linoleic acid diets were fed (such as those containing hydrogenated coconut oil with a low level of safflower oil) hatchability was maintained even without the addition of antioxidants to the diet.

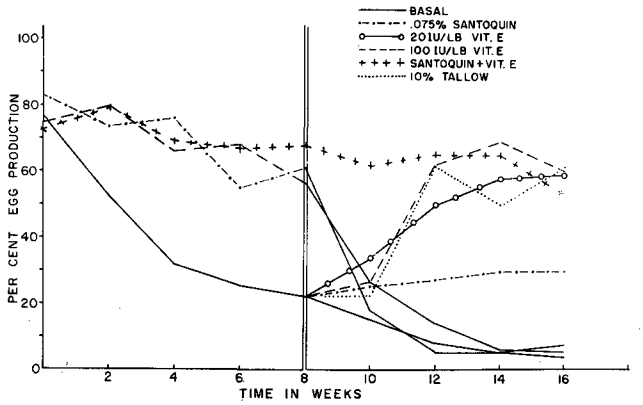

FIG. 1. Effect of unsaturated fat and antioxidants on egg production.

Discussion

Why do unsaturated fatty acids have such a profound effect on E deficiency symptoms? Vitamin E is often destroyed as a result of free radicals produced during the autoxidation of unsaturated fatty acids in the diet or in the intestinal tract. This undoubtedly can explain many old and new reports in the literature. However, in many studies, precautions have been taken to prevent such oxidation. About 20 years ago, Mackenzie (13) showed that E-deficiency symptoms can be precipitated in the rabbit by cod liver oil, although the oil and any dietary source of vitamin E were fed on alternative days. Antioxidants can be added to fats at levels which will result in elimination of autoxidation in the diet, but which will fail to prevent E deficiency symptoms (8). In addition, PUFA-induced symptoms have been prevented by antioxidants in calves (14), chickens (15), and the rat (16), with no concomitant increase in the tocopherol content of the tissues.

It has been known for many years that the unsaturated fatty acid composition of adipose tissue can be increased markedly by feeding of unsaturated fatty acids in the diet (17), and when unsaturated fatty acids are fed without vitamin E, such adipose tissue is unstable (18). Peroxides can be detected in the depot fat of such animals, even when the fat is dissected from the living animal. It now appears that feeding of PUFA to young animals will result in increased proportions of PUFA in non-adipose tissues (19,20,21) which could lead to unstability of the lipid in these tissues, if diets free of vitamin E or other antioxidants are fed (6). Since the young chicken grows at an extremely rapid rate, effects of

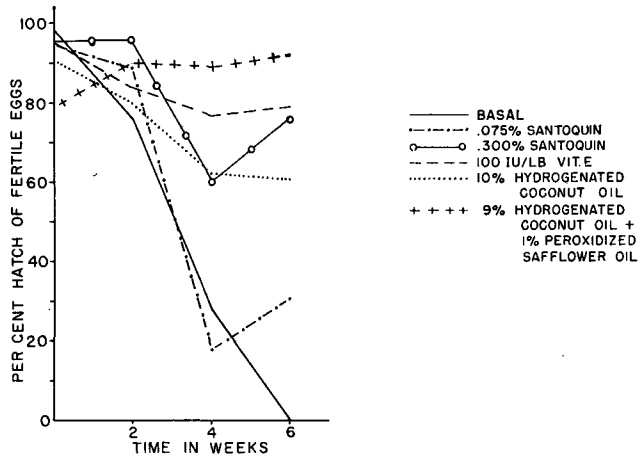

FIG. 2. Effect of unsaturated fat and antioxidants on hatchability.

TABLE I
Effect of Dietary Linoleic Acid on Fatty Acid Composition of Chicken Tissues (13)

	Linoleic (18:2) in diet[a]			
	None		1.5%	
	% of tissue fat			
	18:2	20:4	18:2	20:4
Depot fat	0.0	0.0	13.0	0.0
Heart	4.3	2.1	24.6	18.0
Testis	0.0	1.4	6.6	16.0
Liver	1.1	2.0	17.6	16.4
Cerebrum	0.0	5.5	0.8	15.7

[a] Provided by 2% safflower oil.

diet on fatty acid composition are readily produced. This is illustrated in Table I.

It has also been found that not only the fatty acid composition of the entire tissue is affected by diet but the fatty acid composition of the subcellular particles, such as the mitochondria, can be changed (22,23). It seems reasonable to assume that, if the lipid from mitochondria can be altered, the lipids in cell membranes, lysosome membranes, and other critical structures can also be altered and made less stable when antioxidants are depleted from such structures. Tappel et al. (24) have found that disruption of the lysosomal membrane may be the primary cause of dystrophy in E-deficient rabbits.

Encephalomalacia

When chickens are fed linoleic acid family fatty acids, linoleic and arachidonic acids increase in the cerebellum and appear to reach a maximum proportion at the time of onset of encephalomalacia (22) (Fig. 3). At ca. 10–12 days of age, the linoleic and arachidonic acid content has reached a maximum and about that time the chicks start coming down with the disease. The onset of the disease also appears to coincide with the known depletion of tocopherol from the tissues of the young chick. If tocopherol or other antioxidants are in the diet, the chicken survives even though the brain contains high levels of polyunsaturated fatty acids. The disease can be precipitated in older birds by withdrawal of the antioxidant from a high linoleic acid diet (25) (Fig. 4). In this study, chickens were fed a high-linoleic diet with Santoquin for either 19 or 60 days and at the respective times the antioxidant was withdrawn. In each case, the birds came down with encephalomalacia about two days after withdrawal of the antioxidant. This demonstrates that encephalomalacia can be induced in older birds if they had been previously fed high-linoleic acid diets.

Effectiveness of Antioxidants

It is known that antioxidants vary considerably in their effectiveness in preventing vitamin E deficiency

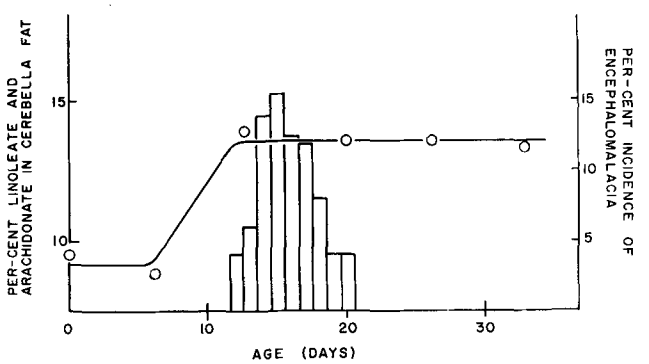

FIG. 3. Encephalomalacia and incorporation of linoleate and arachidonate in chick cerebella.

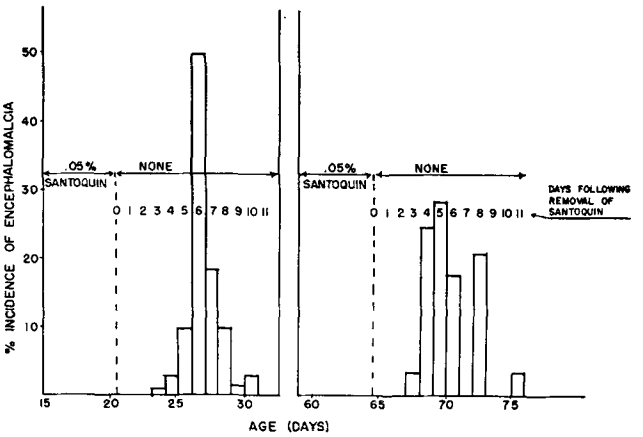

FIG. 4. Encephalomalacia in 10 wk old chickens.

symptoms. As a result, we and others have used the terms *in vivo* and *in vitro* antioxidants. For instance, we have usually though that most of the gallates or hindered phenols were not effective *in vivo*. The last study confirms the fact that they are not very effective but show that, when added at extremely high levels, they can also prevent encephalomalacia (Table II). In this study, a high linoleic acid diet was fed. As can be seen, 0.015% Santoquin completely prevented the incidence of encephalomalacia. It took 0.5% of BHA to completely prevent the disease. There is approximately a 20–30 fold difference in activity between these antioxidants. Other antioxidants, such as dilauryl thiopropionate, were completely ineffective even when fed at an extremely high level (0.5%).

There are several plausible reasons for the difference in effectiveness of various antioxidants in preventing E deficiency symptoms. The first is that they may vary considerably in *in vitro* antioxidant activity. Unfortunately, the correlation between *in vivo* and *in vitro* activity, as measured by a number of tests, has usually been poor. Everyone knows that antioxidant testing is highly empirical and most researchers have probably not used testing conditions which mimic the *in vivo* situation. For example, α-tocopherol is the most effective tocopherol isomer for prevention of E deficiency symptoms. However, most *in vitro* tests of antioxidant activity have indicated that the γ-tocopherol and other isomers are better antioxidants. This has been used as an argument against the antioxidant role of vitamin E in animals. However, recently Lea (26) has shown that by changing the condition for testing, the order of activity is also changed such that the alpha is the most effective *in vitro* antioxidant. Another reason for differences in *in vivo* antioxidant activity could be the difference in the absorption and retention of antioxidants. For instance, α-tocopherol is absorbed and deposited in tissues more readily than the other isomers, and this again correlates with its more effective *in vivo* activity.

The effectiveness of synthetic antioxidants in preventing encephalomalacia, together with other observations previously mentioned, has suggested that encephalomalacia is caused by the peroxidation of a lipid structure in the cerebellum, following the synthesis of lipids high in certain unsaturated fatty acids and following the depletion of antioxidants or vitamin E from the cerebellum.

What would trigger this peroxidation? Free radicals are known to be produced in normal biochemical reactions and this could initiate peroxidation. Hema-

TABLE II
Effect of Synthetic Antioxidants for Prevention of Encephalomalacia

Supplement	Incidence of encephalomalacia [b]
None [a]	16/24
0.010% Santoquin	3/42
0.015% Santoquin	0/14
0.020% BHA	17/18
0.100% BHA	2/14
0.500% BHA	0/14
0.100% Propylgallate	3/14
0.500% Propylgallate	0/14
0.500% Dilauryl thiopropionate	13/14

[a] Basal diet contained 8% ethyl linoleate and 5% methyl myristate.
[b] No. of birds with encephalomalacia/No. of birds observed.

tin-containing proteins are associated closely with unsaturated fatty acids and are known to be catalysts of peroxidation. Peroxides in the blood may also initiate peroxidation in the cerebellum. This is suggested by the work of Nishida et al. (27). They were able to induce encephalomalacia in birds fed linoleic acid diets in as little as 1–5 hr after intravenous injection of linoleic hydroperoxide. They suggest that encephalomalacia is triggered by lipo-hydroperoxides which are in the blood stream as a result of absorption of dietary peroxides. However, dietary peroxides are evidently not absorbed, but are destroyed by intestinal tissue before they can get into the blood (28). This may not rule out dietary peroxide as a trigger since it may take only an extremely minute amount of peroxide in a susceptible animal for peroxidation to ensue, and such low levels may be absorbed but remain undetected by usual procedures.

As mentioned previously (7), hens fed an encephalomalacia-inducing diet for as long as 16 wk do not develop encephalomalacia, although the egg production and hatchability are affected. Why isn't encephalomalacia triggered in these mature animals? We think that an unstable lipid structure must be present in the cerebellum for encephalomalacia to occur (29). This is based on the experiment demonstrating that encephalomalacia can be produced in older birds by deleting an antioxidant from high linoleic diets after birds have been maintained on a high linoleic diet (plus antioxidant) for an extended period of time. We have also been able to demonstrate that, if birds are maintained on diets low in unsaturated fatty acids for a long enough period, they will eventually be completely resistant to the disease. For example (Fig. 5), when birds were fed low-fat diets from 0–76 days, and then fed an encephalomalacia-inducing diet (high linoleic, low antioxidant), about 70% of the birds did not develop symptoms although maintained on this diet for as long as 50 days.

Until recently, there was no chemical evidence for the assumption that peroxidation of lipids in the cerebellum occurs before or during the onset of encephalomalacia symptoms. Dam mentioned several years ago his inability to detect peroxides in the brain of chicks with encephalomalacia (5). We also have failed to detect any difference in peroxides, as measured by the TBA reaction, between normal and encephalomalacia birds. Budowski (30), however, has found definite evidence of free radical damage in the brain of chicks with encephalomalacia compared to normal chicks. Such free radical damage would, of course, be expected if peroxidation occurred.

The problem of providing more direct chemical evidence for *in vivo* peroxidation is difficult for several reasons. One is that peroxides or hydroperoxides are quite unstable, particularly *in vivo*, where there are active systems for their destruction. Thus, the concentration of peroxide at any given time may be extremely small. Secondly, if the peroxidation occurs in a structure such as lysosomal membrane, a small amount of peroxidation may cause tremendous damage as a result of the release of hydrolytic enzymes. It should be mentioned that some research workers, such as Schwarz (31), state that vitamin E does not function solely as an antioxidant and that, even in the case of the unsaturated fatty acids-dependent symptoms, alternative explanations to the *in vivo* peroxidation hypothesis may be plausible. However, it is apparent that unsaturated fatty acids will increase the requirement for biologically-effective antioxidants and it appears that most experimental observations tend to confirm the *in vivo* peroxidation hypothesis.

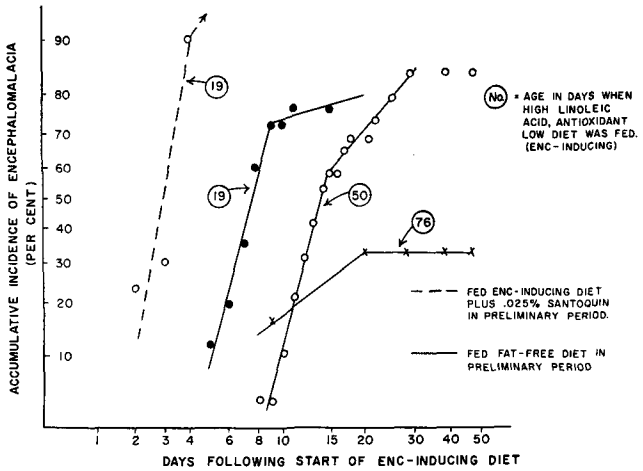

FIG. 5. Delaying encephalomalacia by the feeding of diets low in unsaturated fatty acids.

REFERENCES

1. Agduhr, E., Acta Paedriatica, *5*, 319; *6*, 155 (1926).
2. Evans, H. M., and G. O. Burr, J. Am. Med. Assn., *88*, 1462 (1927).
3. Horwitt, M. K., Bordon's Rev. Nutrition Res., *22*, 1 (1961).
4. Mason, K. E., The Vitamins, *2*, p. 214, ed. by W. H. Sebrell, Jr. and R. S. Harris, Academic Press Inc. (1954).
5. Dam, H., Progress in the Chemistry of Fats and Other Lipids, *3*, 179 (1955).
6. Machlin, L. J., and R. S. Gordon, Proc. Soc. Exp. Biol. Med., *103*, 659 (1960).
7. Machlin, L. J., R. S. Gordon, C. W. Pope, and J. E. Marr, J. Nutr., *76*, 284 (1962).
8. Machlin, L. J., R. S. Gordon, and K. H. Meisky, *Ibid.* *67*, 333 (1959).
9. Crider, Q., P. Alaupovic, and B. C. Johnson, *Ibid.* *73*, 64 (1961).
10. Century, B., M. K. Horwitt, and P. Barley, Arch. Gen Psychiat., *1*, 420 (1960).
11. Century, B., and M. K. Horwitt, J. Nutr., *72*, 357 (1960).
12. Machlin, L. J., and R. S. Gordon, Poultry Sci., *41*, 473 (1962).
13. MacKenzie, C. G., J. B. MacKenzie, and E. V. McCollum, Science, *94*, 216 (1941).
14. Blaxter, K. L., F. Brown, W. A. Wood, and A. M. MacDonald, Brit. J. Nutr., *7*, 337 (1953).
15. Jensen, L. S., J. S. Carver, and J. McGinnis, Proc. Soc. Exp. Biol. and Med., *91*, 386 (1956).
16. Draper, H. A., and A. S. Csallany, *Ibid.*, *99*, 739 (1958).
17. Shorland, B., Progress in the Chemistry of Fats and Other Lipids, *3*, 275 (1955).
18. Klose, A. A., E. P. Mecchi, A. L. Hanson, and H. Lineweaver, JAOCS, *28*, 162 (1951).
19. Machlin, L. J., and R. S. Gordon, J. Nutr., *75*, 157 (1961).
20. Horwitt, M. K., C. C. Harvey, and B. Century, Science, *130*, 917 (1959).
21. Century, B., L. A. Witting, C. C. Harvey, and M. K. Horwitt, J. Nutr., *75*, 341 (1961).
22. Marco, G. J., L. J. Machlin, E. Emery, and R. S. Gordon, Arch. Biochem. Biophys., *94*, 7 (1961).
23. Witting, L. A., C. C. Harvey, B. Century, and M. K. Horwitt, J. Lipid Res., *2*, 412 (1961).
24. Tappel, A. L., *in* Lipids and Their Oxidation, ed. by H. W. Schultz, E. A. Day, and R. O. Sinnhuber, Avi Publ. Co. (1962).
25. Machlin, L. J., G. J. Marco, and R. S. Gordon, JAOCS, *39*, 229 (1962).
26. Lea, C. A., J. Sci. Food Agr. *11*, 212 (1960).
27. Nishida, T., H. Tsuchyana, M. Inoue, and F. A. Kummerow, Proc. Soc. Exp. Biol. Med., *105*, 308 (1960).
28. Mead, J. F., *in* Lipids and Their Oxidation, ed. by H. W. Schultz, E. A. Day, and R. O. Sinnhuber, Avi Publ. Co. (1962).
29. Machlin, L. J., and R. S. Gordon, Fed. Proc., *21*, 474 (1962).
30. Budowski, P., and S. Mokadi, Biochim. Biophys. Acta, *52*, 609 (1961).
31. Schwarz, K., *in* Lipids and Their Oxidation, ed. by H. W. Schultz, E. A. Day, and R. O. Sinnhuber, Avi Publ. Co. (1962).

[Received March 7, 1963—Accepted June 11, 1963]