makes at least probable the presence of mono- and diglycerides.

Additional experiments on hydrolyzed arachis oil are being planned. Furthermore, these investigations are to be applied to other industrial fats.

Summary

1. The presence of mono- and diglycerides in raw and hydrolyzed fats and their effect on the yields of fatty acids obtained by distillation is discussed.

2. Experiments carried out under conditions suitable for the esterification of mono- and diglycerides with fatty acids to form triglycerides indicate the presence of these mono- and diglycerides.

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Interrelationships of Dietary Fat and Tocopherols[1]

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DURING the first decade (1923-33) of researches establishing the existence and nature of vitamin E there occurs frequent reference to the fact that the ability of diets to induce sterility in rats varied considerably with the amount and nature of the dietary fat. The fats concerned were lard, cod liver oil, and butterfat, the latter either as whole fat or as provided by whole milk powder. In the light of present knowledge it is apparent that much of the confusion existing at that time was due to the presence of subminimal amounts of vitamin E in milk-fat and the effect of added lard and cod liver oil in (1) reducing the intake of milk fat, (2) in causing autoxidation of the vitamin in the course of storage or digestion of the diet, or (3) in increasing, in some unknown manner, the tissue requirements for the vitamin.

Evans and Bishop (1), in 1923, observed that omission of lard from their basal diet deficient in the X-substance, later designated vitamin E, resulted in increased fertility of female rats. Confirmation soon came from Mattill *et al.* (2), Sure (3), and Anderegg and Nelson (4) who noted that the reproductive adequacy of diets composed largely of whole milk powder was abolished by additions of lard or cod liver oil to the diets. On the basis of our present knowledge of the low content of tocopherol in butter fat (5), which can be determined quite accurately, it is quite obvious that diets containing about 50% of whole milk powder would provide approximately 0.055 mg. tocopherol, daily—an intake that would be definitely on the border line for reproduction and readily made inadequate by the inclusion of lard or other fats in the diet. Subsequently, Evans and Burr (6), in re-examining the capacity of various dietary fats to promote sterility, observed that the protective action of given doses of wheat germ could be nullified by incorporation of oleic acid or lard in the diet; development of rancidity greatly increased this reaction, and conferred this property upon other fats (such as butterfat and wheat germ oil) which otherwise exhibit anti-sterility activity. About this time (1927), Mattill (7) reviewed the problem of oxidative destruction of vitamins A and E, and provided experimental evidence that oxidative changes accompanying the onset of rancidity in unsaturated fats tend to destroy these vitamins. There was thus established a definite relationship between autoxidation of vitamin E and the sterility-promoting effect of cod liver oil, lard, and other fats when incorporated in diets containing border-line levels of vitamin E.

Later, in connection with the nutritional muscular dystrophy observed in the rabbit and other herbivorous animals, it was soon recognized that the muscle lesions were markedly accentuated by increasing the intake of cod liver oil or lard and, under such conditions, might occur in the face of a reasonable intake of vitamin E (8, 9). On the other hand, if cod liver oil and vitamin E supplements were fed separately and on alternate days, the lesions could be prevented or, if already present, could be repaired (10, 11). In a similar manner it was shown (12) that rancidified fat abolished the effectiveness of partially hydrogenated vegetable shortenings in preventing sterility in rats unless the two fats were fed separately, six hours apart.

It is surprising to note, in retrospect, that, in spite of this vast array of evidence of fat and vitamin E interrelationships, the response of experimental animals to fat-free diets deficient in vitamin E received little attention until 1939 when Mackenzie *et al.* (13) showed that sterility, paralysis, and growth retardation characteristic of vitamin E deficiency occurred in rats fed diets exceedingly low in lipid content (.0078%, plus 25 mg. methyl linoleate daily). They later reported (11) that rabbits exhibit severe lesions of the voluntary muscles when fed E deficient diets containing no more than 0.05% of animal fat. Although dietary fat is not a prerequisite for the production of symptoms of vitamin E deficiency, except possibly in chicks (14), there is now considerable evidence that both the quantity as well as the type of fat, especially its content of unsaturated fatty acids, may accentuate to variable degrees the onset or severity of the symptoms. This becomes a factor of major importance in the bio-assay of vitamin E, and may explain in large part the unexpectedly wide variations in bio-assay results obtained in 1939 when

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investigators in 17 different laboratories in Europe and America collaborated in the assay of a single sample of dl-alpha tocopheryl acetate, being proposed as an International Standard for vitamin E. Results accepted from 9 laboratories indicated a median fertility dose varying from 0.56 to 1.71 mg. (15). Since that time it has been repeatedly demonstrated that the presence of dietary fat (lard or cod liver oil) significantly increases the tocopherol requirement for positive response in bio-assay tests (16, 17, 18). Two other types of interaction between dietary fat and tocopherol deserve comment. In rats subjected to anoxic anoxia the survival time can be significantly increased by oral supplements of tocopherol, while both survival time and the effectiveness of tocopherol are diminished by additions of fat (lard) to the diet (19). In rats deficient in essential fatty acids the efficacy of suboptimal quantities of methyl linolate is definitely enhanced by tocopherol feeding through interactions that are not limited to the gastrointestinal tract (20). The exact mechanisms involved in these phenomena are not clear.

Some of the mechanisms whereby the type of dietary fat, and level at which it is fed, may influence an animal's requirement for vitamin E warrant brief consideration: *First,* dietary fats possessing unsaturation and a low content of natural antioxidants may, under certain conditions, autoxidize tocopherols present in this fat or in other components of the diet. *Second,* through similar interactions in the gut, tocopherols may be inactivated by proöxidants present or be actively expended as intestinal antioxidants, resulting in partial or total loss before they can be absorbed. There is evidence that such interactions are more extensive in herbivorous animals than in omnivorous or carnivorous forms. This provides one explanation for the observations that doses of tocopherol adequate for curing muscular dystrophy in rabbits when fed alternately with cod liver oil at 24-hour intervals are ineffective when both are administered together. *Third,* dietary fats capable of increasing the unsaturation of tissue phospholipids and of stored fats may augment tocopherol needs, or have priority for tocopherol stores, in order to effect adequate stabilization of the body lipids. The latter are known to be influenced by unsaturated fatty acids present in the diet; furthermore, the phospholipids of tissue cells and body fluids have a tendency to select and retain the most highly unsaturated fatty acids offered in the food. Those containing the more highly unsaturated acids are especially adapted for participation in oxidation processes, for formation of semipermeable cell membranes, and for replacement of wear and tear processes; others are utilized for metabolic combustions and energy production (21). The important observations of Burr and his associates (22, 23, 24, 25), concerning the role of tocopherols in stabilizing (and in thereby preventing *in vivo* oxidation of) body fats of the rat and pig, may apply equally well to the tissue phospholipids. Their finding that maximum deposition of a single dose of tocopherol in body fat requires 7 to 10 days (23) suggests a temporary deposition or storage elsewhere, perhaps in the liver, with subsequent and gradual release. They consider depot fats to be a major site of tocopherol storage. While this may be true under certain experimental conditions, bio-assay studies (26) indicate that storage in adipose tissue is not markedly greater than that of other tissues and, at high levels of intake, is much less than that of the liver.

One may perhaps look upon the tocopherols as the only biological antioxidants other than ascorbic acid that, after serving as valuable antioxidants in the plant world, can survive the gamut of storage, digestion, and absorption and continue similar functions in animal cells. Future vitamin E research must endeavor to find an answer to two major questions: *First,* do any of the manifestations of vitamin E deficiency represent secondary reactions resulting from an inadequacy of this irreplaceable, intra-cellular antioxidant; i.e., can they be attributed to abortive oxidations that directly interfere with other cell functions, perhaps those involving phospholipids, or to liberation of oxidation products capable of exerting a damaging effect upon certain tissues? *Second,* in addition to their function as stabilizers of body lipids, do some or all of the recognized tocopherols participate in other, and perhaps more vital functions—such as enzymatic reactions, electrolyte interchange, or amino acid utilization?

There is some evidence available to indicate that alpha tocopheryl phosphate may act in a regulatory fashion to control or inhibit specific enzyme systems (27, 28, 29). Furthermore, the fact that the different tocopherols exhibit rather comparable antioxidant activity, both *in vitro* and *in vivo,* at temperatures approximating normal body temperature (30), and yet show rather marked differences in their biological activity (31) (anti-sterility or anti-dystrophy tests) may be considered as further evidence in support of the dual function of tocopherols—unless it be shown that the biologically less active forms (β, γ, and δ) are less efficiently absorbed than alpha tocopherol, or else require methylation to the alpha form in the body, with variable loss in this conversion, before being able to enter into biological reactions. These, and other aspects of tocopherol functions have received extensive discussion in a recent review by Hickman and Harris (32). The two questions mentioned in the previous paragraph are perhaps more interlocking than they first appear to be. Any new information pertinent to one may, at the same time, contribute much to the other question. The remaining phase of this discussion, concerned with problems under investigation in our laboratory for several years, is more or less related to the first of these questions.

Following the finding of a characteristic brownish-yellow pigment, quite insoluble, inert, and devoid of iron, in smooth and skeletal musculature of vitamin E deficient rats by Martin and Moore (33), it has been demonstrated (34) that a similar pigment occurs in the musculature and, perhaps secondarily, in the reticulo-endothelial system of a large variety of animals reared on diets deficient in vitamin E (rat, mouse, hamster, cotton rat, dog, and monkey). The distribution of this pigment has been followed in tissues chiefly by virtue of its acid-fast staining reactions such as employed in identification of the tubercle and leprosy bacilli. It also shows affinity for Sudan IV and Sudan black in histological sections previously exposed to lipid solvents, suggesting an insoluble lipid complex. It occurs abundantly in smooth muscle cells showing no evidence of degeneration, in skeletal and cardiac muscle usually when degenerative changes are in progress. Although more recent observations indicate that this pigment does not occur when

fat is excluded from E deficient diets, its deposition, as well as the lesions of skeletal and cardiac muscle fibres, are definitely accentuated by fats in the diet, especially by those providing highly unsaturated fatty acids. This pigment may represent a product of undesired oxidation of fatty acids which influences the composition, and possibly the functional capacity, of tissue phospholipids and, under certain conditions of dietary stress discussed below, may also represent polymerized glycerides.

Other studies in this laboratory have demonstrated that vitamin E deficient diets, containing 20 per cent of cod liver oil (35), or its equivalent as highly unsaturated fractions of fatty acids of this oil (36), lead to a similar deposition of pigment in the depot fats, associated with the accumulation of peroxides within the adipose tissue (37). This progressive accumulation of pigment within fat cells, associated with extensive giant cell formation and fibrotic reactions of the connective tissue, leads to a very involved and complicated histological picture (35). It was first suspected that these striking reactions of adipose tissue might be related to fatty acids of rather long chain length and high unsaturation (C_{20} and C_{22}) which are rather characteristic of fish oils. We have been much surprised to find that equally striking changes can be brought about by C_{18} fatty acids with 3 double bonds (linolenic acid), provided as methyl esters of linseed oil fatty acids, whereas C_{18} fatty acids possessing 2 double bonds (linoleic acid), provided as methyl esters of corn oil and administered in a similar manner, produce little more than traces of pigment in fat depots after periods of six months; whereas methyl esters of soy bean oil, providing small amounts of linolenic together with amounts of linoleic acid equivalent to that of corn oil, produce an intermediate degree of pigment deposition. It would seem that rats deprived of vitamin E are unable to prevent autoxidation of the more highly unsaturated fatty acids, especially those with 3 double bonds, during the course of their deposition in their fat depots. Our own observations, which support earlier conclusions of Hass (38) based upon acid fast reactions occurring in the intercellular spaces following injections of certain fats and fatty acids into the subcutaneous tissues, as well as the studies of Endicott (39) concerning the nature of a similar pigment, "ceroid," occurring in the liver and other tissues in nutritional cirrhosis of rats, indicate that the acid-fast pigment under discussion may represent a complex series of polymers arising secondary to abnormal peroxidation of unsaturated fatty acids, prior to or subsequent to their incorporation in body lipids.

It is also worthy of note that the feeding of deficient diets rich in unsaturated fatty acids seems to accentuate the time of onset and severity of most lesions considered typical of vitamin E deficiency, and generally produces a larger amount of acid-fast material in association with such lesions than is commonly observed when such diets contain comparable amounts of lard. Furthermore, we have also observed in animals fed diets containing 20 caloric per cent methyl esters of linseed oil, that the daily intake of either alpha or gamma tocopherol just sufficient to prevent acid-fast changes in adipose tissue are likewise just sufficient to prevent lesions of the skeletal muscle, degeneration of the testis, and the occurrence of acid-fast pigment within the lumen of the intestine.

It thus appears that tocopherols, functioning as antioxidants, play an exceedingly important role in protecting or stabilizing unsaturated fats (a) in the diet before ingestion, (b) in the digestive tract, and (c) especially during their mobilization, metabolic turnover, and storage within tissue cells. Whether the classic histopathologic manifestations of vitamin E deficiency (fetal death, testis degeneration, muscle dystrophy), which it has been pointed out can occur in the absence of dietary fat, but are accentuated by ingestion of unsaturated fatty acids, are due to unstable states arising in tissue lipids synthesized by the animal from other sources, or are due to disturbance of some specific cellular enzyme system in which tocopherols participate by virtue of properties other than those of antioxidants, remains for future researches to determine.

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