Nature of the Cofactor for the Acid Lipase of *Ricinus communis**

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Many plant and animal lipases require emulsifiers, activators, or fatty acid acceptors for optimum activity. The acid lipase of *Ricinus communis*, which requires no additives to catalyze lypolysis, contains a natural cofactor which can be separated from the apoenzyme by cold butanol extraction; its role is not solely that of an emulsifier. Examination of the cofactor by infrared and nuclear magnetic resonance spectroscopy, glass paper chromatography, and appropriate chemical methods shows it to be cyclic polymers of ricinoleic acid—predominantly the tetramer. It may be replaced in varying degrees by fatty acids, monoglycerides, α-tocopherol succinate, and the methyl half ester of dodecenyl succinate.

The castor bean, with about 90% of its fatty acids as ricinoleic acid, is one of the few oilseeds containing a lipase in the quiescent seed capable of hydrolyzing glycerides of long-chain fatty acids. This lipase requires no added emulsifiers or fatty acid acceptors for maximum activity (Ory *et al.*, 1962).

The requirement for emulsifiers or cofactors for lipid hydrolysis in other systems is well documented. Pancreatic lipase requires an emulsified substrate (Desnuelle, 1961). Kates and Gorham (1957) stimulated plastid phospholipase activity on lecithin with organic solvents such as ethyl ether, propyl ketone, and ethyl butyrate. Similar effects of ethyl ether in stimulating the degradation of egg phosphatidyl choline by pancreatic phospholipase A (Hanahan, 1952), and on the action of snake venom lecithinases (Hanahan *et al.*, 1954) and lecithinase D from *Cl. perfringens* (Hanahan and Vercamer, 1954) on synthetic lecithins, have been reported. Weiss *et al.* (1959) isolated a natural activator for phospholipase D from soybean lecithin which catalyzes the hydrolysis of choline from lecithin. This activator appears to be phosphatidyl inositol, the same material which Bangham and Dawson (1960) showed to be necessary for the action of *Penicillium notatum* phospholipase B on lecithin.

A plant lipase which did not require any further additives for the hydrolysis of long-chain (C-18) glycerides raised the possibility that some type of natural cofactor might still be attached to the enzyme after the various buffer and ether extractions used in the preparation. Extraction of this partially purified lipase with ice-cold 1-butanol removed a lipid cofactor which is required for full activity on long-chain unsaturated glycerides but not on tributyrin (Ory and Altschul, 1962b). The cofactor could be replaced by α-tocopherol succinate but not by the antioxidant Ionol (Ory and Altschul, 1962a).

The purpose of this paper is to report the isolation and identification of this cofactor and to describe other lipid materials which can replace it.

EXPERIMENTAL

Seed.—Castor beans (*Ricinus communis*) of the Baker 296 variety were a gift of W. E. Domingo of the Baker Castor Oil Company.

Materials.[1]—Silicic acid was 100-mesh Mallinckrodt, chromatography grade; glass fiber paper was Reeve Angel, type 934-AH; organic solvents were Baker Analyzed Reagents used without further purification; soybean lecithin was from the Glidden Co., technical grade; cottonseed oil was commercial Wesson oil; α-tocopherol succinate was from Distillation Products, Inc., Control K-315; ricinoleic acid (sodium salt) was from Eastman Organic Chemicals, practical grade; mono-, di-, and triricinolein were technical grade products of Baker Castor Oil Co.; oleic acid (99% purity) was a gift of W. S. Singleton; methyl half ester of dodecenyl succinate was prepared by controlled methylation of dodecenyl succinic anhydride, Baker & Adamson, technical grade.

Methods.—Lipase activity is reported as micromoles of fatty acids released from 1000 μmoles of glyceride substrate at *p*H 4.2 by 1.3 mg of enzyme protein in 5 ml reaction volume in 10 minutes by titration on a Radiometer *p*H-stat (Altschul *et al.*, 1963). Infrared spectra were determined in KBr disks or as the pure liquid between NaCl plates in a Perkin-Elmer 21 spectrophotometer. Nuclear magnetic resonance spectra (NMR) were made on approximately 10% solutions in CCl_4 with a Varian Associates Model A-60.

Isolation of Cofactor.—Castor bean lipase was prepared by lyophilizing the extracts of macerated rehydrated castor beans as described earlier (Altschul *et al.*, 1963). This dry preparation, now free of neutral fats and of water-soluble and salt-soluble components, was split into a particulate fraction with greatly reduced activity (apoenzyme) and a thick oil (cofactor) by extraction in an ice bath with 1-butanol as described by Ory and Altschul (1962b). The oil is readily soluble in the usual lipid solvents; it contains no nitrogen, phosphorus, or sulfur.

Glass Paper Chromatography.—Glass fiber paper sheets for chromatographic analysis of the cofactor and its possible replacements were treated with silicic acid according to the method of Dieckert *et al.* (1958).

RESULTS AND DISCUSSION

Components of Cofactor.—Best analytical separation of the cofactor into its components is by chromatography on silicic acid–treated glass paper developed in isooctane-ether-acetone 200:10:10. There is a trace spot at the solvent front plus two major spots with R_F values of 0.70 and 0.32 (Fig. 1).

Attempts at separation of larger quantities by chromatography on a silicic acid column yielded two spots

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[1] Mention of a trade name does not imply approval or recommendation of the product to the exclusion of others which may be also suitable.

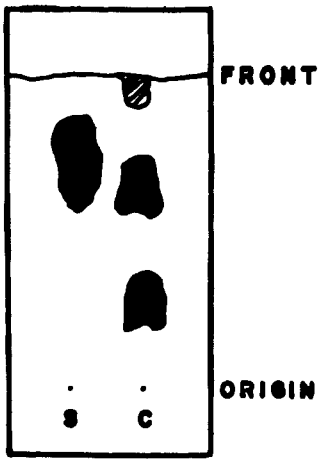


FIG. 1.—Glass paper chromatogram of cofactor before and after saponification. Silicic acid–treated paper; solvent, 200:10:10 isooctane-ether-acetone; spots developed by H_2SO_4 charring; S, saponified cofactor; C, nonsaponified cofactor.

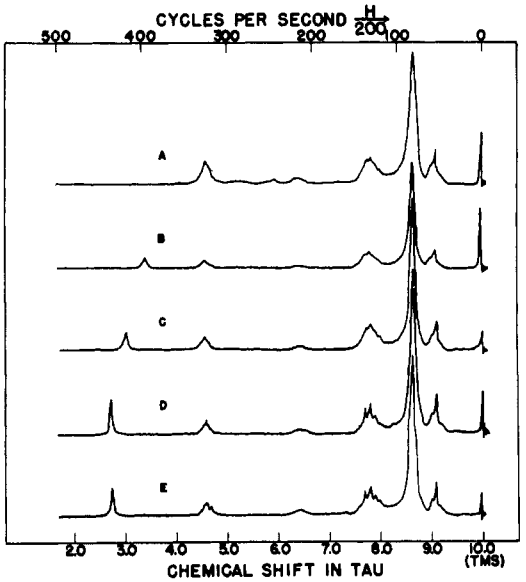


FIG. 2.—NMR spectra of cofactor and related compounds. A, fresh cofactor; B, first component eluted from silicic acid column; C, second component eluted from silicic acid column; D, authentic ricinoleic acid; E, alcoholic KOH extract of lipase.

which appeared on paper chromatograms to be the same as the original cofactor components. However, subsequent spectroscopic studies showed that the material from the column contained free hydroxyl and carboxyl functions (Fig. 3B and 3C) not present in the original mixture. This apparent hydrolysis on the column precluded further attempts to identify the individual cofactor components. It was interesting to note that upon standing the slowest component from the column was quickly and completely transformed into the faster-moving material, and this transformation was not reversed by reduction with sodium borohydride, sodium hydrosulfite, or hydrogen sulfide gas.

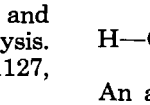
The transformation of a large slow-moving lipid to a faster-moving product without a quantitative loss of material suggested some form of depolymerization. A similar observation was made on the original cofactor mixture. Molecular weights for three different preparations of cofactor were determined by vapor pressure osmometry. One sample was fresh cofactor prepared immediately before the analysis, the second was cofactor prepared one day before the analysis, and the third was the R_F 0.32 component eluted from the column and kept at room temperature for a month before analysis. The average molecular weights, in order, were: 1127, 974, and 824.

Saponification of Cofactor.—Biological activity of the unpurified cofactor is not destroyed by heating on a steam bath for 20 minutes under a nitrogen atmosphere, nor by KOH saponification for 30 minutes.

Though saponification does not destroy biological activity of the cofactor, it does produce a change in composition as shown on the glass paper chromatograms. The entire mixture is converted into a single component having an R_F value of 0.75 (Fig. 1).

Cochromatography of the cofactor before and after saponification with ricinoleic acid, glycerol, mono-, di-, and triricinolein, and methyl ricinoleate excluded the three glycerides on the basis of R_F values but suggested that the cofactor saponification product was ricinoleic acid. No glycerol was detected in the saponified material by various extraction procedures, by attempted conversion to acrolein, nor by cochromatography with authentic glycerol. Experiments on synthetic mixtures of glycerine and fatty acids showed that glycerine is detectable under these conditions. The absence of glycerol after saponification, the different R_F values, and the NMR spectra obtained in the comparison studies exclude glycerides as components of the cofactor.

Structure of Cofactor.—The fact that saponification produced only one product from the original two components was the basis for determining its structure, first by identification of this hydrolysis product. Direct extraction of the lipase preparation with 2 N alcoholic KOH and decolorization with activated charcoal yield a single acidic material which has the same chromatographic properties as the saponification product of the cofactor. The NMR spectrum of this acid in CCl_4 (Fig. 2E) shows a distorted triplet at 9.08 τ, attributed to a single terminal methyl group. The long unbranched chain is indicated by the broad peak at 8.68 τ, and the methylene group adjacent to the carboxyl function and the four allylic protons by the multiplet at 7.88 τ. Broad peaks at 6.42 τ and 4.60 τ indicated

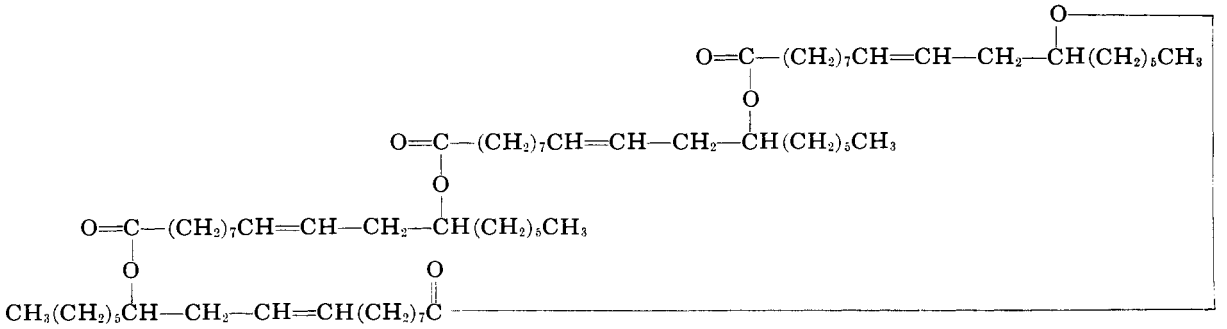


and —CH=CH— functions, respectively. An additional peak is present in the vicinity of 2.75 τ but the exact position of this signal seems to be dependent upon both the solvent and the concentration of the sample. Addition of D_2O to the sample causes this signal to vanish, leading to the conclusion that it is due to the rapidly exchanging carboxyl and hydroxyl protons. Such a pattern would be consistent with any of a number of isomeric straight-chain fatty acid structures containing 36 protons. However, since the castor bean is the major source of ricinoleic acid and this acid contains a hydroxyl and a double bond, the unknown long chain acid was assumed to be ricinoleic acid.

This was confirmed by infrared spectral analysis of the unknown acid. The infrared spectrum of this product (Fig. 3E) is essentially superimposable upon the published spectrum of ricinoleic acid[2] (Sadtler No. 21992.)

Final confirmation of the identity of this acid was obtained by the alkali cleavage method of Ackman

[2] Sadtler Standard Spectra No. 21992, published by Sadtler Research Laboratories.



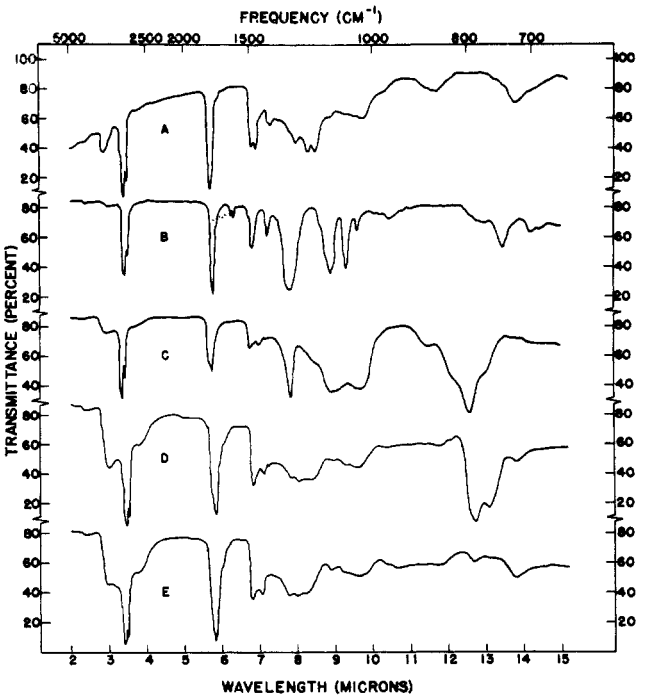


FIG. 3.—Infrared spectra of cofactor and related compounds. A, methyl ricinoleate; B, first component eluted from silicic acid column; C, second component eluted from silicic acid column; D, fresh cofactor; E, alcoholic KOH extract of lipase. Curve A determined on KBr disk; all others as pure liquid between NaCl plates.

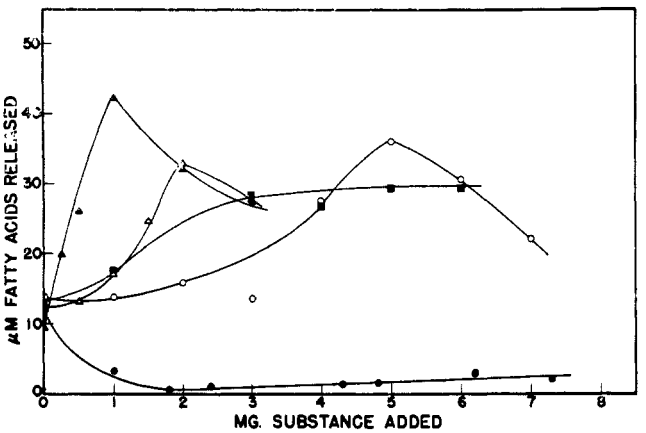


FIG. 4.—Biological activity of possible cofactor substitutes. Reaction conditions: substrate, 1000 μmoles cottonseed oil; 1.3 mg protein per test; reaction volume, 5 ml; *p*H 4.2; reaction time, 10 min.; ●, soybean lecithin; ○, diolein; ▲, monoricinolein; △, oleic acid; ■, methyl half ester of dodecenyl succinate.

et. al. (1960). The cleavage product was isolated, decolorized, and recrystallized from water to yield small white plates of sebacic acid whose identity was established by melting point (127–129°, Kofler micro hot stage) and mixture melting point with a sample similarly derived from authentic ricinoleic acid.

The infrared and NMR spectra show a close relationship between the cofactor and ricinoleic acid. Figure 2A shows the NMR spectrum of the crude cofactor. Peaks at 9.06 τ, 8.68 τ, 7.81 τ, and 4.60 τ are essentially the same as those in the spectrum of ricinoleic acid. The peak at 6.42 τ is less intense for the cofactor than for the free acid, suggesting that some hydroxyls are esterified in the cofactor. The signal at 5.95 τ, for a H—C—O—C=O moiety, strengthens this interpretation. Since the intensity of this peak is approximately one-fifth that expected for monoricinolein, this signal appears to be due to partial esterification of the hydroxyl group of ricinoleic acid rather than to the presence of any glyceride. A low-field peak due to rapidly exchanging carboxyl and hydroxyl protons as found in the free acid is completely absent. In fact, no carboxyl protons can be found within 1000 cps of tetramethylsilane and no hydroxyl protons are observable even though a small peak is seen at 6.42 τ.

If one compares these spectra with those of triricinolein or methyl ricinoleate, the latter show signals due to free hydroxyl protons in the region 7.5–8.2 τ. Thus, if only a small amount of free hydroxyl is present in the cofactor, the resonance signal for hydroxyl protons might very easily be lost in the large broad peak present at the same point. The decrease in intensity of this peak on treatment with D_2O would be considerably less than one-seventh of the original and would be very difficult to detect.

As expected, the infrared spectrum of the crude cofactor (Fig. 3D) exhibits absorption maxima at 2.97 μ (hydroxyl) and 5.7–5.8 μ (ester) indicating varying degrees of esterification in the cofactor mixture.

These data are not sufficient for unequivocal assignment of structures to the various components of the cofactor. However, ricinoleic acid, with a free hydroxyl and a nearby double bond, contains a structure which is properly adapted to the formation of polymers. The lack of end-groups shown by NMR and the molecular weights of cofactor preparations determined immediately after extraction and after aging seem to favor cyclic structures such as the tetramer and/or lower polymers. However, the presence of the 6.42 τ peak in the NMR spectrum excludes a monomeric lactone as the major constituent of the cofactor.

The apparent decrease in molecular weight upon standing suggests that the cofactor *in vivo* and prior to butanol extraction is predominantly tetramer. Once the coenzyme is separated from the apoenzyme

this stability is decreased, the net result being a gradual depolymerization to more stable but still biologically active lower molecular weight forms. Thus a molecular weight of this material at any time after disruption of its natural bond to the protein would be an average of the cyclic esters present.

Bolley (1964) observed the formation of a somewhat similar complex mixture of lactones, lactides, simple esters, and linear and cyclic polyesters. This mixture, which he termed "estolides," was formed from ricinoleic acid upon heating or from methyl ricinoleate upon addition of sodium methylate catalyst and mild heating under vacuum. This reaction is not spontaneous, however, and must be catalyzed by the alkali catalyst and heat.

In preparation of the lipase and the cofactor all steps are conducted at 0–4°; the evaporation of butanol is the only point which involves heat. A chromatographic analysis of the butanol extract before and after removal of the solvent by heat yields the same pattern shown by C, Figure 1. Therefore, we may conclude that the components observed are not heat-induced artifacts. The natural occurrence of large cyclic structures such as those proposed here is by no means new. Woodward (1957) and Hochstein *et al.* (1960) have found such systems in a variety of natural sources.

Biological Activity of Related Compounds.—It was of interest to determine whether materials other than ricinoleic acid derivatives would also exhibit cofactor activity and what properties or functional groups they might have in common. The results in Figure 4 show that monoricinolein, diolein, oleic acid, and the methyl half ester of dodecenyl succinate exhibit varied degrees of cofactor activity. It was shown earlier that α-tocopherol succinate can replace the cofactor (Ory and Altschul, 1962a). However, this compound is completely inactivated by saponification. Thus, it appears that both some type of carboxyl function and a long hydrocarbon chain are required for cofactor activity. The fact that triglycerides are not themselves active as cofactors might suggest that surface activity is another requirement.

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Estriol Biosynthesis by Sow Ovary

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Incubation of 16α-[4-^{14}C]hydroxyprogesterone with minced sow ovary has led to the formation of [4-^{14}C]estriol. A pathway is presented for the genesis of estriol based on the compounds isolated and identified. The results are in agreement with the biosynthetic route as proposed for progesterone. The presence of an active C-16 dehydrase enzyme system is found which appears to operate in preference to the 17α-hydroxylase enzyme system.

The formation of estriol from estradiol-17β by human fetal liver slices has been demonstrated by Engel *et al.* (1958) and also with isolated rat livers (Hagopian and Levy, 1958). Surprisingly enough, no estriol biosynthesis has been observed from estradiol-17β in adult liver (Engel *et al.*, 1958; Ryan and Engel, 1953). Ryan (1959) has been able to show conversion of C-16-oxygenated steroids to estriol in human placenta, while in a preliminary note the conversion of 16α-hydroxyprogesterone to estriol in sow ovary was reported (Kadis, 1964). It is the purpose of this report to elucidate further the nature of the latter reaction through the identification of intermediate products.

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EXPERIMENTAL

Incubation techniques used and isolation of compounds have been described elsewhere (Warren and Salhanick, 1961a) with one major change. For removal of fats, a partition between 90% methanol and Skellysolve B was used.

Eight per cent sodium hydroxide was used to separate the incubated material into neutral and phenolic fractions which were chromatographed separately (Kadis, 1964). The neutral fraction was chromatographed in a hexane-benzene system for 20 hours; this left all steroids on the paper except 16-dehydroprogesterone (Table I). To the overrun known 16-dehydroprogesterone was added and chromatographed according to the outline in Table I. Of the compounds remaining on the paper, the 4-androsten-16α-ol-3,17-