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Lipid-mediated hemagglutination

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LIPID-MEDIATED HEMAGGLUTINATION

by

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A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

LIPID MEDIATED HEMAGGLUTINATION

by

IOANNIS S. PATRIKIOS

Advisor: Professor Charlotte S. Russell

Contradictory reports in the literature about the hemagglutinating activity of dioleoylphosphatidylethanolamine (DOPE) were partially explained in this laboratory by Pi-Shiang Lai. Pure DOPE does not agglutinate red cells, but aged or heated samples agglutinate several species of red cells. It was also found that oleic acid (cis-9-octadecenoic acid, OA) exhibited the same hemagglutinating activity under the same conditions and presented a simpler system to investigate.

OA itself has a very low agglutination titer but lyses red cells at higher concentrations. When mono-unsaturated fatty acids are heated in air, they form hemagglutinins. Octadecenoic acid with a cis-6 or cis-9 double bond has a higher agglutination titer than cis-11-octadecenoic acid. Stearic acid does not become a hemagglutinin on heating. Hydroxy-monounsaturated fatty acids, such as ricinoleic (cis-12-hydroxy-Δ-9) and ricinelaidic (trans-12-hydroxy-Δ-9) hydroxy acids are not hemagglutinins unless they are heated. Rabbit,

rat and chicken erythrocytes (RBCs) give the highest titers but RBCs of other species are also agglutinated. OA was chosen for further study. Its specific titer against rat RBCs increases with time of heating in air. Sialidase treatment of the red cells increases the titer. Removal of cations does not alter the titer but addition of Ca^{2+} or Mg^{2+} lowers the titer. Thin-layer chromatography (TLC) and mass spectrometry (MS) show that higher molecular weight compounds are formed on heating OA and that hemagglutination activity is associated with these materials.

Synthetic linear oleic dimers (obtained by oxidation of OA with di-tert-butyl peroxide) and commercial OA dimers (Emery, Cincinnati, OH and Unichema, Chicago, IL) and a commercial preparation of OA trimer mixed with polymer (Emery) which contained linear cycloaliphatic and aromatic dimers and trimers all have high hemagglutination titers against rat RBC. A cyclic, long-chain dicarboxylic acid, 5(6)-carboxy-4-n-hexyl-2-cyclohexene-1-octanoic acid (Westvaco, North Charleston, SC), gives no lysis and a very low titer unless heated. Filtration of all of these fatty acids through a 0.2 μm membrane does not change the titer.

Light microscopy was used to characterize and visualize the agglutination process with rat RBCs. Large multilamellar vesicles are observed when OA is dispersed in saline at pH 7.4 at concentrations of 1-4 mg/mL, sonicated and filtered through Whatman 41 paper. Agglutination without lysis or fusion is observed for low concentrations of heated OA and C18 dimers and C18 trimer-polymer preparations and no large vesicles are seen. A

pH study (pH 6-9) of heated OA, C18 dimer and C18 trimer-polymer against rat RBCs shows that the specific titer increases with pH from 6 to 9, beyond which range at either end the RBC are not stable. It is concluded that oligomeric fatty acids with two or more hydrophobic chains of seven or more carbons and more than one carboxyl group are agglutinins at physiological pH. Near infrared studies with dimers at high and low concentrations suggest that the carboxyl and carboxylate groups form intermolecular hydrogen-bonds. This suggests that they can form hydrogen-bonded aggregates of various kinds, possibly networks. Agglutination by the dimer may result from the insertion of two hydrophobic side chains into adjacent RBC membranes or from complete insertion of dimer into RBC membranes and alteration of their properties. The carboxyl groups may also play a role in the process by interacting with polar headgroups of phospholipids in the RBC membrane.

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This thesis is dedicated to my father, Stylianos Patrikios, who died last year from prostate cancer at the age of 57 without having the joy of seeing me graduate.

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ABBREVIATIONS

C-22 : dimer of behenic acid

CI : chemical ionization mass spectroscopy

DOPE : dioleoylphosphatidylethanolamine

EI : electron impact mass spectroscopy

HPLC : high pressure liquid chromatography

HEPES : N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid

IR : infrared spectrometry

LM : light microscopy

MS : mass spectrometry

MW : molecular weight

NIR : near infrared spectroscopy

NMR : nuclear magnetic resonance spectroscopy

OA : oleic acid

OA Δ10D : oleic acid heated for 10 days

OA Δ 24 hrs : oleic acid heated 24 hrs

PBS : phosphate buffered saline

PBS-N_3 : phosphate buffered saline with 0.01% NaN_3

RBC : red blood cells

rRBC : rat red blood cells

SA : sialidase

SGC : silica gel column

TBA : thiobarbituric acid

TLC : thin-layer chromatography

INTRODUCTION

Lectins

Lectins are specific proteins capable of binding to or agglutinating certain cells. Certain plant proteins, sometimes called phytohemagglutinins, can bind to and agglutinate red blood cells. It has been found that they will bind to the surfaces of many other kinds of animal cells. They are especially abundant in plants, particularly of the legume family, and are also found in many invertebrate tissues. Concanavalin A from jack bean and ricin from castor bean were among the first hemagglutinins discovered [1].

The term 'lectin' (Latine legere, "to pick or choose") was first applied by Boyd and Shapleigh (1954) [2] to seed extracts of plants, which could agglutinate and distinguish among human blood groups. Most remarkable and significant is the fact that some lectins preferentially agglutinate malignant tumor cells. Tumor cells must therefore have a different surface structure than normal cells. Materials which could clump red blood cells were then called hemagglutinins. Hemagglutinins have been found particularly in the seeds of plants, but also in other parts of plants, such as roots, leaves, and bark [3]. In addition, agglutinins have been found in bacteria, fungi, lichens, fish roe, fish, snails, insects, invertebrates, and mammals [4]. Lectins of plants and

invertebrates appear to be defensive proteins that protect these organisms, which have no immune system and thus no antibodies to fight the invasion by microbial parasites. Hemagglutinins may be membrane-bound or soluble in aqueous solution. Some lectins are known to agglutinate not only red blood cells but also other kinds of cells such as lymphocytes, fibroblasts (connective-tissue precursors), spermatozoa, bacteria, and fungi [5].

Stein and Cooper [6] classified agglutinins into three categories: antibodies, lectins and other receptor-specific substances. (a) Antibodies are proteins of the immune system, are produced by lymphocytes or plasma cells, and may be elicited in response to antigens. They are glycoproteins with highly specific molecular structure composed of multiples of basic subunits consisting of heavy and light polypeptide chains. They have a wide range of binding specificities, with two or more binding sites per molecule. They have been found only in vertebrates. (b) Lectins are glycoproteins or proteins, are probably of nonimmune origin, and are usually present as constitutent molecules, i.e., are not generally increased by the presence of inducing substances. Their binding specificities are confined to carbohydrates and, as a general rule, they have two or more binding sites per molecule. They are present ubiquitously in viruses, bacteria, plants and animals. (c) Other receptor-specific substances are those agglutinins excluded from the other two categories by their relatively strict definition. These include the

inducible, non-immunoglobulin heteroagglutinins of vertebrates and invertebrates and other lectin-like but possibly nonprotein (carbohydrate or lipid) agglutinins in invertebrates.

Invertebrate lectins

It has been proposed that agglutinins participate in the defense mechanism of invertebrates by aiding phagocytosis [7,8]. Invertebrates do not possess the adaptive immune systems of the vertebrates. Therefore, the protective mechanisms of invertebrates are the less immune-like phenomena of lysis and agglutination by components in coelomic fluid, and phagocytosis by coelomocytes. This property of coelomocytes is well documented [9]. Invertebrate lectins may play a role in immune systems, perhaps as (a) a sugar configuration-specific antibody-like molecule which can agglutinate bacteria, viruses, fungi, sperm and other parasites; (b) an opsonin which enhances hemocyte phagocytosis of foreign particles; (c) a perivitilline protector to maintain sterility inside eggs. Investigation of annelid coelomocytes point to their participation in some form of specific "self" recognition and cellular immunity [10,11]. Cooper and coworkers [12] found that coelomocytes in Lumbricus are involved in some form of specific immune response.

Many invertebrate lectins have been characterized [7,8,13-30] and have been shown to vary in their physical and chemical properties. However, most of the invertebrate agglutinins have high molecular weights and the agglutinins are usually composed

of subunits (2 to 18) held together by noncovalent binding. Most of the invertebrate agglutinins are proteins or glycoproteins with carbohydrate-directed specificity. Calcium and occasionally magnesium is required for activity. Some agglutinins vary from these patterns and they are shown to have relatively low molecular weights, with the subunits of agglutinin covalently bound and with no requirements for divalent cations (Axinella polypoides) [25,26].

Russell and coworkers [28, 29, 30] found that the coelomic fluid from Nereis virens, the common sandworm, contains three classes of hemagglutinins: (1) four low molecular weight glycoproteins, (2) a mixture of lipid agglutinins which have heat-insensitive activity, and (3) a high molecular weight agglutinin whose activity is partially heat-sensitive. Each class had a unique erythrocyte (RBC) specificity and inhibitor profile. It was suggested [30] that the hemagglutinins from Nereis recognize specific receptors on susceptible RBC surfaces, or the lipid agglutinins from Nereis might agglutinate RBC by a combination of hydrophobic interactions between lipid agglutinin and the red cell surface [30].

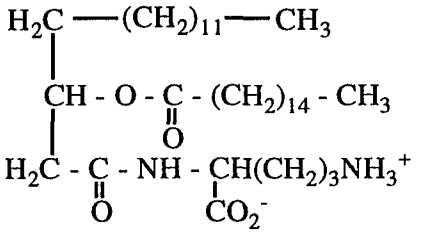
Proteoglycan hemagglutinins

Fuke and Sugai [27] found that hemagglutinins in the coelomic fluid of the ascidians, Styela plicata and Halocynthia hilgendorfi, are high molecular weight substances which are heat stable, resistant to trypsin digestion, but sensitive to periodate. They

thought that the isolated hemagglutinin is a polysaccharide or mucopolysaccharide. Russell et al. [30] reported a hemagglutinin which is a lipid-associated sulfated proteoglycan isolated from Nereis coelomic fluid and it was suggested that the proteoglycan recognizes specific receptors on RBC surfaces which causes agglutination.

Lipid hemagglutinins

Stone [31] observed that mixtures of pure lipids (phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, cardiolipin, OA, stearic acid and glycol distearate) showed agglutinating properties similar to organic extracts of a wide variety of tissues. Tsivion and Sharon [32] reported that OA, dioleoylphosphatidic acid, a polar lipid fraction from rat thymocytes, and certain bovine-brain lipids agglutinated some kinds of erythrocytes (RBC). It was not clear whether this activity was due to oxidized materials. Kawai and coworkers [33,34] found that an ornithine-containing lipid from Bordetella pertussis strongly agglutinated human (type A and B), rabbit, green monkey, and BALB/C mouse RBC, but weakly agglutinated horse, sheep, chicken and guinea pig RBC. The proposed structure of this amphipathic aminolipid was 3-hydroxyhexadecanoic acid, amide-linked to ornithine and esterified to hexadecanoic acid.



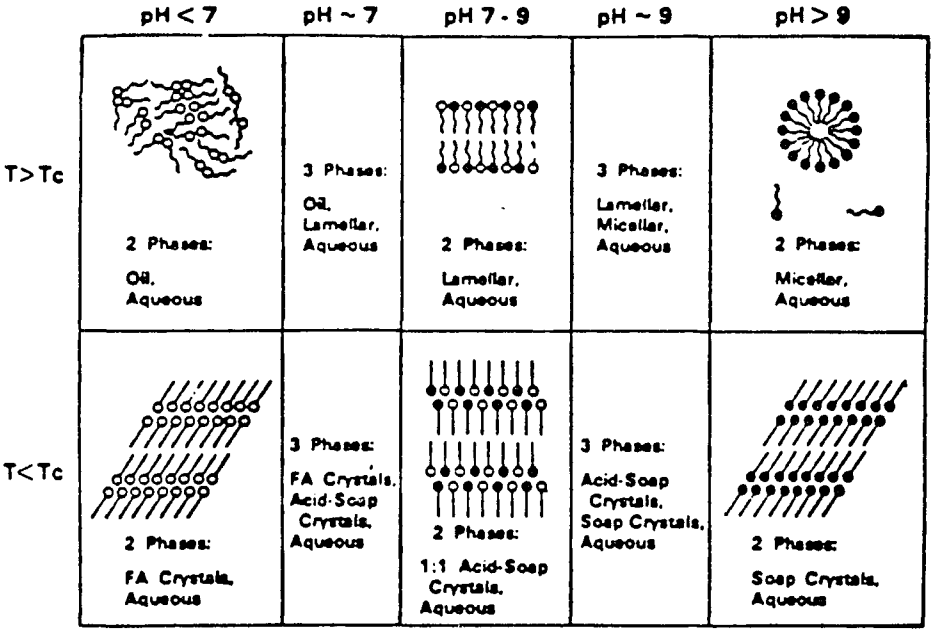
Kawai [35] reported that bovine-brain phosphatidylserine which has a structure similar to the proposed structure of the ornithine-containing lipid of Bordetella pertussis strongly agglutinated human, rabbit, green monkey, chicken and BALB/C mouse RBC. He also found that dioleoylphosphatidylserine agglutinated rat RBC. Forbes and coworkers [36] reported that egg phosphatidylethanolamine (PE) and dioleoylphosphatidyl-ethanolamine (DOPE) agglutinated mouse RBC. Kawai reported that PE (of unspecified origin) did not agglutinate human (types O, A and B), rabbit, green monkey, sheep, horse, guinea-pig, chicken and mouse (BALB/C) RBC. This contradiction has not been resolved. Lai et al. [37] reported that fresh commercial DOPE did not have agglutination activity while samples which had "aged" or been heated in water for 1 hour or been heated dry for 3-6 hours became powerful agglutinins. When distearoylphosphatidyl-ethanolamine (DSPE), which has no double bonds, was treated in the same way no hemagglutination activity was found. It was concluded that unsaturation is necessary for PE to be converted to a hemagglutinin. It was also observed that as the degree of unsaturation increases, hemagglutination becomes more facile,

and it was suggested that oxidation or polymerization products are responsible for the generation of hemagglutination activity.

Properties of fatty acid dispersions

OA is one of the major components of lipids constituting biological membranes. The aqueous chemistry of OA and its soaps has been extensively studied over many years but its complex nature is not yet fully elucidated [38]. Cistola and coworkers [39-40] examined the phase behavior of several medium and long-chain (18-carbon) fatty acids in water as a function of the ionization state of the carboxyl group by the generation of equilibrium titration curves above and below fatty acid and acid-soap chain melting temperatures and above and below critical micelle concentrations (cmc). The phases formed were characterized by X-ray diffraction, ^{13}C NMR spectroscopy and phase-contrast and polarized light microscopy. Figure 1 (figure 7 of ref. 40) summarizes the physical states formed by medium-chain and long-chain fatty acids (0.08 M) in excess water as a function of pH (ionization states) and temperature. At temperatures above critical temperature (Tc), at pH values lower than 7, long-chain fatty acids in excess water form an oil phase, whereas at pH between 7 and 9 a lamellar phase (stacked multilayers or large vesicles) is formed, and at pH values greater than 9 a micellar phase is observed. At temperatures lower than Tc, fatty acids in excess water form fatty acid crystals at pH values lower than 7, they form 1:1 acid-soap (half neutral)

Figure 1 Schematic summary of the physical states formed by medium-chain ($\geq$ 10 carbon atoms) and long-chain fatty acids in excess water as a function of pH and temperature (T). Tc represents the hydrocarbon chain melting temperature in excess water, and Tc differs for fatty acids, 1:1 acid-soaps, and soaps. The closed circles represent ionized (anionic) carboxylate groups and the open circles protonated carboxyl groups. The straight lines represent ordered hydrocarbon chains and the curved lines disordered (liquid) hydrocarbon chains. [Reprinted with permission from ref. 40]



crystals at pH values 7-9, and they form soap crystals at pH values greater than 9 [40].

Tc values vary depending on the hydrocarbon chain length, degree of saturation and fatty acid ionization state [40]. For example, the Tc values of oleic acid and decanoic acid are 16°C and 32°C, respectively [40]. It was concluded that the predominant phase formed at physiologic pH and temperature is the lamellar fatty acid/soap phase and that the micellar phase is not formed below pH 9 [40]. Hence, it is unlikely that local accumulations of unesterified fatty acids could exert detergent effects on cellular membranes, as has been widely suggested.

Knowledge of the physical states formed by fatty acids in water at a given pH aids in predicting the ionization and physical states formed by nonesterified fatty acids during their transport and metabolism in vivo [39-40].

Haines [41] stated that two phase changes occur during a titration of fatty acids in water. Above pH 10, the fatty acids are exclusively in the micellar form; below pH 7, they form oil droplets. Between these pH values vesicular oligolamellar liposomes are formed. It is also proposed that anionic lipid headgroups in biological membranes share protons as acid-anion dimers in which one proton is shared by two carboxylate groups [42-43].

Hamilton (private communication) found that a C18 dimer acid (Emery, Cincinnati, OH), when added to phospholipid vesicles

[44], equilibrates or "flip-flops" rapidly or spontaneously into both halves of the bilayer as measured by acidification of the internal aqueous volume. However, this flip-flop process, while taking place in seconds, is slower than the same process with OA by a factor of five. We know of no other work on the effect of dimers on biological membranes.

Iwahashi [45] studied the dissociation of hydrogen-bonded associated OA into its monomeric species by NIR spectroscopy over a temperature range of 8.6-84.4°C and a concentration range of 0.301-3.15 mol/L. Molecules of monomeric fatty acids in the liquid state associate to form dimers through hydrogen-bonding between their carboxyl groups and the hydrogen bond has been believed to be very strong and not to break even at high temperatures [45]. The band at 1445 nm, which is attributed to the free hydroxy group of the free fatty acid molecules, increased in absorbance with increasing temperature but appeared even at low temperatures. This means that the dissociation of the acid dimers occurs even at low temperatures and increases with increasing temperature. The same absorption band at 1445 nm was also observed in carbon tetrachloride solution at very low concentrations. The extent of hydrogen bonding between fatty acid carboxyl groups is temperature and concentration dependent. As the concentration decreased, the extent of free hydroxy group of the free fatty acid molecules increased, and the same was observed as the temperature was increased. In the above study

two break-point temperatures were shown at 30° and 55°C in a plot of degree of dissociation vs temperature. The transition of the liquid crystal to a more disordered liquid crystal was shown at 30°C, and the transition to the liquid state (presence of the monomeric molecules produced by the dissociation of the dimeric acid) was shown at 55°C. A similar study with dimeric C18 acids was initiated in this laboratory.

Dimerization and polymerization

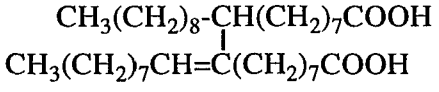
Processes in which polyunsaturated fatty acids are polymerized by heating to a high temperature are disclosed in the patent literature [46-47]. There are a variety of methods to dimerize unsaturated fatty acids (mostly linoleic, but sometimes oleic) including thermal, clay-catalyzed, and peroxide-catalyzed methods but only two of these have been commercialized extensively: thermal and clay-catalyzed dimerizations. Oligomers (C36, C44 and C54) are used commercially in many different applications, including solid and liquid polyamide resins, urethane resins, corrosion inhibitors, varnishes, soaps, polymer modifiers, oil additives, and epoxy curing agents. These oligomers are liquids probably because of the presence of so many structural isomers. For thermal dimerization of polyunsaturated fatty acids a high temperature (270-290°C) in a closed system under an atmosphere of inert gas or steam is used. Thermal dimerization has been largely replaced by the clay-catalyzed method which, with some modifications (small amounts of alkali

in the clay-catalyzed reaction mixture), can give higher dimer/trimer ratios of fatty acids [48-50]. Various types of natural or synthetic clays have been specified, such as a neutral bentonite clay from Alabama, an alkaline earth metal salt-modified clay, a specific natural acid clay found in Texas, etc.

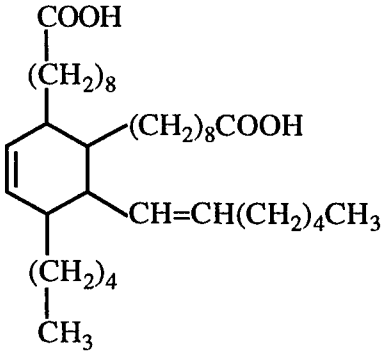
One of the reactions (thermal or clay-catalyzed) common to all unsaturated, straight-chain aliphatic acids and their alkyl esters is self-condensation to form high-molecular-weight dibasic and polybasic acids. One molecule of an unsaturated fatty acid will react with another to form a dicarboxylic acid with double the original molecular weight. The resulting dimer is not a single entity, but is a collection of isomers grouped together under the designation "dimer acids". These commercial dimer acids are mostly mixtures of 36-carbon dibasic acids, smaller amounts of 54-carbon tribasic acids, still higher-molecular-weight polybasic acids, and trace levels of monomer [51]. It has been shown [52] that different precursors for dimer acid formation give different structures (see Table 4 of ref. 53). The methyl ester of OA was thermally polymerized (in the absence of clay) by Sen Gupta [54]. The principal component of the dimer mixture was shown to be the straight chain (linear) dimeric acid. Clay catalysis of oleic acid by den Otter [55] indicated that a large amount of saturated fatty acids is formed, most likely by hydrogen transfer. According to this assumption, dienoic acids must also be formed, which dimerize to cyclic dimers. The

thermal polymerization of oleic acid mostly yields the acyclic form (by a free-radical mechanism), but when linoleic acid is the precursor the monocyclic cycloaliphatic structures predominate (by a Diels-Alder mechanism). The clay-catalyzed dimerization of oleic and/or linoleic acid on a commercial scale produces a 60:40 mixture of dimer acids (C36 and higher oligomeric acids) and monomer acids (C18 cyclized, aromatized, and isomerized fatty acids) [52]. One of the mechanisms for thermal dimerization proposed by Meyers [48] involves a free-radical mechanism. A free-radical mechanism for thermal dimerization is very plausible when oleic acid is the starting material. A transfer of a hydrogen atom from one oleate molecule to another can lead to the formation of two organic radicals that can combine and form a dimer [53]. The dimer was postulated to be either acyclic or a six-membered ring cyclic dimer. More extensive work done on clay-catalyzed dimerization of oleic and linoleic acid by McMahon [52] showed that dimer acids from oleic acid contain a higher percentage of linear components than dimers obtained from linoleic acid. Linoleic dimerization gives mostly a six-membered ring cyclic dimer. There is still disagreement about the structures of the dimers and trimers obtained by clay or thermal dimerization of unsaturated fatty acids. Possible structures of dimer acids, showing only a single isomer of the many that are possible are shown below (I-III) [52]. The acyclic structure (I) when the Δ9-C18 acid is the source,

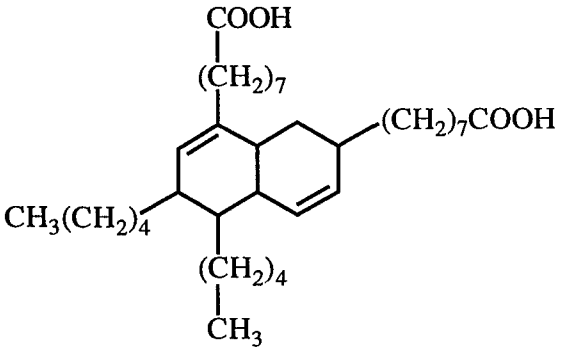
monocyclic (II) when Δ9,11-C18 acid is the source and bicyclic (III) when the Δ9,11,13-C18 acid is the source. There are a myriad of possible isomers, positional and geometrical isomers of the double bond, as well as structural isomers resulting from head-to-head or head-to-tail alignment of the starting material as well as for the cyclic dimers [52]. Head-to-head means that both carboxyl groups are at the same side and head-to-tail means that the carboxyl groups are at the opposite side. When the oligomer is in the linear form, the hydrocarbon chains are free to rotate about the connecting C-C bond and the carboxyl groups can be at any position; but when the oligomer is in the ring form the carboxyl groups are frozen in only one relative position.



I Acyclic

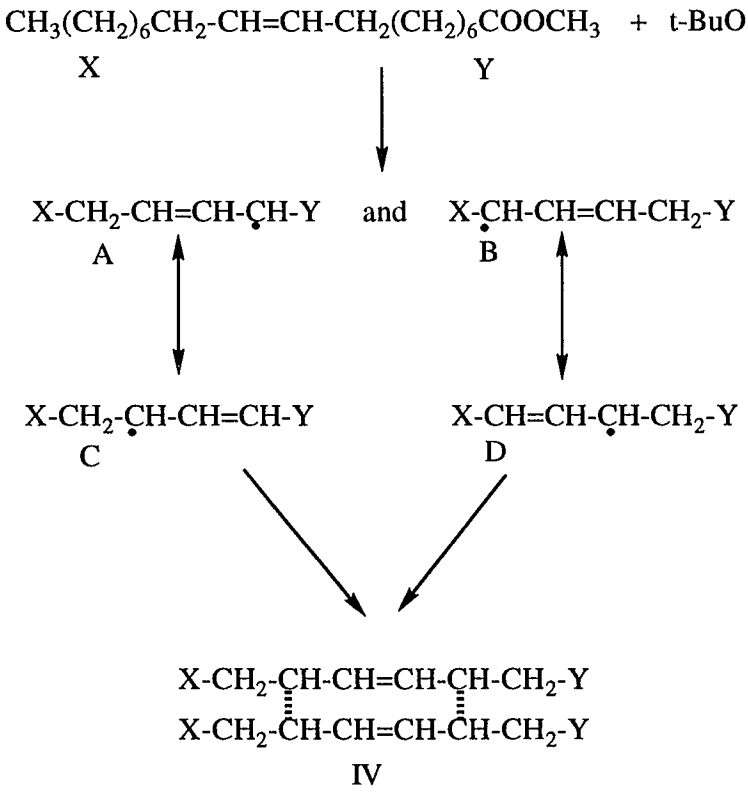


II Monocyclic



III Bicyclic

Methyl oleate can be dimerized by di-tert-butyl peroxide to yield a mixture of isomers of an acyclic dimer [56]. The tert-butoxy radical could remove a hydrogen atom from the 11 or 8 carbon atom of oleate. These allyl radicals would have two resonance structures each, and all could couple to give dimers. The following mechanism was proposed by Paschke et al. [56].

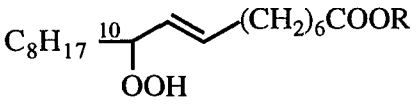


The free radical species A, B, C, and D couple randomly to form dimer, resulting in 10 species of dimer, AA, AB, AC, AD, BB, BC, BD, CC, CD, and DD. These dimers represent joining to the same

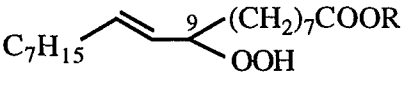
extent at positions 8, 9, 10 and 11 of the oleate segments of the dimer [56].

Monoene autoxidation

The study of the autoxidation by heating of monoene fatty acids such as OA is hampered by the fact that product analysis is difficult. At least six simple hydroperoxide products are formed in the autoxidation of methyl oleate 1-6 [57]:



1



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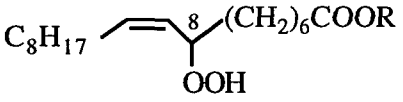

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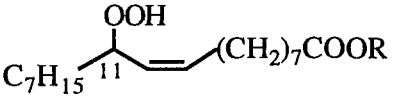

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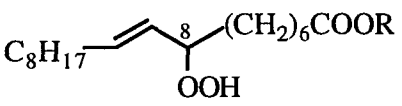

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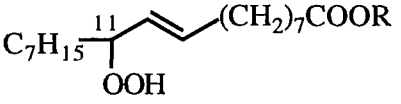
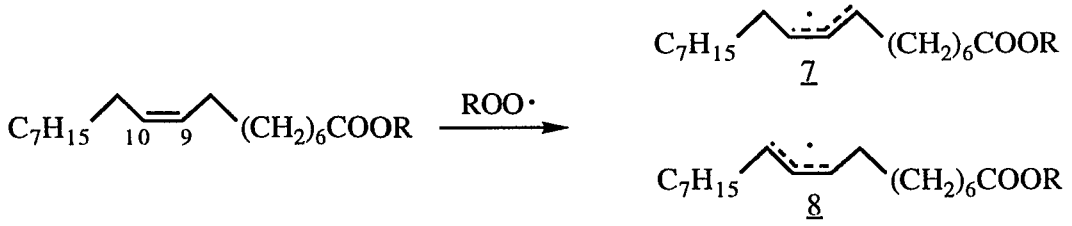


6

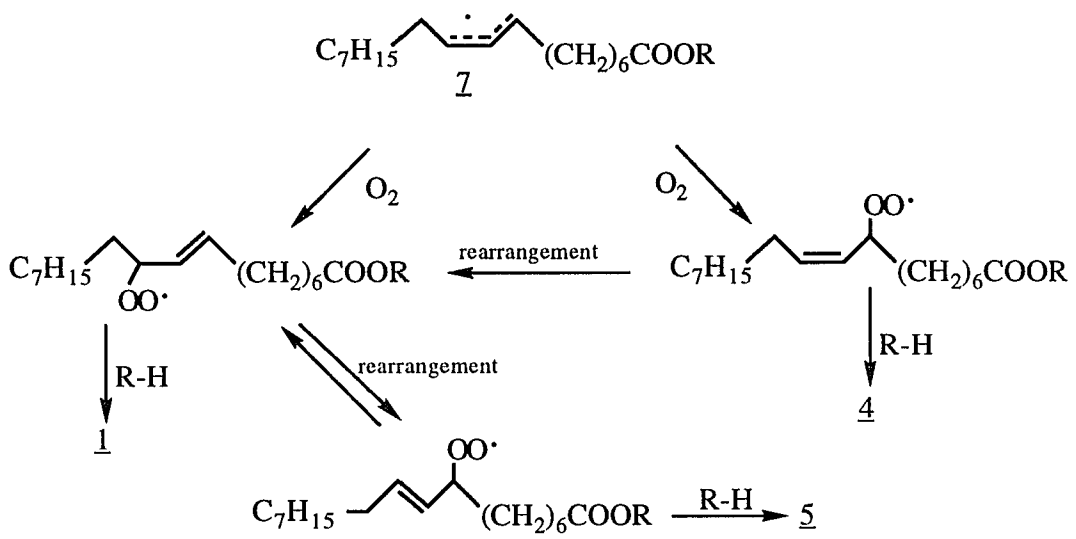
The products are substituted on the 18 carbon chain at positions 8, 9, 10, and 11 [57].

The most accepted mechanism for oleate autoxidation [57] involves hydrogen abstraction at carbon-8 and carbon-11 since the hydrogen atoms at positions 8 and 11 are both allylic and, thus, both would be expected to be reactive. The interactions

between the unpaired electron on carbon-8 and carbon-11 and the π-electrons of the adjacent double bond produce two resonance-stabilized allylic radicals (similar to di-tert-butyl peroxide oxidation mechanism) with delocalized electrons distributed over three carbon atoms, as shown below (7,8).



The radicals have partial double bond character between the carbons in the allylic system, and these radicals can exist as *trans-trans*, *trans-cis*, *cis-trans*, and *cis-cis* isomers. Addition of oxygen to allylic radicals 7 and 8 would give rise to four different peroxy radicals formed from addition at either end of the two radicals (see below) [57, 59-61].



These radicals could be interconnected by rearrangement and products 1, 4, and 5 would result from hydrogen-atom abstraction by these peroxyl radicals. The expected product distribution would be based on the availability of abstractable hydrogen-atoms. Similar chemistry should occur for peroxy radical 8 and accounts for the formation of products 2, 3, and 6 [57]. It should be noted that this is a hypothetical mechanism as a thorough mechanistic investigation of oleate oxidation has not been carried out.

Oxidation and thermal degradation of lipids

There is a great deal of interest in the oxidation and thermal degradation of lipids. (a) These changes can occur in aging [62]. Oxidized lipids can alter the normal physiological activities of lipids, including regulation and membrane properties [58].

(b) The fatty acid components of lipid foodstuffs undergo oxidative and thermal changes during storage, processing and cooking. These changes alter the nutritional properties of lipids. The biological effects that consumption of heated and/or oxidized fats may exert on human health have stimulated extensive research in the past years. Overall, reports on digestibility, which was measured in experiments in which male Wistar rats were fed diets supplemented with unheated, heated, and a 1:1 mixture of unheated/heated olive oils at 6, 12, and 20% w/w of diet. Fecal lipids were extracted after 14 days and analyzed by a combination of adsorption and high-performance size exclusion chromatographies. Significantly lower digestibility values of thermally oxidized oils compared to fresh oils were found, and this fact generally has been attributed to the presence of polymeric products [63]. Both dietary oils and fecal lipids were analyzed according to an analytical procedure which includes saponification of lipid samples and removal of unsaponifiable matter. It was concluded that the excreted levels of altered compounds (dimers plus polymers) were two-fold higher for the heated-oil diet compared to the unheated/heated oil diet.

Other experiments carried out on compounds derived from thermally oxidized oils have reported digestibilities ranging from 30-70% [64-65]. Complex mixtures of lipid degradation products occur in our daily diet, and further investigation is needed in this regard [64]. Because of the complex mixture of such alteration

products and the lack of specificity of the analytical methods used, determination of specific compounds has been difficult, to date [63].

(c) Polymerization of fatty acids in particular, under oxidative or thermal treatments, are the subjects of intense interest in the synthetic chemical industries as well, especially as precursors for higher polymers.

(d) The recent literature also suggests that fatty acids, particularly OA, may function as a biological regulatory molecules. Some examples are: OA stimulates secretion of apolipoprotein B in Hep G2 cells [66]. OA is the most potent agent for inducing translocation of phosphocholine cytidylyltransferase from cytosol (inactive) to the membrane (active form) [67]. OA has been identified as a potent inhibitor of lipid peroxidation in intestinal mucosa [68]. OA has been implicated in phosphokinase C-mediated [69] and non-phosphokinase C-mediated [70] signal transduction. OA blocks epidermal growth factor-activated early intracellular signals without altering the ensuing mitogenic response [71]. There are no reports of the effects of free fatty acid oligomers on biological materials.

This work describes results with unsaturated fatty acids which suggest that thermal oxidation converts them into species which interact with erythrocyte membranes different from oleic acid, i.e. cause hemagglutination rather than lysis. In addition the

behavior of some heated fatty acids and oligomeric fatty acids toward several species of RBCs is presented.

Purposes of this work

We became interested in studying the hemagglutinating activity of OA when it was observed in this laboratory by P.-S Lai that DOPE samples, which had shown no hemagglutinin activity when fresh, exhibited hemagglutination activity after being kept frozen or refrigerated over a long period. The same result could be obtained by heating a fresh sample in water. It was found that oleic acid exhibited the same hemagglutinating activity under the same conditions and presented a simpler system to investigate.

The main goals of this study were:

(1) To define the conditions under which OA is converted to a hemagglutinin.

(2) To characterize the various species formed by thermal oxidation of OA.

(3) To determine if other fatty acids are agglutinins before or after thermal oxidation.

(4) To synthesize agglutinins of better defined structure.

(5) To characterize the red cell specificity of the agglutination and responses to divalent cations, pH and sialidase alteration of the red cell membrane.

(6) To visualize the red cell-fatty acids interactions by microscopy.

MATERIALS AND METHODS

A. Chemicals and Biologicals

Chemicals

HPLC grade solvents and fatty acids and esters were obtained from Sigma (St. Louis, MO). Di-tert-butyl peroxide was obtained from Aldrich (Milwaukee, WI). 3-Octadecanone, 3-hexadecanone, 4-ketopimelic acid, 7-oxooctanoic acid, trans-2-nonenal, undecenal, dodecanal, tetradecanal, acetaldehyde, cis-9,10-epoxyoctadecanoic acid and trans-9,10-epoxyocta decanoic acid were purchased from Aldrich and decanal and hexadecanol dimethyl acetal were from Sigma. Dimer acid 1010, a mixture of C18 fatty acid oligomers (3% monomer (C18), *94% dimer (C36)*, 3% trimer (C54) and traces of tetramers and other oligomers; all containing cycloaliphatic, aromatic and acyclic structures) to be designated in the text as C18 dimer (Emery), and trimer acid 1040, a mixture of C18 acid oligomers (2% monomer (C18), 18% dimer (C36), *60% trimer (C54)* and 20% higher oligomers, also containing cycloaliphatic, aromatic and acyclic structures) to be designated in the text as C18 trimer (Emery), were gifts from Henkel Corporation (Emery Group, Cincinnati, OH). Pripol 1008, a C18 acid dimer (0.1% monomer (C18), 1% intermediate, *98% dimer (C36)* and 1% trimer (C54); 54% cycloaliphatic, 37% acyclic and 9% aromatic structures) to be designated in text as C18 dimer

(Unichema) and Pripol 1004, a C22 acid dimer with a very high content of linear isomers (0.1% monomer, 4% C36 dimer + intermediates, *95% dimer* and 1% trimer) to be designated in text as C22 dimer (Unichema), were gifts from Unichema North America (Chicago, IL). C-21 Diacid (5(6)-carboxy-4-n-hexyl-2-cyclohexene-1-octanoic acid) was a gift from Westvaco Corporation (North Charleston, SC). Sialidase was purchased from Sigma. Solvents for lipid extraction, thin-layer chromatography (TLC), and HPLC were HPLC grade. All other chemicals were reagent grade or better. Dialysis tubing Spectrapor #1 and microtiter plates (round well not tissue- culture treated) were from Fisher (Springfield, NJ). Polygram Sil G silica gel plates on plastic for TLC was purchased from Brinkmann Instruments, (Westbury, NY) and from Fisher (Springfield, NJ). Silica gel for column chromatography was purchased from Pharmacia (Piscataway, NJ).

Erythrocytes

RBC were obtained from etherized live Sprague-Dawley rats (Taconic Farms, Germantown, NY) by cardiac puncture using 3.8% sodium citrate, pH 7.2, as anticoagulant. Sheep, rabbit, dog and cat RBC were purchased from Pel-Freez Biologicals (Rogers, AK), guinea pig, chicken, bovine, goat and human (O) RBC were purchased from Rockland (Gilbertsville, PA). Erythrocytes were washed with PBS-N_3 (0.9% NaCl-1 mM phosphate, pH 7.2, 0.01% NaN_3) three times at room temperature, spun down each time for

3 minutes in a table-top International Clinical centrifuge, and suspended in PBS-N_3 to make a 5% suspension. Erythrocytes were stored in citrate solution and could be used within a week. Erythrocytes that were washed and suspended in PBS-N_3 had to be used the same day.

B. Analytical methods

(1) Microtiter assay for hemagglutination

All species of RBC were washed with PBS-N_3 twice at room temperature. The volume of packed RBC was noted and diluted to 5% RBC (v/v) with PBS-N_3. PBS-N_3 (100 μL) was placed in the control well, #1, of a U shaped microtiter plate and 50 μL of PBS-N_3 was placed into the wells #2 to 12. Lipid preparation (50 μL) (0.2-0.5 mg/mL) was serially diluted in the wells #2 to 12. These dilutions were done in duplicate. A second 50 μL aliquot of PBS-N_3 was added to the serially diluted wells, making a total of 100 μL, followed by 25 μL of 5% RBCs to all wells. The plates were sealed and agitated on a Tektator Unit (model 4138, Scientific Products) for 3 minutes, then incubated at room temperature. The plates were read at 30-min intervals by tilting them and observing the bottoms of the wells. The first well of each row served as control. Hemagglutination was recognized as the button-like settling of cells or a film at the bottom of the well in contrast to flowing in the control wells. Titers were performed in duplicate, but if the results differed by one well, the titer was presented as the average value (fractional titer). If

titers differed by more than one well the determination was repeated. Titer is expressed as the highest dilution of test sample which still gives agglutination. Specific titer is defined as titer per mg lipid per mL.

(2) Quantitative determination of double bonds (Iodine value)

The iodine value [72 (p.121)] was used to determine unsaturation in the unheated and heated (0-24 hours) sample of oleic acid. To an aliquot of 5.0 mL lipid solution in chloroform containing a known amount (2-5 mg) of lipid, 5.0 mL of pyridine dibromide solution (0.05 N) was added. The solution was mixed and left at room temperature in the dark for 15 min. Then 0.5 mL of potassium iodide solution (10%), 0.5 mL of water and a few drops of starch indicator (1%) were added. The liberated iodine was titrated with standard 0.020 N thiosulfate solution.

Reagents: Dam's reagent (0.05 N pyridine dibromide solution): 2.06 mL of pyridine in 5 mL of glacial acetic acid was added to a solution of 1.85 mL of concentrated sulfuric acid in 5 mL of glacial acetic acid. It was cooled to 27°C and 2.0 g (0.63 mL) of bromine was added; the solution was diluted to 500 mL with glacial acetic acid. Potassium iodide solution (10%) was made by dissolving 10 g KI in 100 mL of water. Starch indicator solution (1%) was made by dissolving 1g soluble starch in 100 mL of 13% potassium chloride solution; brought to a boil and cooled. Standard thiosulfate solution was 0.02 N.

(3) Neutralization equivalent for carboxyl group determination

For carboxyl group determination the method of Kates [72 (p.128)] was used. Methanolic sodium hydroxide (0.025 N) was prepared by dissolving 2.0 g NaOH pellets in 35 mL methanol. The solution was centrifuged and the supernatant was diluted to 50 mL with methanol. To 5.0 mL of this solution 20 mL of distilled water was added and then the solution was diluted to 200 mL with methanol. The solution was standardized by titration against standard 0.010 N HCl (see below). o-Cresol red (1%) (0.5 g of o-cresol red in 50 mL of 90% methanol) was used as indicator. The experiment was performed on OA (control) and on OA Δ24 hours.

An aliquot of chloroform solution of the fatty acid containing 25-250 milliequivalents acid was placed in a 50-mL Erlenmeyer flask, and the solvent was evaporated with nitrogen. The residue was dissolved in 5 mL methanol:water (9:1 v/v) and 2 drops of indicator were added. The solution was warmed on a hot plate almost to boiling and titrated hot with methanolic 0.025 N NaOH to a yellow to red end-point. As a blank, 5 mL of solvent alone was titrated in the same way.

(4) Group-specific reagents used on Sil G plates for TLC peroxide determination

The KI/starch test [73 (p.82)] was used to determine whether there were any peroxides in the samples. The fatty acid

samples were spotted on the plate followed by application of 10% aqueous KI to each spot. Then 1% aqueous starch solution was added to each spot. To see if the solvent had any effect on the KI test, the solvent ($CHCl_3$) was also heated under the same conditions as the OA samples. The heated solvent ($CHCl_3$) was also spotted on the plate as a control. Hydrogen peroxide was used as a standard to compare the positive results, which should be brown when 10% KI was added and blue when 1% starch solution was added.

C. Spectroscopy

(1) UV spectra

UV spectra were obtained with a Perkin-Elmer Lambda 3B spectrophotometer. The samples were measured in cells containing 1 mL of sample (1 mg/mL of ethanol). The range of scanning was 200-350 nm.

(2) Infrared spectroscopy (IR)

A Perkin-Elmer Model 247 spectrophotometer was used and the samples were measured in 13-mm KBr disks containing 0.5 mg/100 mg of KBr.

(3) Near-infrared spectroscopy

A Perkin-Elmer Lambda 19 UV/VIS/NIR spectrophotometer, controlled by a PC Dec 316, was used to obtain spectra in the range of wavelengths 1100 to 1700 nm. Two quartz cells with 1-cm or 3-cm path lengths, depending on concentration, were used. The samples were run under different conditions of concentration

and temperature [45]. For high concentration (3.278 mol/L, 2.375 mol/L, 1.354 mol/L, 0.682 mol/L and 0.377 mol/L) studies the 1-cm cell was used and for the low concentration (0.038 mol/L, 0.127 mol/L, 0.347 mol/L and 0.520 mol/L) studies the 3-cm cell was used. The temperature dependent studies were run at 5°, 25°, 41°, 60°, 71°, 81° and 105°C. The samples were maintained at constant temperature in a dri-bath heating block (Thermolyne #DB17615, Dubuque, IA).

(4) Nuclear magnetic resonance (NMR) spectra

Proton and ^{13}C-NMR spectra were recorded on an FT NMR spectrometer NR/300 (Bruker). Samples were dissolved in 1 mL of $CDCl_3$ (99.8%) + 1% TMS (Cambridge Isotope Lab., Woburn, MA) and transferred to NMR tubes (507-PP) (Wilmad, Buena, NJ) and filled to 3 cm. The spectral width was 2994 Hz for ^{1}H and 17241 Hz for ^{13}C NMR. The delay time between pulses was 1.0 s for ^{1}H NMR and 4.0 s for ^{13}C NMR. The number of scans collected for ^{1}H NMR was 8 and for ^{13}C NMR was 500.

(5) Mass spectrometry (MS)

Mass spectra were obtained with a Finnigan MAT SSQ70 spectrometer using chemical ionization (CI) with ammonia (134 EV) for molecular ions and electron impact (EI) (70 EV) for fragmentation patterns. The temperature program ran from room temperature to 600°C at 60° per minute.

D. Separations

(1) TLC.

Silica gel G plates (20 x 20 cm) (Brinkmann Instruments, Westbury, NY) were prewashed in the proper solvent system: isooctane:isopropyl alcohol:acetic acid (95:5:1 v/v/v) for fatty acids (discriminates by the number of carboxyl groups) and hexanes:diethyl ether (70:30 v/v) for methyl esters (discriminates by polarity), air dried for 30 min and activated by heating for one hour at 120°C in a vacuum oven at 15 mm. A sample (20-30 μg) from a stock solution of 0.5 mg/ml in methanol was spotted onto silica gel plates using 2 μl Microcap pipettes (Drummond). Before development, the plates were dried using a hand-held dryer on a cool setting for 5 minutes. The chromatography chamber, 26 cm x 7 cm x 24 cm (d x w x h), was saturated with vapor from the solvent system for 30 min before development of plates. The plates were allowed to develop until the solvent front was about 2 cm from the top, removed and air-dried for 30 min. The plates were visualized in iodine vapor. For extraction from the plates the bands were visualized by a strip exposed to iodine and each band was scraped off and eluted with methanol. For fatty acids, a part of each residue was dried under nitrogen, dissolved (a) in pure ethanol or (b) neutralized and pH-adjusted to 7.4-8 in PBS-N_3, then filtered through a 0.2 μm Acrodisc (Gelman) to remove particulate matter and titered

against rat RBC. Methyl esters were hydrolyzed before dispersion in PBS-N_3 (see below).

(2) HPLC

HPLC was performed (Analytical Manual, Emery Group, Cincinnati, OH) on a Waters instrument comprised of a 600E Millipore System Controller, a U6K Millipore Injector, a Lambda LC spectrophotometer, an SE-120 BBC Goerz Metrawatt recorder and a 2112 LKB Redirac Fraction Collector. Two Rainin Dynamax 8 m silica columns (4.6x250 mm, pore size 60A) were used in series. The mobile phases were 94% Solvent A and 6% Solvent B, where Solvent A was 99.8% cyclohexane:0.2% acetic acid and Solvent B was 84.8% cyclohexane:15% isopropyl alcohol and 0.2% acetic acid. The flow rate was 0.5 mL/min, 1mL/tube (200 tubes). Samples were prepared fresh each day at a concentration of 50 mg/mL in solvent B. The solvents were mixed fresh every two days. The collected fractions were analyzed by TLC in isooctane:isopropyl alcohol:acetic acid (95:5:1 v/v/v) as above.

(3) Silica gel column chromatography

The column used was 50-cm long and 2-cm i.d. Silica gel 300 mesh (Fisher Scientific Company, Springfield, N.J.) was swollen in hexanes:diethyl ether (1:1 v/v) (elution solvent) and the column was packed in the same solvent system. When two bed volumes of solvent had come off the column the sample was applied (200 mg/mL hexanes:diethyl ether 1:1 v/v). The collected fractions (1 mL/min/tube, 600 tubes) were analyzed by TLC in hexanes:

diethyl ether (1:1 v/v) (separation by polarity) and in isooctane: isopropyl alcohol:acetic acid (95:5:1 v/v/v) (separation on the basis of functionality). All of the monocarboxylic acids, regardless of chain length, will move as one spot and will include all monomers or monocarboxylic dimers/trimers. An aliquot from the collected fractions was evaporated with N_2, the residue was dissolved in 80% ethanol/water and titered against rat RBC.

E. Fatty acid preparations

(1) Thermal oxidation of oleic acid

Approximately 3 mL of OA (Sigma, O-3879) was poured into each of three separate tubes. The first tube was covered with Whatman filter paper which protected the sample from dust particles but allowed access to air. Nitrogen was used to flush the sample in tube #2 for approximately 3 minutes, at the end of which time the tube was covered with an air-tight stopper. Tube #3 was connected to an apparatus with an inlet and outlet. The inlet allowed the intake of air into the tube and therefore, it was also covered with Whatman filter paper. The outlet valve was connected to a peristaltic pump (Buchler Instruments) set at speed 6. The action of the pump resulted in air being sucked through the intake valve and passed through the sample. All three tubes were placed in a dri-bath heating block (Thermolyne, Dubuque, IA) and heated at 100°C for ten days. Aliquots of the samples were taken from tubes #1 and #3 every 24 hours from the start of the heating period until the completion of the study.

Aliquots were removed from tube #2 for the first six days of the heating and then on the tenth day. The aliquots from tube #2 were taken from the tube by piercing the rubber stopper with a non-sterile, deflected point, stainless steel needle (Popper & Sons, Inc., New Hyde Park, NY). The plunger of the syringe was depressed just before piercing the rubber stopper to ensure that all excess air was removed from the needle allowing only the minimum into the system. However, it appeared that air did get into the system toward the end of the experiment.

The absorption spectrum of OA and OA Δ10D showed a new peak appearing at 217 nm for the OA Δ10D. This peak is most likely evidence for the presence of conjugate double bonds [72, 77].

The following peaks were found in the IR spectra of OA: 3010 cm^{-1} for sp^2 C-H stretching, 2936 and 2853 cm^{-1} for $-CH_2$ and $-CH_3$ (C-H stretching), 1465 cm^{-1} for $-CH_2$ (C-H bending), 1400 cm^{-1} for $-CH_3$ (C-H bending). The peak near 2990 cm^{-1} indicates the presence of a double bond, and one at 1720 cm^{-1} is indicative of a carboxylic acid. The IR bands were assigned as in Kates [72 (pp 172-175)].

The following peaks were found in the IR spectra of OA Δ10D: 3010 cm^{-1} for sp^2 C-H stretching, 2936 and 2853 cm^{-1} for $-CH_2$ and $-CH_3$ (C-H stretching), 1200 cm^{-1} for ester C-O stretch, 1465 cm^{-1} for $-CH_2$ (C-H bending), 1400 cm^{-1} for $-CH_3$ (C-H bending). The peak near 2990 cm^{-1} indicates sp^2 C-H stretching,

at 1720 cm^{-1} indicates carboxylic acid dimer and a peak at 1730-35 cm^{-1} indicates C=O, ester or a ketone.

The ^{1}H and ^{13}C NMR spectra for OA showed the following signals (δ): 0.8 ppm (triplet) for CH_3-, δ 1.2 ppm for -CH_2-, from carbon #4 to carbon #15 (acyl C-4 to C-(ω-3) except the allylic -CH_2-, carbon #8 and #11 which give different signals), 2.0 ppm (m) for -CH_2-C=C (allylic methylene), 2.3 ppm (m) for -CH_2-COOH, and 5.0-5.4 ppm (m) for R-CH=CH-R. The ^{13}C NMR spectrum showed signals at: 14.0 ppm for CH_3-, 22.6 ppm for CH_3CH_2-, 24.9 ppm for -CH_2CH_2COOH, 29.1-29.6 ppm for -CH_2-, acyl C-4 to C- (ω-3), 31.9 ppm for CH_2 (ω-2), 34.1 ppm for -CH_2COOH, 130-134 ppm for R-CH=CH-R, and at 174.2 ppm for COOH. The NMR bands were assigned as in Kates [72 (pp 177-178)].

The ^{1}H and ^{13}C NMR spectra for OA Δ10D showed the following signals (δ): 0.8 ppm (triplet) for CH_3-,1.2 ppm for -CH_2-, acyl C-4 to C-(ω-3), 1.6 ppm for -CH_2-, carbon #3 (acyl C-3), 2.0 ppm (m) for -CH_2-C=C (allylic methylene), 2.3 ppm (m) for -CH_2-COOH, 3.4-3.7 ppm for -CH_2O-, a very weak peak at 3.6 and 3.75 ppm for -CH_2OH (at 3.5-3.6 ppm also for -CH_2OR) and -CHOH respectively, 4.2 ppm for -CH_2OCOR, 5.1 ppm for -CHOCOR, and 5.0-5.4 ppm (multiplet) for R-CH=CH-R. The ^{13}C NMR spectrum showed signals at: 14.0 ppm for CH_3-, 22.6 ppm for CH_3CH_2-, 24.9 ppm for -CH_2CH_2COOH, 29.1-29.6 ppm for -CH_2-, acyl C-4 to C- (ω-3), 31.9 ppm for CH_2 (ω-2), 34.1 ppm for -CH_2COOH, 64 ppm for -CH_2OCOR, 70-72 ppm for -CHOCOR (at 71-72 ppm also

for $-CH_2OR$), 130-134 ppm (multiplet) for R-CH=CH-R, 171-174 ppm for R-CO- and at 174.2 ppm for COOH. The methine proton was very difficult to identify because of its very small quantity in the crude sample. The same is true for the methine carbon in the ^{13}C NMR spectra.

(2) Preparation of fatty acid dispersions

Several methods were used to disperse fatty acids in PBS-N_3. Samples dissolved well in ethanol, but high concentrations yielded precipitates or separation of phases when mixed with PBS-N_3.

(2-a) Neutralization

Dispersions of 0.5 mg/mL were prepared by neutralizing 5-10 mg of fatty acid with 1 M NaOH, diluting the sample to 0.5 mg/mL and adjusting the pH to 7.4-8. Dispersions of oleic acid, heated oleic acid, dimers, trimers and other fatty acid preparations were subjected to filtration with LC PVDF Acrodisc membranes (Gelman) with size cut-off 0.2 μm to remove particulate matter.

(2-b) Ethanol

Samples were dispersed in absolute ethanol by simply adding 1 mL of ethanol to a preweighed sample. The weight of the samples was restricted to 1-4 mg/mL.

F. Syntheses

(1) Synthesis of methyl esters

Diazomethane was generated by using the Mini-Diazald apparatus (Technical Bulletin AL-180)(Aldrich, Milwaukee, WI).

The yellow ether distillate containing the diazomethane was added slowly to the fatty acid to be esterified and the solution was allowed to stand at room temperature in a hood until the ether evaporated. The TLC developing system for the esters was hexanes:ethyl ether (70:30 v/v).

(2) Hydrolysis of methyl esters

To methyl esters dissolved in methanol, KOH (0.1 M) in methanol was added in 4 molar excess. The solution was allowed to stand at room temperature overnight. Then the methanol was evaporated with nitrogen and the resulting salt was dissolved in PBS-N_3. The pH was adjusted to 7.4-8.

(3) Synthesis of acyclic oleic dimer

The method of Paschke et. al [56] was used. Methyl oleate (28.7 g, 0.097 mol) and di-tert-butyl peroxide (1.8 g, 0.012 mol) were heated under nitrogen with stirring at 135°C for 48 hr. Volatiles were removed in a short-path distillation apparatus under vacuum (0.1 mm) up to 80°C pot temperature, with the receiver in dry ice. Monomer came off up to 250°C pot temperature. The residue was then distilled in a molecular still (Lurex, South Vineland, NJ) to give monomer in the receiver and dimer in the residue. Further purification of the dimer was achieved by TLC on silica plates. The developing solvent was hexanes:ethyl ether (70:30 v/v) which separates monomer from dimer and polar from nonpolar. Bands with R_fs of 0.17-0.26 (dihydroxydimer ester), 0.34 (monohydroxydimer ester), 0.50-0.57

(trimer ester with some tetramer ester), and 0.70 (dimer ester) were eluted with methanol (see above), hydrolyzed, and titered. The dimer ester moved as one spot before hydrolysis in the developing system mentioned above, which discriminates by polarity and, after hydrolysis, moved as one spot (R_f of 0.32) on silica gel TLC developed with isooctane:isopropyl alcohol:acetic acid (95:5:1 v/v/v), which discriminates by the number of carboxyl groups. In addition, the mass spectrum (a single peak in the ion chromatogram and correct mass for acyclic structure) and ^{1}H and ^{13}C NMR showed that only acyclic C18 dimer acids were present.

The CI (ammonia) mass spectrum of the synthetic dimer dimethyl ester purified by TLC showed a single parent peak at m/e 590, as expected for a carbon-carbon linked dimer with two double bonds. The numerical mass values given for all CI and EI mass spectra is the observed m-18. Another minor peak appears at 296 (M/2) since one of the bonds which is particularly prone to cleavage is the bond joining the two oleate fragments. This linkage is between two tertiary carbons, and is allylic to two double bonds. Similarly the EI shows a single parent peak at m/e 590. The parent peak is weak, since there are many bonds where cleavage could occur. Very strong peaks were seen at 295 (base peak, M/2) and 294 (hydrogen rearrangement). Peaks were seen at m/e 477 and 491, corresponding to loss of C_7H_{15} and C_8H_{17} from the parent mass of 590. Similarly, peaks were seen at m/e 447

and 433, corresponding to loss of $C_6H_{12}COOCH_3$ and $C_7H_{14}COOCH_3$ from the parent mass, which are also attached to tertiary and allylic carbons. Figure 9 compares the ^{13}C NMR spectra of methyl oleate, dimethyl dioleate and dimethyl distearate.

The ^1H NMR spectrum showed the following resonance signals: 0.8 ppm (m) for CH_3-,1.2 ppm for -CH_2-, acyl C-4 to C-(ω-3), 1.6 ppm for R_3C-H and -CH_2-, acyl C-3, 2.0 ppm (m) for -CH_2-C=C (allylic methylene), 2.3 ppm (m) for -CH_2-COOR, 3.6 ppm for CH_3-O- methyl ester and 5.0-5.4 ppm (m) for R-CH=CH-R. The ^{13}C NMR spectrum showed signals at: 14.0 ppm for CH_3-, 22.6 ppm for CH_3CH_2-, 24.9 ppm for -$CH_2CH_2COOCH_3$, 27.4 ppm for -CH_2-C=C- allylic methylene (<u>cis</u>), 29.1-29.6 ppm for -CH_2-, acyl C-4 to C- (ω-3), 31.9 ppm for CH_2 (ω-2), 32.6 ppm for -CH_2-CH=CH- allylic methylene (<u>trans</u>), 34.1 ppm for -CH_2COOR, 51.3 ppm for CH_3-O- methyl ester, at 130-134 ppm for R-CH=CH-R and at 174.2 ppm for $COOCH_3$. R_3C-H was detected at 47.7 ppm using 90° distortionless enhancement by polarization transfer (DEPT), NMR (^1H and ^{13}C) signals were assigned according to Kates [72 (pp. 177-178)].

The IR spectrum showed the following major peaks: 1750 cm^{-1} for ester C=O stretch, 3010 cm^{-1} for sp^2 C-H stretching, 2936 and 2853 cm^{-1} for -CH_2 and -CH_3 (C-H stretching), 1200 cm^{-1} for ester C-O stretch, 1465 cm^{-1} for -CH_2 (C-H bending), 1400 cm^{-1} for -CH_3 (C-H bending), 970 cm^{-1} for -CH=CH (<u>trans</u>)

(CH out-of-plane deformation). As in oleic acid, there was no distinctive peak in the 650-750 cm^{-1} region for -CH=CH (cis). The IR bands were assigned as in Kates [72 (pp 172-175)].

The CI (ammonia) mass spectrum of the oligomer esters purified by TLC showed the following parent peaks: m/e 624 for dihydroxydimerate, m/e 608 for monohydroxy dimerate, m/e 884 (major) for trimerate, m/e 890 for the addition of monomer to the dihydroxydimerate, m/e 1178 (minor) for tetramerate and traces of monohydroxyoleate at m/e 312.

(4) Synthesis of stearic dimer

Oleic dimer dimethyl ester, purified by TLC as above, was hydrogenated in a Parr apparatus at room temperature and 50 psi pressure. The catalyst used was 5% Pd on charcoal (Engelhard) and the solvent was ethanol (50 cc/g of ester). Twice the weight of catalyst based on the weight of dimer was used and the hydrogenation was complete in 18 hr [56]. The catalyst was removed by filtration and the ethanol was removed on the Rotavapor (Brinkmann).

The CI (ammonia) mass spectrum gave a single parent peak at m/e=594, the expected mass for a saturated dimer with one carbon-to-carbon linkage joining the two C_{18} chains. The EI mass spectrum was the same as reported by Paschke et al. [56] and confirmed their assignment of structure. Peaks at M-CH_3O and M-CH_3OH, and M-2CH_3O were seen, and would be expected for the methyl ester of a dibasic acid. Cleavage at the bond joining the

two chains is shown by the peak at mass 297 (M/2), which is the strongest peak (base peak) in the spectrum. This is expected, since this is a bond between two tertiary carbons. Peaks at M/2-32 and M/2-33 are related to the same cleavage, with simultaneous loss of CH_3OH and CH_3OH+H. One homologous series had peaks of equal intensity at m/e 495, 481, 467 and 453. This series corresponds to loss of C_7H_{15}, C_8H_{17}, C_9H_{19} and $C_{10}H_{21}$, from the parent mass of 594, indicating joining at atoms 8, 9, 10 and 11. A second series of peaks of equal intensity was at masses 451, 437, 423 and 409, corresponding to loss of $C_6H_{12}COOCH_3$, $C_7H_{14}COOCH_3$, $C_8H_{16}COOCH_3$ and $C_9H_{18}COOCH_3$ from the parent mass of 594, again indicating joining at carbons 8, 9, 10 and 11. A third series of peaks of equal intensity was at m/e 463, 449, 435 and 421. These correspond to loss of $C_nH_{2n+1}+CH_3OH$ from the parent mass of 594, where n = 7, 8, 9 and 10, again indicating the points of joining to be at carbons 8, 9, 10 and 11. Weak peaks at 477 and 491 correspond to joining at carbons 12 and 13. A fourth series of peaks of equal intensity was at m/e 431, 417, 403 and 389. These correspond to loss of $C_nH_{2n+1}+2CH_3OH$ from the parent mass of 594, where n = 7, 8, 9 and 10, again indicating the points of joining to be at carbons 8, 9, 10 and 11. Weak peaks at 445 and 459 correspond to joining at carbons 12 and 13. A fifth series of peaks at m/e 419, 405, 391 and 377 corresponds to loss of $C_nH_{2n}COOCH_3+CH_3OH$ from the parent mass of 594, where n = 6, 7, 8 and 9, again indicating

joining at carbons 8, 9, 10 and 11 [56]. The $^{1}H,^{13}C$ NMR and IR spectra show the same peaks as for oleic dimer but the following peaks are missing, indicating completion of hydrogenation: from ^{1}H spectrum, 2.0 ppm (m) for $-CH_2-C=C$ (allylic methylene) and 5.0-5.4 ppm (m) R-CH=CH-R. The following resonances were not seen in the ^{13}C NMR spectrum; 27.4 ppm for $-CH_2-C=C-$ allylic methylene (cis), 32.6 ppm for $-CH_2-CH=CH-$ allylic methylene (trans) and 130-134 ppm (m) for R-CH=CH-R. R_3C-H was detected at 39.4 ppm using 90^{o} DEPT. From the IR spectrum, peaks at 3010 cm^{-1} for C=C (aliphatic C-H stretching), 970 cm^{-1} for -CH=CH- (trans), CH out-of-plane deformation and 720 cm^{-1} for -CH=CH- (cis) and CH out-of-plane deformation were missing.

G. Characterizing lipid-mediated hemagglutination

(1) Size-selective filtration

All samples that were titered for agglutination were also titered after filtration through a 0.2 μm membrane to see if this treatment affected the specific activity and to remove any particulate matter.

(2) Concentration studies

The specific activity for hemagglutination by heated oleic acid was also tested by using different concentrations of the sample (0.5-5.0 mg/mL).

(3) RBC specificity profile

Different standard fatty acids were heated in air for ten days as for OA (see above) and tested for agglutination activity

against different species of RBC. The samples were fully neutralized as above and the final concentration was 0.5 mg/mL PBS. All species of RBC were treated in the same way as was rRBC.

(4) <u>The effect of cations on hemagglutination activities</u>

The test samples were OA, OA Δ10D, C18 dimer (Emery), and C18 trimer (Emery). (a) 1 mL of each sample (0.5 mg/mL) was in PBS-N_3 as control. (b) 1 mL of each test sample was dialyzed against N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffered saline (4.76 g/L HEPES, 0.9% NaCl, pH 7.5, HBS) for 3 hr at 4°C. (c) 1 mL of each test sample was dialyzed against HBS with 1 mM ethylene-bis-(beta-aminoethylether)-N,N'-tetraacetic acid (EGTA) for 3 hr at 4°C to chelate Ca^{2+}. (d) 1 mL of each test sample was treated as in (c) with 10 mM EGTA. (e) 1 mL of each test sample was treated as in (d), then dialyzed against HBS with 1 mM $CaCl_2$ for another 3 hr at 4°C. f) 1 mL of each test sample was treated as in (d), then dialyzed against HEPES saline buffer with 1 mM $MgCl_2$ for another 3 hr at 4°C. The volume of dialyzing buffer was 500 mL in each case. All samples were titered against rRBC. For determining titers, PBS-N_3 buffer was used for (a), HBS for (b), HBS with 1 mM EGTA for (c), HBS with 10 mM EGTA for (d), HBS with 1 mM $CaCl_2$ in (e), and HBS with 1 mM $MgCl_2$ (the affinity of EGTA for Mg^{2+} is 10^{-3} of that for Ca^{2+}) for (f). In (a) the rRBC were suspended in PBS-N_3 but for the rest the rRBC were suspended in HBS.

(5) pH effect on agglutination

By using 0.1 N HCl in PBS and 0.1 N NaOH in PBS, PBS at the following pH values was prepared: 6, 6.5, 7, 7.5, 8, 8.5, and 9. At higher pH than 9, or lower than 6, rRBC's were unstable. Lipid sample at 0.5 mg/mL was prepared in PBS of appropriate pH and diluted for titration with PBS of the same pH.

(6) Light microscopy

Photographs were taken using an Olympus SC 35 mm camera (with an exposure and ASA setting of -1 and 125, respectively) loaded with Kodak TMX 135-24 film, and attached to an Olympus CH-1 microscope. Agglutinated cells were taken from microtiter plates containing samples which were being titered and allowed to develop for approximately 30 min. The following preparations were used: (1) untreated red cells; (2) standard OA (1 mg/mL); (3) oleic acid heated in air for ten days at 100°C (1 mg/mL); (4) standard C18 dimer (Emery) (1 mg/mL); (5) standard C18 trimer (Emery) (1 mg/mL) and standard OA without red cells. All samples were totally dissolved in ethanol. Fresh blood was drawn from a Sprague-Dawley rat and immediately stored in 3.8% sodium citrate, pH 7.2. The cells were washed once with citrate. The cells retain their shape much better in citrate than in PBS and, therefore, allow better photographs to be taken. However, it should be noted that cells washed in citrate will only maintain their integrity for approximately two hours at room temperature

under the microscope and, for that reason, only that portion of drawn blood needed for a single titer plate is washed.

(7) Gel filtration

A column packed with Sepharose CL-2B (Pharmacia, Piscataway, NJ) in PBS-N_3 was 30 cm long and 2 cm i.d. Sepharose CL-2B has a molecular mass fractionation up to 40 x 10^6 daltons and bead diameter ranging from 60 to 250 μm. The column was calibrated using blue Dextran (2000 kD), thyroglobulin (669 kD), and ferritin (440 kD) and detected at 254 nm using a 2238 UVICORD SII recorder. The fatty acid samples (1 mL) (1 mg/mL) were applied in PBS-N_3. The collected fractions (1 mL/min/tube, 70 tubes) were analyzed by TLC in isooctane:isopropyl alcohol:acetic acid (95:5:1 v/v/v) and visualized by spraying with 20% $(NH_4)_2SO_4$ and heating at 180°C.

(8) Sialidase treatment of rRBC [74]

Rat RBC (100 μL packed cells) were suspended in PBS-N_3 containing 30 mU sialidase (SA) (Sigma) to a final volume of 250 μL and incubated at 37°C for 45 min with occasional gentle shaking. The same amount of rRBC with SA was also incubated with the SA inhibitor, 2,3-dehydro-2-deoxy-N-acetyl-neuraminic acid (Boehringer Mannheim), at a final concentration of 10 μM. In the control, buffer was added instead of SA. The erythrocytes were washed 3 times, each time in 100 volumes of PBS and used to determine titers of fatty acid samples.

(9) Agglutination activity of aldehydes, ketones, epoxy acids and dicarboxylic acids related to oleic acid oxidation

Several aldehydes, ketones, epoxy acids and dicarboxylic acids were tested for activity against rRBC. The concentration of the samples was 1 mg/mL of 80% ethanol in water and the previous method for agglutination titers was used.

H. Ionization of fatty acids in water

pH studies of C18 dimer (Emery)

A 100-mL solution of 0.08 M potassium oleate and a 100-mL solution of 0.04 M potassium C18 dimerate (Emery) were prepared, and 2.5 mL of these solutions were pipetted into each of 40 clean glass vials. An appropriate amount of titrant (0.94 N HCl) was added to each vial so that each vial corresponded to a point on a titration curve [40]. HCl solutions were standardized against potassium acid phthalate using phenophthalein and pH measurements as end-point indicators. The samples were equilibrated at 6°C for 10 days, at 25°C for 2 days and overnight at 40°C and pH measurements for each sample at each temperature were made.

RESULTS

Thermal oxidation of oleic acid

OA lyses rRBC but when it is heated at 100°C in air, OA agglutinates rRBC. The titer increases with time of heating (Table I). The change is faster if air is bubbled through and much slower if heating is conducted under nitrogen.

The concentration of the samples does not affect the specific titer obtained when the samples are dispersed by full neutralization (Table II). However, there is pronounced cloudiness when concentrated samples in ethanol (greater than 4 mg of acid per mL of ethanol) are used, which implies that the solution is heterogeneous.

Heating OA for 3-12 hrs had no apparent effect on the iodine value. On the other hand, the iodine value for the OA Δ24 hrs was reduced to 75%. This observation suggests that the percentage of double bonds in the sample molecules had been reduced and secondary oxidation products or cleavage products had formed. Since tertiary allylic hydrogens in this compound can be substituted by halogens, some of the iodine uptake may be due to reaction of dimers with iodine.

The neutralization equivalent remained the same after 24 hrs of heating (data not shown).

Table I. Hemagglutinin activity by OA depends on time of heating and the presence of oxygen.

Time of heating[a] (hr)	Specific activity		
	Air	N_2	Bubbled air[c]
24	2.9×10^2	2.5×10^2	5.8×10^2
48	3.6×10^2	5.1×10^2	1.1×10^3
72	5.7×10^2	2.5×10^2	2.5×10^3
96	5.7×10^2	2.5×10^2	5.1×10^3
120	1.0×10^3	2.5×10^2	1.1×10^3
144	6.4×10^2	5.1×10^2	5.1×10^3
168	1.2×10^3	7.2×10^2	9.6×10^3
192	___ [b]	____	9.8×10^3
216	2.3×10^3	____	1.0×10^4
240	2.3×10^3	5.7×10^3	2.0×10^4

[a] OA was heated at 100°C. All samples were fully neutralized with NaOH-PBS and filtered with Whatman 41 filter paper before they were titered.

[b] (____) means that no assays were performed on these samples.

[c] See thermal oxidation of OA, page 31.

Table II. The specific activity for hemagglutination by heated OA does not change with concentration.

Concentration (mg/ml)	Specific activity[a]	
	Ethanol[b]	Fully neutralized[c]
0.5	2.9×10^4	2.9×10^4
1.0	2.0×10^4	2.0×10^4
3.0	2.7×10^4	2.7×10^4
4.0	4.1×10^4	2.9×10^4
5.0[d]	1.3×10^5	1.6×10^4

a All samples were filtered with Whatman 41 filter paper before they were titered.

b Dissolved in ethanol

c Dispersed in phosphate buffered saline at pH 7.4.

d Solutions became cloudy.

This means that the carboxyl groups were not involved in a chemical change, such as the formation of oligomers through ester formation between one OA molecule and another OA hydroxylated molecule, during this heating period. Small traces of peroxides were detected during the early stages of heating (2-12 hrs).

Spectroscopy

The absorption spectrum (Figure 2) of OA and OA Δ10D showed a new peak appearing at 217 nm. This indicates some formation of conjugated double bonds and/or presence of linoleic acid but not ketones, aldehydes, or esters (275, 290, and 211 nm, respectively).

Figure 3 shows the IR spectra of OA and OA Δ10D. The only difference between the two spectra is the new peak at 1730-35 cm^{-1} for the OA Δ10D which may show the presence of an extra carboxyl group located in a position that causes the peak to be shifted. This peak might also be indication of an ester (there is a small peak at 173 ppm for ^{13}C spectra of OA Δ10D, indicating the presence of a small amount of ester).

The OA Δ10D ^{1}H NMR spectrum (Figure 4) showed oxidized materials between 2.0 and 5.0 ppm which are not present in the OA ^{1}H NMR spectrum; at 3.4-3.7 ppm for $-CH_2O-$ (aliphatic saturated alcohol or ether), 3.6 ppm for $-CH_2OH$, 3.75 ppm for -CHOH, 4.2 ppm for $-CH_2OCOR$, and at 5.1 ppm for -CHOCOR. However, the methine proton such as in a dimer was very difficult

Figure 2. Absorption spectrum of OA and OA Δ10D.

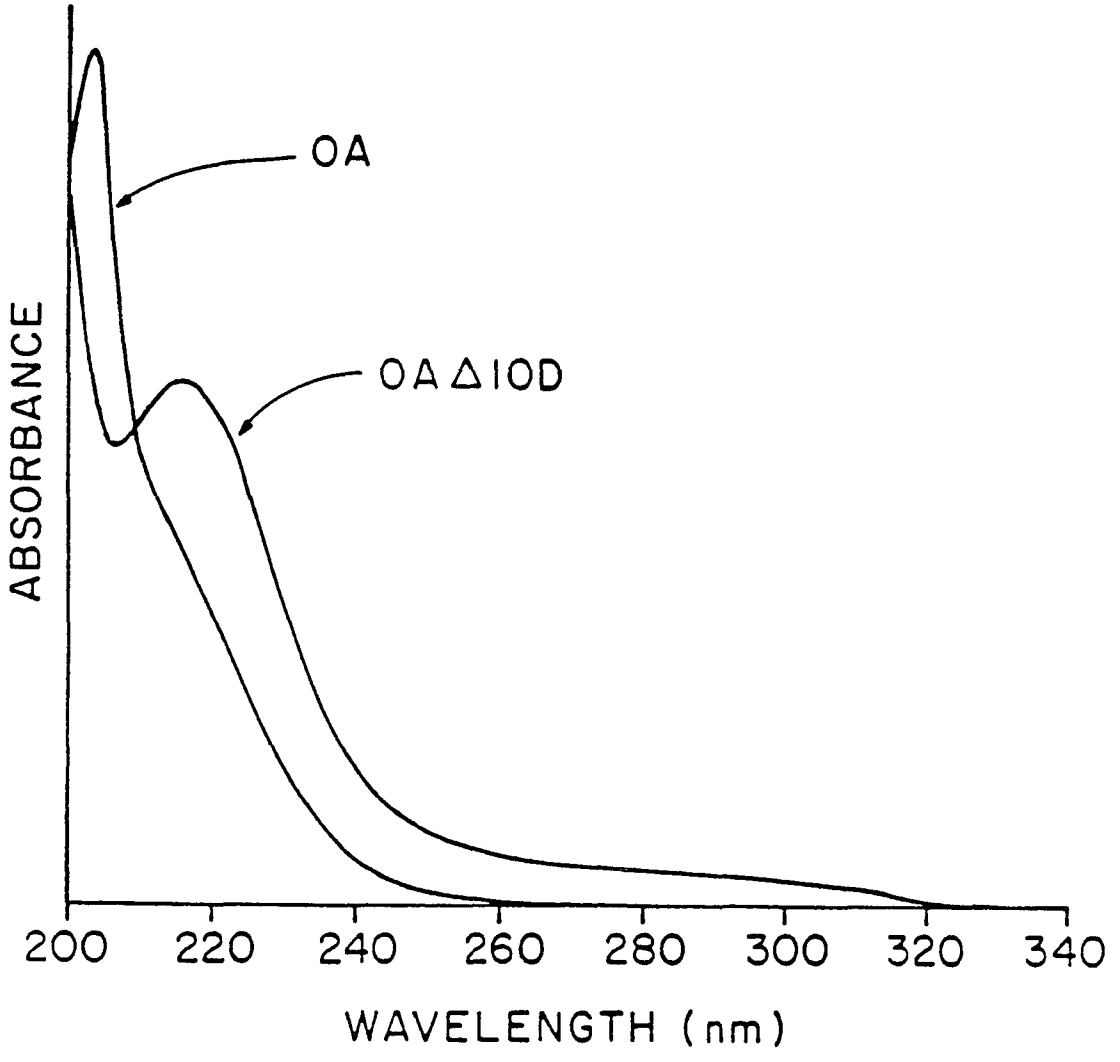


Figure 3. IR spectra of OA and OA Δ10D.

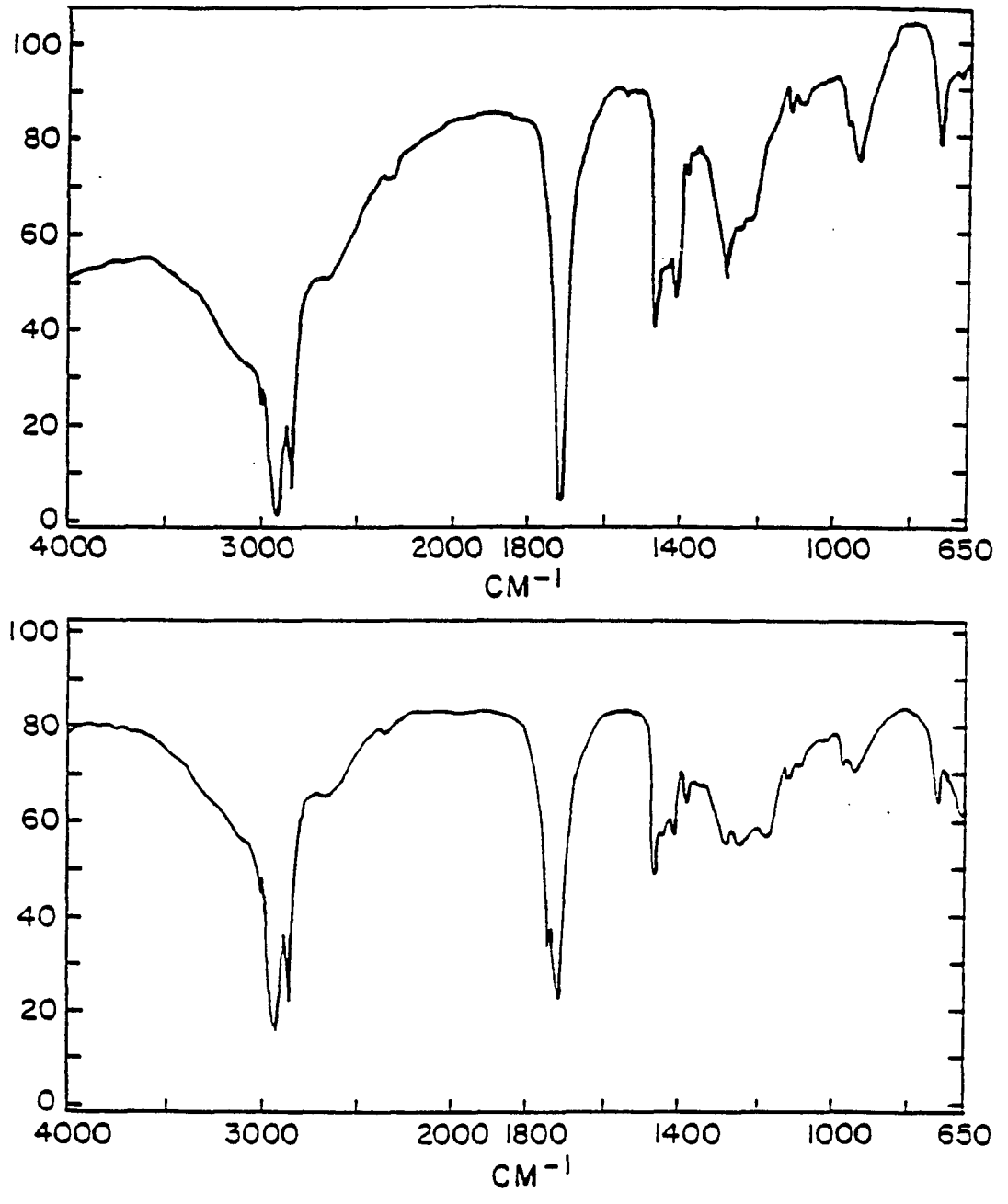
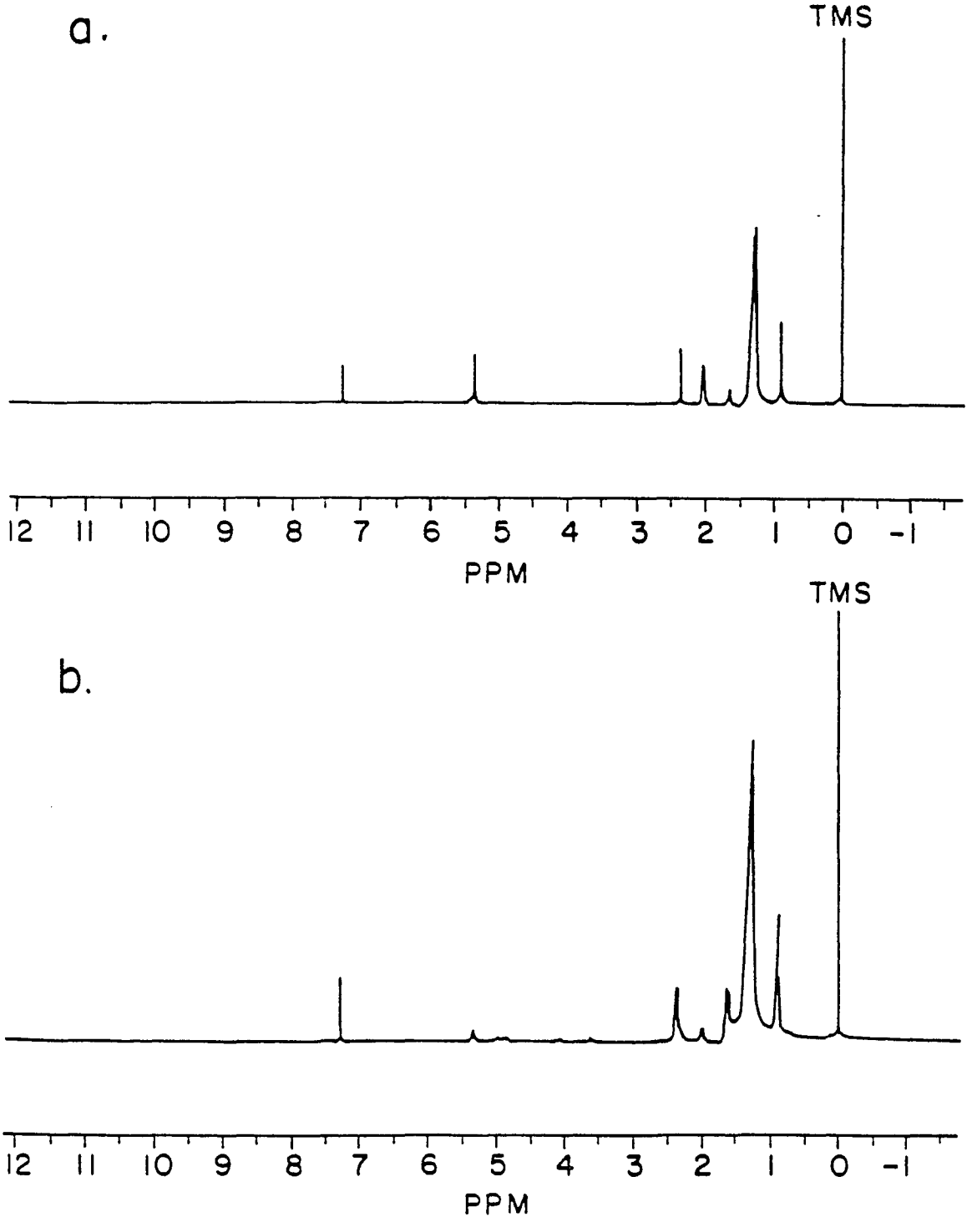


Figure 4. ^{1}H NMR spectra (a) OA, (b) OA Δ10D.



to identify because of the strong peaks between 1.0-1.7 ppm (R_3CH should give a peak at 1.4-1.7 ppm). Another difference between the OA and the OA Δ10D ^1H NMR is the peak at 5.4 ppm for the double bond, where the OA Δ10D showed lower unsaturation.

In the ^{13}C NMR spectrum of OA Δ10D (Figure 5), the following peaks differed from OA: at 64 ppm for $-CH_2OCOR$, at 67-70 and 73-76 ppm for $-CH_2O-$ (aliphatic saturated alcohol or ether) and at 171-174 ppm for RCO- (acyl ester carbonyl) but no ketone peak.

Structural requirements for hemagglutination activity

To determine whether other fatty acids are hemagglutinins or can be converted to them, a series of fatty acids were evaluated before and after heating.

The titers against rRBC for a series of mostly C18 fatty acids before and after heating at 100°C are shown in Table III. The results show that all unheated monomers, even the hydroxylated fatty acids, are inactive until heated. Heated Δ-6,7 C18 and Δ-9,10 C18 acids have higher titers than heated Δ-11,12 C18 acids. Stearic acid is not an agglutinin and does not become one on heating. Hydroxylation as in hydroxystearic acid or ricinoleic acid does not confer agglutinating ability unless the fatty acid is also heated. When 12-hydroxystearic acid is heated, it probably undergoes dehydration to give an unsaturated fatty acid which undergoes further reaction. However, oligomeric fatty

Figure 5. ^{13}C NMR (a) OA, (b) OA Δ10D .

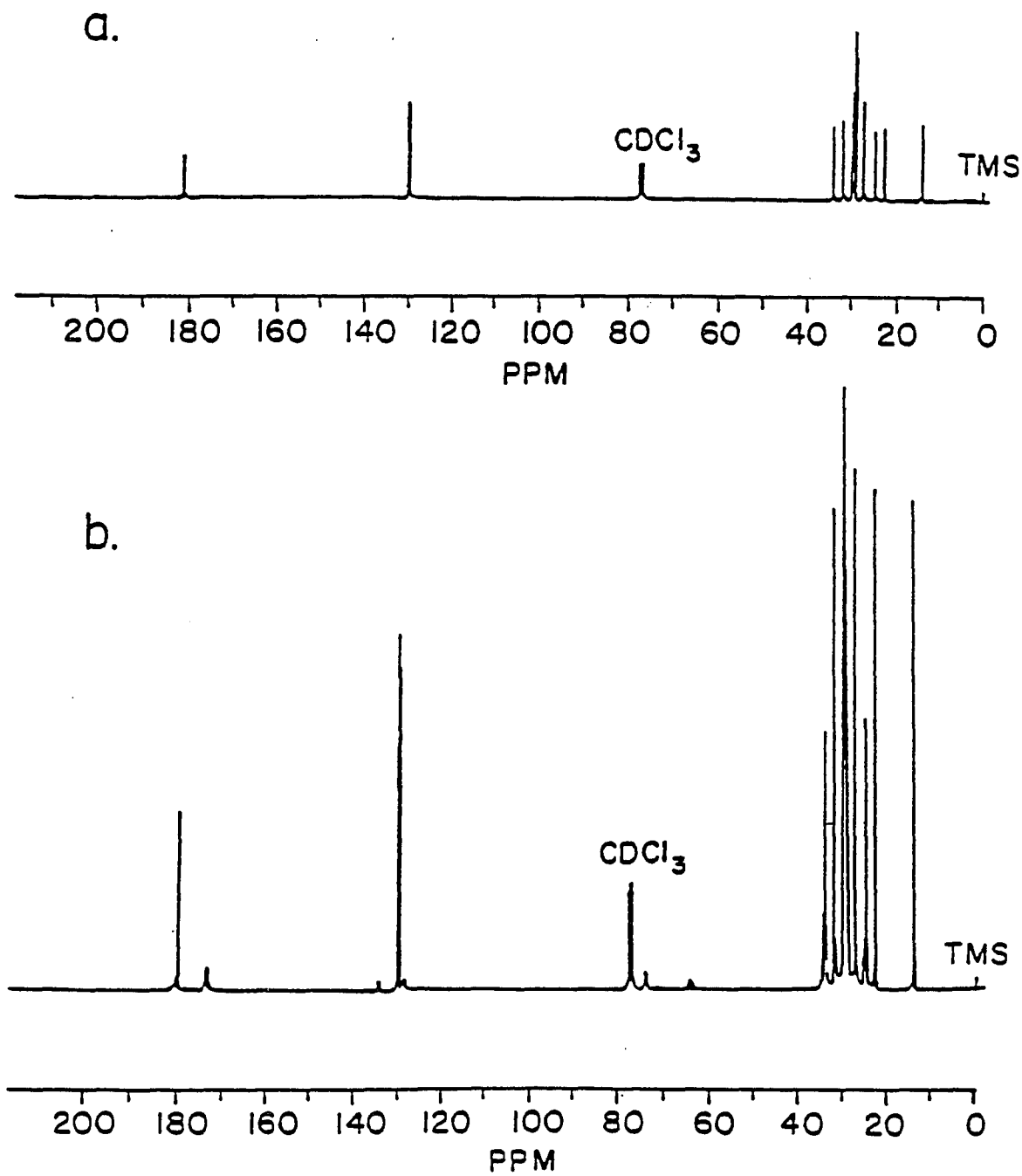


Table III. Hemagglutination, lysis and specific titers of rRBC by unsaturated fatty acids before and after heating in air at 100°C for 240 hours[a].

Fatty Acid	Titers Unheated			Titers Heated		
	Lysis	Agglutination	Specific activity	Lysis	Agglutination	Specific activity
Oleic (cis-Δ9)	2^6	2^7	2.0×10^2	2^4	2^{14}	3.2×10^4
Elaidic (trans-Δ9)	2^{6b}	0^b	------	2^{3b}	2^{13b}	-------
C18 dimer (Emery)	0	2^{14}	2.0×10^4	0	2^{14}	2.0×10^4
C18 dimer (Unichema)	0	2^{14}	2.0×10^4	0	2^{14}	2.0×10^4
C22 dimer (Unichema)	0	2^{14}	2.0×10^4	0	2^{14}	2.0×10^4
Oleic linear dimer(saturated)[c]	0	2^{14}	2.0×10^4	0	2^{14}	2.0×10^4
Oleic linear dimer(unsaturated)[c]	0	2^{14}	2.0×10^4	0	2^{14}	2.0×10^4
Linear trimer + tetramer	0	2^{14}	2.1×10^4	–[d]	–	------
Monohydroxy oleic dimer[c]	0	2^{13}	1.0×10^4	–	–	------

Table III continued.

Dihydroxy oleic dimer[c]	0	2^{13}	1.0×10^4	–	–	------
C21 Diacid	0	2^4	1.0×10	2^4	2^{11}	5.0×10^3
C18 trimer (Emery)	0	2^{15}	4.0×10^4	0	2^{15}	4.2×10^4
Ricinoleic (12-OH, Δ9)	0	2^6	1.5×10^2	0	2^{14}	1.0×10^4
Ricinelaidic (12-OH, Δ9)	0[b]	2^{7b}	------	0[b]	2^{14b}	------
Vaccenic (trans-Δ11)	0	2^5	8.0×10	2^5	2^{12}	4.2×10^3
Vaccenic (cis-Δ11)	2^{3b}	2^{6b}	------	2^3	2^{12}	4.0×10^3
Palmitic	0	2^4	4.0×10	0	2^4	4.0×10
Stearic acid	0[b]	2^{2b}	------	0[b]	2^{2b}	------
12-Hydroxystearic	0[b]	2^{2b}	------	0[b]	2^{8b}	------
Palmitoleic (cis-Δ9)	2^4	2^5	7.5×10	2^5	2^{10}	2.5×10^3
cis-Δ9,12-Octadecadienoic	2^5	2^7	3.0×10^2	2^3	2^{13}	1.0×10^4
Petroselinic (cis-Δ6)	2^4	2^7	3.0×10^2	0	2^{13}	1.2×10^4
Petroselaidic (trans-Δ6)	0[b]	2^{6b}	------	0	2^{13}	1.2×10^4
Linolenic (cis-Δ9,12,15)	2^5	2^6	1.5×10^2	0	2^{12}	4.0×10^3
γ-Linolenic (cis-Δ6,9,12)	2^5	2^7	3.2×10^2	2^5	2^{12}	4.0×10^3

Table III continued.

erythro-9,10-Dihydroxystearic	0^{b}	2^{2b}	_____	2^{3}	2^{10}	2.4×10^{3}
threo-9,10-Dihydroxystearic	0^{b}	2^{3b}	_____	2^{2}	2^{10}	2.2×10^{3}

a Samples were fully neutralized with NaOH in PBS, then pH-adjusted to 7.4 and filtered through a 0.2 μm filter. The concentration of the solutions was approximately 0.5 mg/mL in PBS.

b Not completely dissolved. All titers are for filtered samples.

c Synthesized dimers by the method of Paschke et al. (1964).

d (____) means that no assays were performed.

acids are good agglutinins before heating. Thus it appears that unsaturation is required and that heating of the unsaturated fatty acid causes the formation of oligomers. The only exceptions are the dimers, trimers-oligomers which show the same activity even after heating. Also, the specific activity of OA heated for 10 days is comparable to the specific activities of the dimers and the trimers-oligomers. Thus it appears that fatty acid oligomers are hemagglutinins.

A series of standard aldehydes, ketones, epoxy acids and dicarboxylic acids was also tested for hemagglutination activity and was shown to be inactive. A low activity was also found with unheated aldehydes and with epoxy acids but not comparable to the activity found in the OA Δ10D (Table IV).

Size exclusion study

Table V shows that the specific activity of the unfiltered material of all samples gave the same titer as the materials which were passed through the 0.2 μm Acrodisc membranes. This means that the species or its aggregate responsible for the activity is smaller than 0.2 μm or if it is larger it can change its size and form and can pass through the pores under the pressure which is applied on the sample by the syringe.

Analysis of OA Δ10D by TLC, HPLC, SGC and MS.

The components of heated OA were resolved by TLC on silica gel in a system which discriminates by number of carboxyl groups (Figure 6). Bands were cut out, eluted and titered against rRBC.

Table IV. Agglutination activity of unheated aldehydes, ketones, epoxy acids and dicarboxylic acids against rRBC.

Sample[a]	Lysis	Tite	Specific activity
3-Octadecanone	2^4	0	0
3-Hexadecanone	2^5	0	0
4-Ketopimelic acid	2^6	0	0
7-Oxooctanoic acid	2^4	2^6	8.0 x 10
trans-2-Nonenal	2^6	0	0
Undecenal	2^4	2^6	7.5 x 10
Decanal	2^4	2^6	8.0 x 10
Dodecanal	2^8	0	0
Tetradecanal	2^9	0	0
Acetaldehyde	2^3	0	0
Hexadecanol dimethyl acetal	2^2	0	0
cis-9,10-Epoxy octadecanoic acid	2^4	2^9	6.4 x 10^2
trans-9,10-Epoxy octadecanoic acid	2^4	2^9	6.4 x 10^2
Dodecadienoic acid	0	2^2	1.0 x 10
Hexadecadienoic acid	0	2^5	6.5 x 10

[a] The concentration of the solutions was approximately 1 mg/mL in 80% ethanol / 20% water.

Table V. OA, OA Δ10D, and OA oligomers were subjected to size-selective filtration and titered.

Sample[a]	Filter[b]	Specific titer
OA	None	$2x10^2$
	Acrodisc	$6x10^2$
OA Δ10	None	$3x10^4$
	Acrodisc	$3x10^4$
C18 dimer (Emery)	None	$2x10^4$
	Acrodisc	$2x10^4$
C18 dimer (Unichema)	None	$2x10^4$
	Acrodisc	$2x10^4$
C21 Diacid	None	1x10
	Acrodisc	1x10
C18 Trimer (Emery)	None	$4x10^4$
	Acrodisc	$4x10^4$
Oleic linear dimer[c]	None	$2x10^4$
	Acrodisc	$2x10^4$
Stearic linear dimer[c]	Acrodisc	$2x10^4$
Linear trimer + tetramer[c]	Acrodisc	$2x10^4$
Monohydroxy oleic dimer[c]	Acrodisc	$1x10^4$
Dihydroxy oleic dimer[c]	Acrodisc	$1x10^4$

a Fully-neutralized with NaOH, pH adjusted to 7-8.

b Filtered trough a 0.2 μm acrodisc.

c Synthesized by the method of Paschke et al. (1964).

Figure 6 also shows the accumulation of oligomeric material with increasing time of heating. The identification of the component of the fraction was by comparison with R_fs of the components of standard dimers and trimers. The highest titers for heated oleic acid are associated with dimeric, oligomeric and polymeric fractions (Table VI). Even though HPLC and SGC showed very poor resolution, the more active fractions contained some high molecular weight (MW) structures corresponding to the MW of dimers and trimers (Table VII).

This common denominator might be the reason for the hemagglutinating activity since polymerization is apparently necessary and sufficient for the activity. Therefore, dimers and oligomers of C18 and C22 acids were tested. A commercial dimer obtained from Emery, C18 dimer (Emery) composed of 3% monomer (C18), 94% dimer (C36), 3% trimer (C54) and traces of tetramers and other oligomers; all containing cycloaliphatic, aromatic and acyclic structures, a "hydrogenated dimer" from Unichema, C18 dimer (Unichema) composed of 0.1% monomer (C18), 1% intermediate, 98% dimer (C36) and 1% trimer (C54) [54% cycloaliphatic, 37% acyclic and 9% aromatic structure], a C22 dimer (Unichema), with a very high content of linear isomers (0.1% monomer, 4% C36 dimer + intermediates, 95% dimer and 1% trimer) and a trimer-oligomer mixture, C18 trimer (Emery) composed of 2% monomer (C18), 18% dimer (C36), 60% trimer (C54) and 20% higher oligomers, also containing cycloaliphatic,

Figure 6. Thin layer chromatogram of OA heated at different time intervals. Visualization was by I_2.

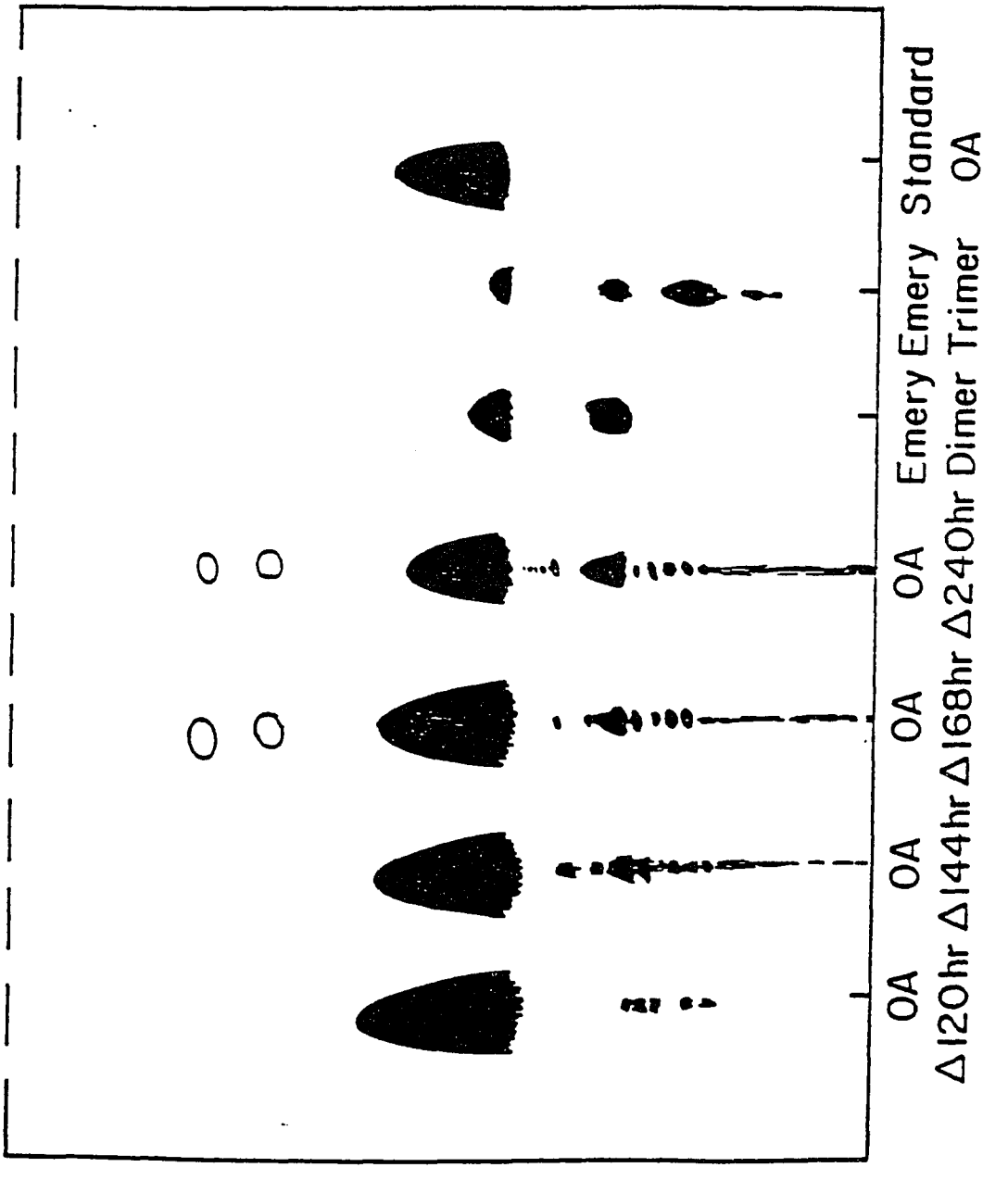


Table VI. OA and oligomers were purified by TLC and the hemagglutination activity of the components against rRBC was measured.

Oleic acid (OA) or derivative		R_fs of components [a]				
		Monomer	Dimer	Trimer	Polymer	Specific activity
Oleic acid		0.52				5.8×10^2
C18 (Emery)		0.48				1.0×10^2
			0.32			1.5×10^4
				0.21		2.0×10^4
C18 trimer (Emery)		0.48				1.0×10^2
			0.31			2.0×10^4
				0.18		3.9×10^4
					0.12	1.5×10^5
					0.00	1.6×10^5
OA Δ10D	0.87[b]					1.0×10
		0.47				1.0×10^2
			0.30			1.0×10^4
			0.28			
			0.26			
				0.18		1.0×10^4
					0.16-0.00 (smear)	1.5×10^5

[a] R_fs of oleic acid samples on silica gel TLC in isooctane : isopropyl alcohol : acetic acid (95 : 5 : 1 v/v/v).

[b] R_f higher than OA. Not identified

Table VII. Mass spectra of the most active samples isolated by different methods.

Sample	Method of isolation	Molecular weight	Interpretation
OA Δ10D	SGC	282	oleic acid
(Most active)[a]		562	oleic dimer
		580	oleic dimer + H_2O
		842	oleic trimer
		860	oleic trimer + H_2O
OA Δ10D	HPLC	298	hydroxystearic
(Most active)[a]		562	oleic dimer
		580	oleic dimer + H_2O
		842	oleic trimer
		860	oleic trimer + H_2O
C18 dimer (Emery)			
	HPLC	562	
		844	
		846	
C18 trimer (Emery)			
	HPLC	562	
		844	
		1126	
		1124	

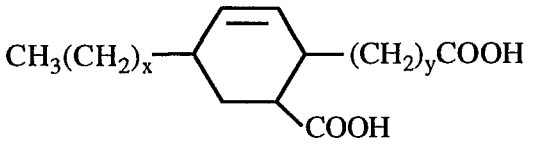
Table VII continued.

OA Δ10D(smear)[a] TLC[b]	298	hydroxystearic
	562	oleic dimer
	842	oleic trimer
OA (fresh)	282	
OA (bench)	282	oleic acid
	564	oleic dimer

a Most active part of OA Δ10D.

b The TLC was developed in isooctane : isopropyl alcohol : acetic acid (95 : 5 : 1 v/v).

aromatic and acyclic structures, showed specific titers as high as heated oleic and no lysis. A C21 dicarboxylic acid with the structure shown below,

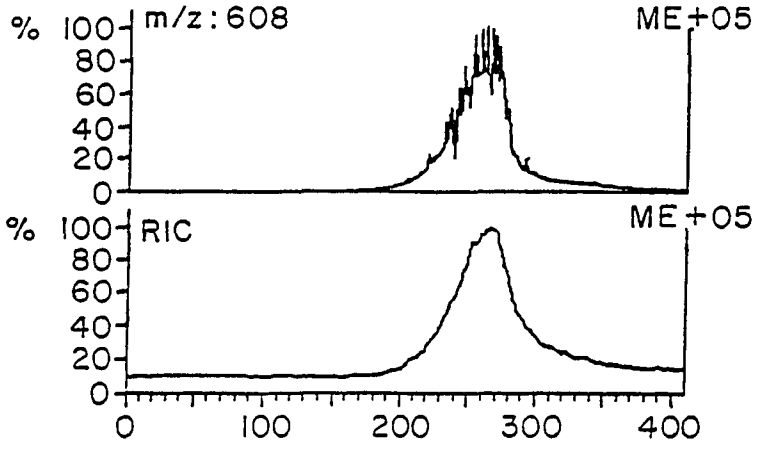


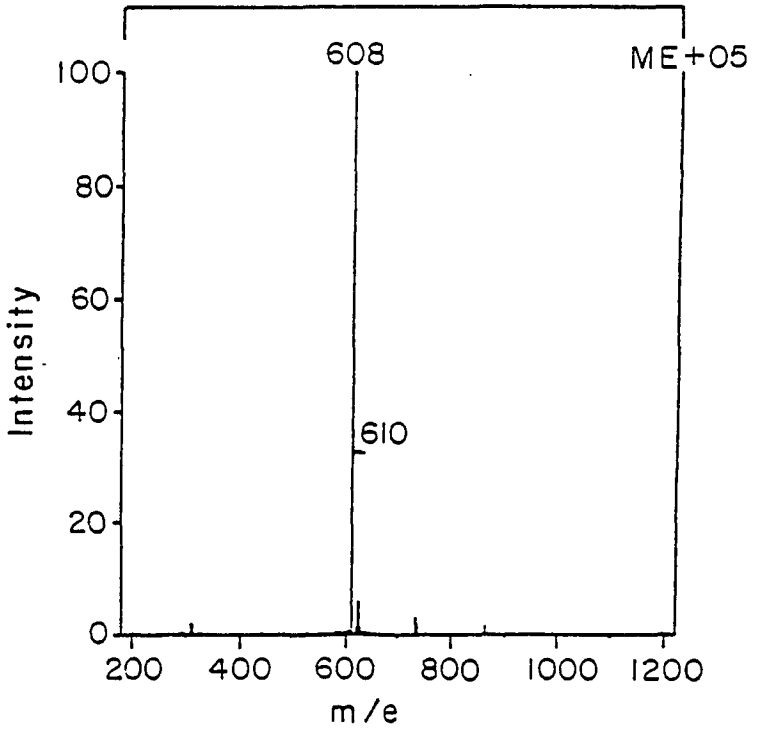
$x + y = 12$

gives a very low titer and no lysis before heating but becomes active after 10 days of heating. In fact the trimers showed higher specific titers than dimers (Table III). Since the mixtures in heated OA were complex and the oligomers available contained cycloaliphatic, linear and aromatic structures, linear dimers of methyl oleate and methyl stearate were synthesized. In order to get fatty acid oligomers of more well-defined structure we synthesized the linear C18 dimers first described by Paschke et al. [56]. Methyl oleate was oxidized with di-tert-butyl peroxide to form a mixture of linear C18 dimer esters. Some of the dimethyl dioleate was hydrogenated to dimethyl distearate.

Figure 7 shows the ion and mass chromatogram (CI mode in ammonia) of dimethyl dioleate with a single parent peak at m/e 590 (after subtracting 18 for NH_4^+), as expected for a carbon-carbon linked dimer with two double bonds. Figure 8 (CI mode in ammonia) shows the ion and mass chromatogram of methyl

Figure 7. Ion and mass chromatograms of dimethyl dioleate. (Since CI was with ammonia, the actual m/e is observed mass-18).





distearate with a single parent peak at m/e 594 (fully saturated). From the EI mass spectra the cleavage at the bond joining the two chains is shown by the peak at mass 297 (M/2), which is the strongest peak (base peak) in the spectrum. The points of attachment are also confirmed by the homologous series that has peaks of equal intensity at m/e 495, 481, 467 and 453. This series corresponds to loss of C_7H_{15}, C_8H_{17}, C_9H_{19} and $C_{10}H_{21}$, from the parent mass of 594, indicating joining at atoms 8, 9, 10 and 11. A second series of peaks of equal intensity is at masses 451, 437, 423 and 409, corresponding to loss of $C_6H_{12}COOCH_3$, $C_7H_{14}COOCH_3$, $C_8H_{16}COOCH_3$ and $C_9H_{18}COOCH_3$ from the parent mass of 594, again indicating joining at carbons 8, 9, 10 and 11.

Figure 9 shows the ^{13}C NMR spectra of (a) methyl oleate, (b) dimethyl dioleate and (c) dimethyl distearate. The dimers have signals due to tertiary carbons at 47.7 ppm (for dimethyl dioleate) and 39.4 ppm (for dimethyl distearate). The peak of the dimethyl distearate due to tertiary carbon is shifted compared to the peak of the dimethyl dioleate because of the different adjacent carbons; in dioleate, a vinyl carbon is adjacent but in distearate, aliphatic carbons are adjacent. Dioleate differs from distearate in the allylic carbon signals at 27.4 ppm (cis) and 32.6 ppm (trans), which are missing in the distearate spectra and also in the vinyl carbons at 130-134 ppm (multiplet) for R-CH=CH-R which are also missing. For the ^1H NMR spectra the peaks at 2.0 ppm (m) for $-CH_2-C=C$ (allylic methylene) and at 5.0-5.4 ppm (m)

Figure 8. Ion and mass chromatograms of dimethyl distearate. (Since CI was with ammonia, the actual m/e is observed mass-18).

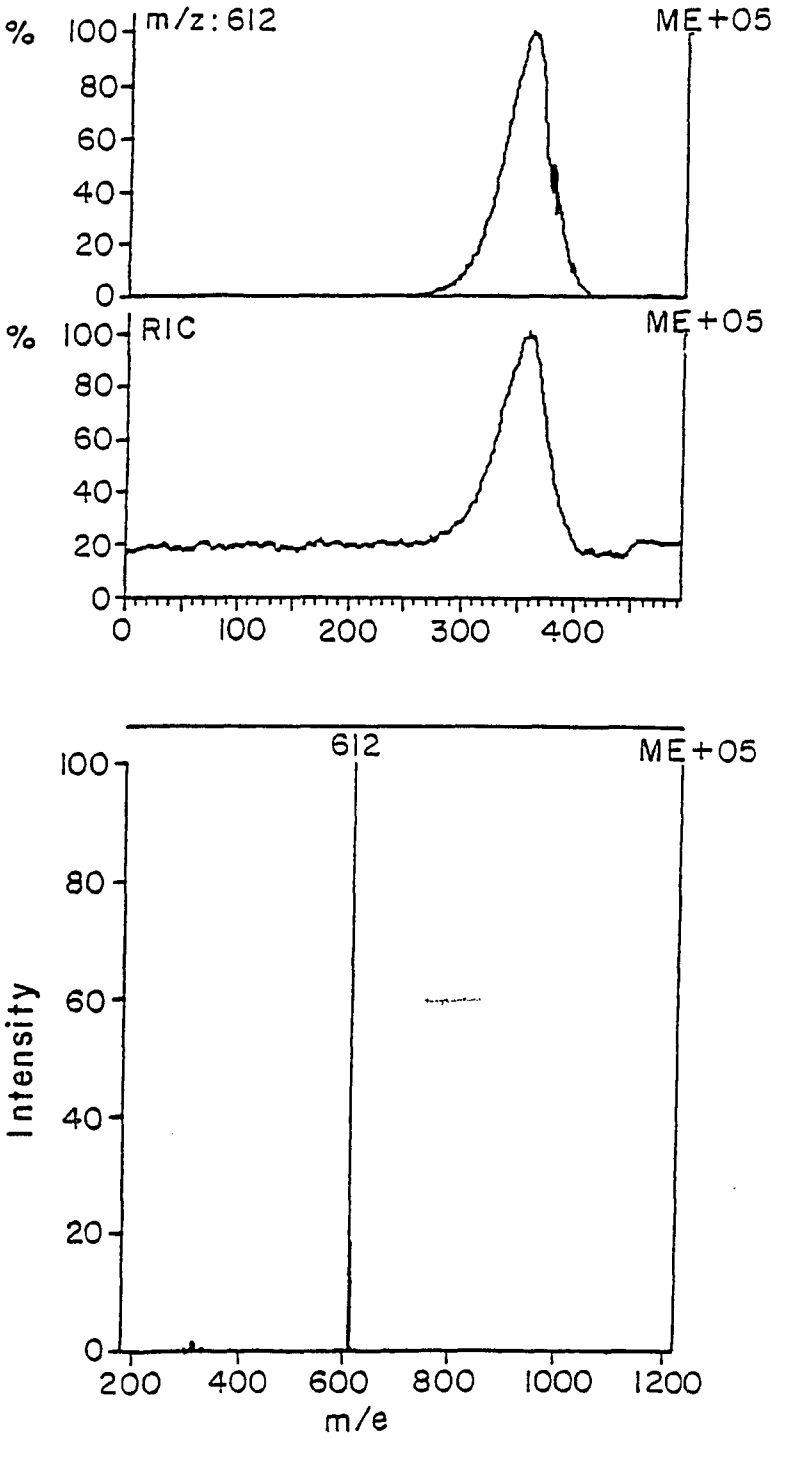
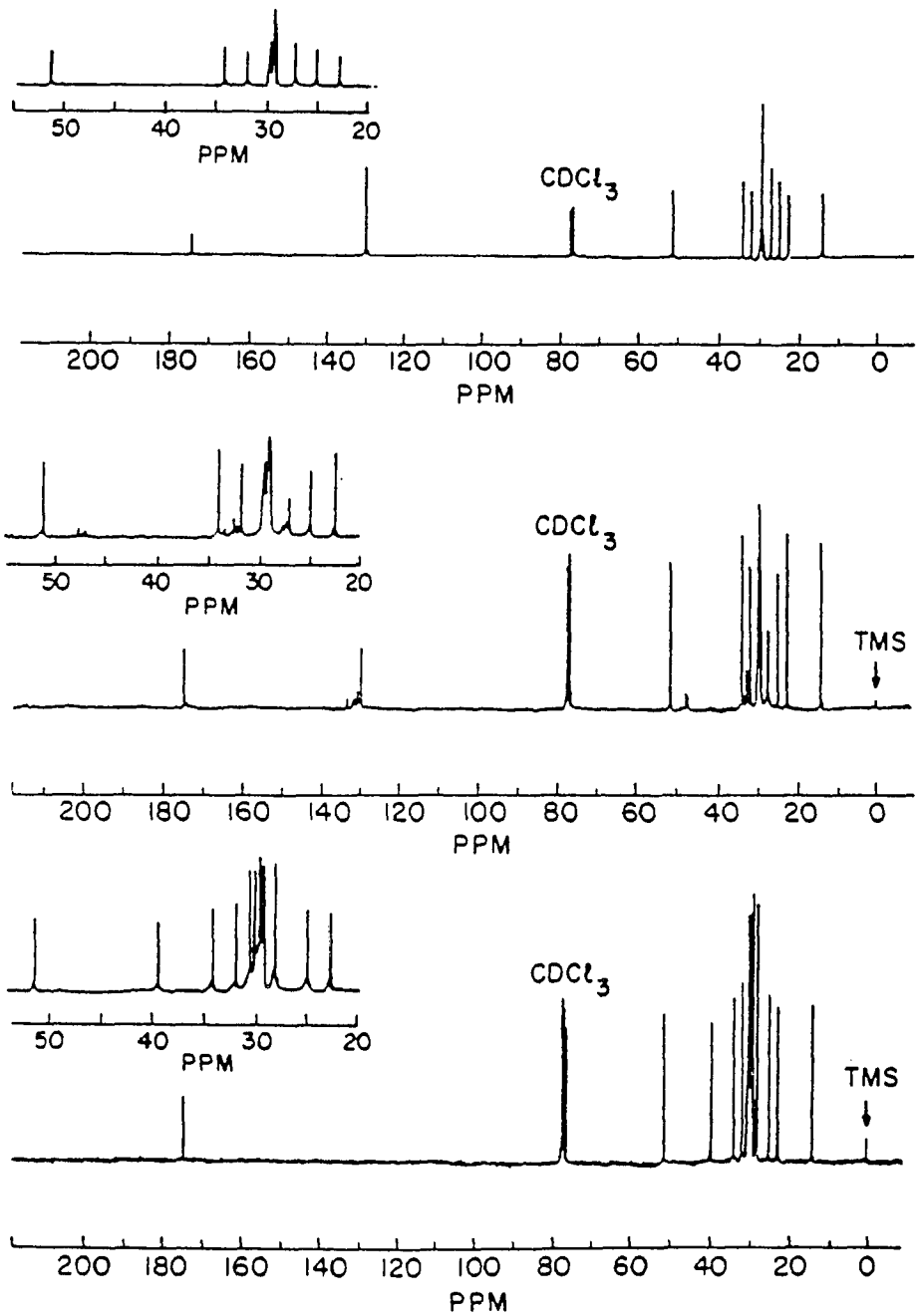


Figure 9. ^{13}C NMR spectra of (a) methyl oleate, (b) dimethyl dioleate and (c) dimethyl distearate.



for R-CH=CH-R which were present in the dioleate ^1H NMR spectra, were also missing in the distearate ^1H NMR spectra, confirming once again the corresponding dimer structure.

These synthesized compounds are considered a mixture of linear dimers with points of attachment at C8, 9, 10 and 11 from the fact that they move as a single spot on TLC in (a) hexanes:diethyl ether (70:30 v/v) in the methyl ester form and (b) isooctane:isopropyl alcohol:acetic acid (95:5:1 v/v/v) in the acid form (obtained by hydrolysis of the methyl esters) and because they produce one peak in the MS ion chromatogram corresponding to the correct masses.

These linear dimers had the same specific activity as the commercial mixtures of dimers (Table III). Since distearic acid had the same specific activity (Table V), double bonds are not required in the dimer for activity.

Characteristics of oligomeric fatty acid-mediated hemagglutination

Each active heated acid shows a different RBC specificity profile, although they all show high titers for rat and rabbit RBC (Table VIII) with rabbit being higher or equal to rat RBC. The order of RBC specificity for OA Δ10D, C18 dimer (Emery), C18 trimer (Emery), and synthetic linear C18 dimer was rabbit > rat > chicken > dog = guinea pig > human > goat = sheep = bovine RBC. OA Δ10D had the same pattern of erythrocyte specificities as the dimers and trimer-oligomer.

Table VIII. Erythrocyte specificities of lipid agglutinins (relative titers)[a]

Heated Lipid	Rat	Rabbit	Human	Cat	Dog	Guinea Pig	Goat	Sheep	Bovine	Chicken
Oleic acid	2^6(L)	2^8(L)	2^5(L)	2^5(L)	2^5(L)	2^6(L)	2^5(L)	2^6(L)	2^5(L)	2^7(L)
OA Δ10D	2^{14}	2^{14}	2^6	2^5	2^6	2^7	2^5	2^6	2^6	2^8
Linear Dimer[c]	2^{14}	2^{14}	2^8	2^7	2^8	2^8	2^6	2^6	2^7	2^9
C18 dimer (Emery)	2^{14}	2^{15}	2^9	2^8	2^{10}	2^{10}	2^7	2^7	2^7	2^{10}
C18 trimer (Emery)	2^{15}	2^{15}	2^9	2^8	2^{10}	2^{10}	2^7	2^7	2^7	2^{11}
Ricinoleic	2^6(L)	2^8(L)	2^4	2^2(L)	2^2(L)	2^4(L)	2^2(L)	2^3(L)	2^2(L)	2^4(L)
ΔRicinoleic	2^{14}	2^{13}	2^6	2^4(L)	2^4(L)	2^6	2^4	2^4	2^4	2^6
Ricinelaidic[b]	2^6	2^6	2^3	2^2(L)	2^2(L)	2^2(L)	2^2(L)	2^2(L)	2^4	2^3(L)
ΔRicinelaidic[b]	2^{13}	2^{12}	2^4	2^3(L)	2^5(L)	2^6	2^3	2^4	2^4	2^7
t-Vaccenic	2^5	2^7	2^3	2^3	2^3	2^3(L)	2^2(L)	2^2(L)	2^4(L)	2^2
Δt-Vaccenic	2^{12}	2^{13}	2^8	2^6	2^8	2^7	2^5	2^5	2^6	2^7
c-Vaccenic[b]	2^6	2^7	2^4	2^5(L)	2^6(L)	2^6(L)	2^5(L)	2^5(L)	2^5(L)	2^5
Δc-Vaccenic	2^{11}	2^{11}	2^7	2^4	2^7	2^7	2^2(L)	2^5	2^2(L)	2^7

Table VIII continued.

Stearic[b]	2^2(L)	2^2(L)	2^2(L)	0	0	0	0	0	0	0
ΔStearic[b]	2^2(L)	2^4(L)	2^2(L)	0	0	0	0	0	0	0
12-OH-Stearic[b]	2^2(L)	2^3(L)	2^2(L)	0	0	0	0	0	0	0
Δ12-OH-Stearic[b]	2^8	2^{10}	2^5	2^3	2^5	2^5	0	0	0	2^3
Palmitoleic	2^4	2^5	2^4(L)	2^4(L)	2^4(L)	2^5(L)	2^4(L)	2^4(L)	2^4(L)	2^4(L)
ΔPalmitoleic	2^{11}	2^{12}	2^5(L)	2^6(L)	2^5(L)	2^6(L)	2^5(L)	2^6(L)	2^5(L)	2^6
γ-Linolenic	2^7	2^8	2^5(L)	2^5(L)	2^7(L)	2^6(L)	2^5(L)	2^5(L)	2^5(L)	2^6
Δγ-Linolenic	2^{13}	2^{14}	2^8	2^3	2^7	0	0	2^4(L)	0	2^7
cis-Δ9,12 Octadecadienoic	2^7	2^8	2^4(L)	2^5(L)	2^6(L)	2^6(L)	2^4(L)	2^3(L)	2^4(L)	2^5
Δcis-Δ9,12 Octadecadienoic	2^{12}	2^{13}	2^8	2^4	2^8(L)	2^5	2^3(L)	2	2^4(L)	2^6
Petroselinic	2^7	2^9	2^5(L)	2^5(L)	2^6(L)	2^6(L)	2^5(L)	2^5(L)	2^5(L)	2^6
ΔPetroselinic	2^{12}	2^{13}	2^9	2^4	2^8	2^7	2^4(L)	2^5(L)	2^5(L)	2^7
Petroselaidic[b]	2^6	2^7	2^3(L)	2^3	2^3(L)	2^4(L)	2^2(L)	2^3(L)	2^5(L)	2^4
ΔPetroselaidic	2^{11}	2^{12}	2^9	2^3(L)	2^8	2^6	2^2(L)	2^4(L)	2^6	2^6

Table VIII continued.

Linolenic	2^6	2^7	2^5(L)	0	0	0	0	2^2(L)	0	0
ΔLinolenic	2^{12}	2^{13}	2^8	2^4(L)	2^9(L)	2^5(L)	2^2(L)	2^5(L)	2^2(L)	2^5
erytho-9,10-										
Dihydroxystearic[b]	2^2(L)	2^3(L)	0	0	0	0	0	0	0	0
Δerythro-9,10-										
Dihydroxystearic	2^8	2^9	2^4(L)	2^3(L)	2^5(L)	2^5	2^4(L)	2^5(L)	2^4(L)	2^6
threo-9,10-										
Dihydroxystearic[b]	2^3(L)	2^3(L)	0	0	0	0	0	0	2^3(L)	0
Δthreo-9,10-										
Dihydroxystearic	2^7	2^9	2^4(L)	2^2(L)	2^4(L)	2^4	2^3	2^4	2^4	2^7
Palmitic[b]	2^4	2^4	2^3	0	0	0	0	0	2^4	0
ΔPalmitic	2^4	2^4	2^4	0	0	0	0	0	2^5	0
Elaidic[b]	2^5(L)	2^4	2^5	2^3	0	2^3(L)	0	0	2^4	2^4
ΔElaidic[b]	2^9	2^8	2^7	2^4	2^3	2^3(L)	0	0	2^7	2^5

[a] Samples were fully neutralized with NaOH in PBS, then pH-adjusted to 7.4 and filtered through a 0.2μm filter. The concentration of the fatty acid dispersions was 0.5 mg/mL in PBS.

Table VIII continued.

[b] Not completely dissolved. All results reported are for filtered samples.

[c] Linear dimer (hydrogenated) was synthesized by the method of Paschke et al. (1964).

L: Lysis.

Δ: Heated for 240 hr.

Sialic acid was removed from the rRBC surface by sialidase (SA) treatment, thus lowering surface negative charge [74]. This treatment increases the titer for OA Δ10D, C18 dimer (Emery) and C18 trimer (Emery) (Table IX). When an inhibitor of sialidase was present in the solution the activity was the same as for no sialidase treatment.

Removal of Ca^{2+} with EGTA had no effect (Table X), but adding Ca^{2+}(1 mM) or Mg^{2+} (1 mM) lowered the specific titer.

Increasing the pH of OA Δ10D, C18 dimer (Emery) and C18 trimer (Emery) increased the specific activity (Table XI). When the pH was raised from 6 to 9, the specific activity for OA increased 2.5-fold, for OA Δ10D it increased 30-fold, for oleic C18 dimer (Emery) it increased 58-fold and for oleic C18 trimer (Emery) it increased 63 fold.

Equilibrium titration curves, using the procedure of Cistola et al. [40] at 5°, 25°, 37°C were obtained for the potassium salt of OA and C18 dimer (Emery) (Figure 10). For potassium oleate at 5°C, between 0 and 5 μL of 0.94 N HCl, the pH decreased from 10.1 to 9.8, and the samples were optically clear. Between 5 and 60 μL of 0.94 N HCl, the pH remained almost constant at 9.8 and the samples contained crystals suspended in a clear liquid. Between 60 and 120 μL of 0.94 N HCl the pH decreased from 9.8 to 8.1. However, between 110 and 170 μL of 0.94 N HCl, samples contained a white pasty material and the pH decreased from 8.1 to 7.1. Between 170 and 210 μL of 0.94 N HCl the pH decreased

Table IX. Effect of sialidase (SA) on fatty acid mediated agglutination of rRBC[a].

Fatty Acid	rRBC treatment	Titer		Specific Titer
		Lysis	Agglutination	
OA	+ PBS	2^5	2^7	2.0×10^2
	+ SA	2^6	2^7	2.0×10^2
	+ SA + I[b]	2^5	2^7	2.0×10^2
OA Δ10D	+ PBS	2^3	2^{13}	2.0×10^4
	+ SA	2^4	2^{15}	1.6×10^5
	+ SA + I[b]	2^3	2^{13}	2.0×10^4
C18 dimer (Emery)	+ PBS	0	$2^{13.5}$	2.9×10^4
	+ SA	2^3	2^{16}	1.6×10^5
	+ SA + I[b]	0	2^{14}	4.0×10^4
C18 trimer (Emery)	+ PBS	0	$2^{14.5}$	5.8×10^4
	+ SA	2^3	2^{19}	1.3×10^6
	+ SA + I[b]	0	2^{15}	8.0×10^4

[a] Samples were fully neutralized with NaOH in PBS, then pH-adjusted to 7.4 and filtered through a 0.2 μm filter. The concentration of the dispersions was 0.5 mg/mL in PBS. 30 mU of SA were used for every 100 μL of packed rRBC and incubated at 37°C for 45 min.

[b] I: Sialidase inhibitor: 2,3-dehydro-2-deoxy-N-acetyl-neuraminic acid (10 mM).

Table X. The effects of cations on fatty acid mediated agglutination of rat erythrocytes (rRBC).

Medium[a]	Specific Titer			
	Oleic Acid	OA Δ10D	C18 Dimer (Emery)	C18 Trimer (Emery)
PBS-N_3	3.0×10^2	1.6×10^4	3.0×10^4	6.0×10^4
HEPES	3.0×10^2	1.6×10^4	1.5×10^4	3.5×10^4
1 mM EGTA + HEPES	3.0×10^2	1.5×10^4	1.0×10^4	2.1×10^4
10 mM EGTA + HEPES	3.0×10^2	1.7×10^4	1.0×10^4	2.1×10^4
1 mM $CaCl_2$ + HEPES	1.5×10^2	3.3×10^3	2.6×10^3	7.3×10^3
1 mM $MgCl_2$ + HEPES	7.7×10	1.7×10^3	2.6×10^3	7.3×10^3

[a] The concentration of fatty acid dispersions was ~ 0.5 mg/ml in HBS.

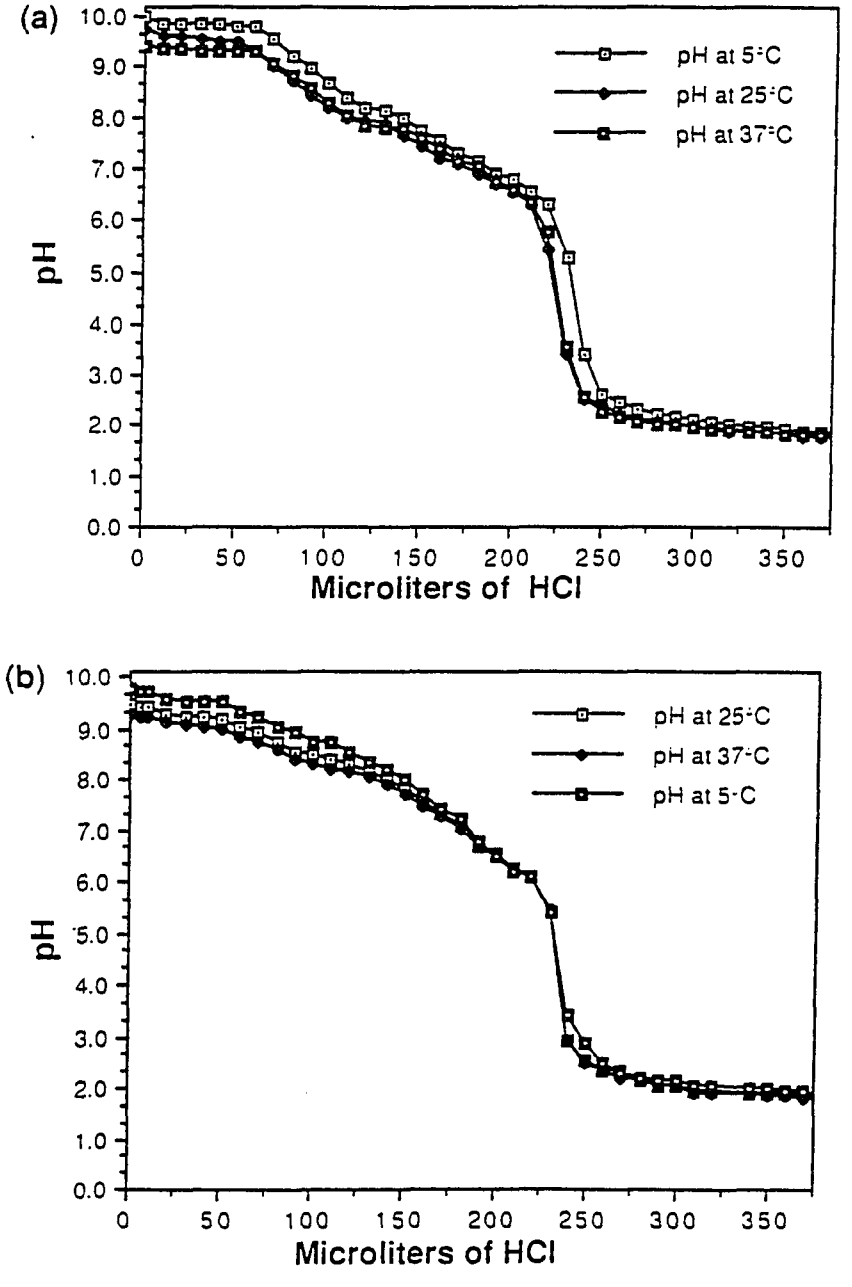
Table XI. Effect of pH on fatty acid mediated agglutination of rRBC[a].

	Specific titer			
pH	OA	OA Δ10D	C18 dimer (Emery)	C18 trimer (Emery)
6[b]	2.0×10^2	1.4×10^3	1.0×10^3	1.5×10^3
6.5[b]	2.0×10^2	3.9×10^3	4.0×10^3	5.7×10^3
7	2.1×10^2	2.1×10^4	1.2×10^4	1.1×10^4
7.5	2.4×10^2	3.0×10^4	2.1×10^4	2.3×10^4
8	2.4×10^2	4.5×10^4	4.6×10^4	6.4×10^4
8.5	4.8×10^2	4.5×10^4	4.6×10^4	9.0×10^4
9	5.0×10^2	4.3×10^4	5.8×10^4	9.4×10^4

[a] The concentration of the lipids in these dispersions was 0.5 mg/mL in PBS.

[b] The fatty acid dispersions were cloudy.

Figure 10 Equilibrium titration curves (a) for 0.08 M potassium oleate and (b) 0.04 M potassium C18 dimer (Emery) with added microliters of 0.94 N HCl at 5°, 25°, and 37°C.



from 7.1 to 6.3, and the sample contained crystal suspended in a clear liquid. Above 210 μL of HCl, pH values decreased from 6.3 to 2.1.

For the potassium salt of C18 dimer (Emery) at 5°C, between 0 and 5 μL of 0.94 N HCl, the pH decreased from 9.8 to 9.6, and the samples were clear. Between 5 and 135 μL of 0.94 N HCl, the pH dropped from 9.6 to 8.2, and the samples were clear with some crystals at the bottom. Between 165 and 275 μL of 0.94 N HCl, the pH dropped to 2.3, and the samples contained a white pasty material. The calculated pH at 1/4 neutralization point was 9.4, the 1/2 (midpoint) 8.3, 3/4 point 6.9 and the endpoint 3.2. The same system at 25°C and 37°C showed lower pH values at each partial neutralization point as the temperature increased. The 1/4 neutralization point at 25°C was calculated at pH 9.2 and 8.8 at 37°C; the midpoint was calculated at pH 8.3 and 8.2, respectively; the 3/4 at pH 6.7 and remained the same for both temperatures; and the endpoint at pH 3.0 and 2.8, respectively (Table XII).

A near infrared (NIR) study of OA at different temperatures and concentrations showed that OA in the liquid state associates to form dimers through hydrogen bonding between carboxyl groups [45]. The dissociation of dimers of OA into monomeric species in pure liquid was studied by NIR over a temperature range of 8.6-88.4°C [45]. An absorption band at 1445 nm, which is due to the free hydroxy group of the fatty acid molecules, appeared and its

Table XII. Neutralization end points of potassium oleate and C18 dimer (Emery).

Neutralization point	pH at °C			
	5°	25°	37°	
1/4	9.9	9.6	9.6	Potassium oleate
	9.4	9.2	8.8	Potassium salt of C18 dimer
1/2	8.3	8.2	8.1	Potassium oleate
	8.3	8.3	8.2	Potassium salt of C18 dimer
3/4	7.2	7.0	7.0	Potassium oleate
	6.9	6.7	6.7	Potassium salt of C18 dimer
4/4	3.5	3.2	3.1	Potassium oleate
	3.2	3.0	2.8	Potassium salt of C18 dimer

peak height increased with increasing temperature and was also dependent on the concentration. As the concentration decreased the dissociation of the acid dimers into the monomeric species increased. We performed the same experiment on the C18 dimer (Emery) in order to see if the hydrogen-bonding is intermolecular or intramolecular. The 1445 nm peak is expected to increase with dilution if hydrogen-bonding is intermolecular and stay the same if it is intramolecular. Of course, hydrogen-bonding may be intermolecular at high concentrations and intramolecular at low concentrations.

Figure 11 shows the variations of the near-infrared spectrum in the region of 1100-1700 nm of neat C18 dimer (Emery) with temperature. The absorption band at 1209 nm corresponds to the CH stretching mode for methylene groups. Bands at 1390, 1410 and 1430 nm are bands of the CH stretching mode with deformation modes of CH. The peak heights of these bandsgradually decreased with increasing temperature. This comes from the lowering of the density of the samples with increasing temperature. The intense absorption above 1600 nm is due to the stretching vibrations for hydrogen-bonded OH and/or CH in methyl and methylene groups. In order to clarify the temperature effect on the 1445-nm band, we obtained the difference spectra at 81°C and at 60°C by subtracting the spectrum at 25°C from each of them (Figure 12). With increasing temperature, a shoulder at 1445 nm is observed (Figure 12),

Figure 11. NIR of neat C18 dimer (Emery) at different temperatures. Arrows indicate the directions of intensity change of absorption bands with increasing temperature.

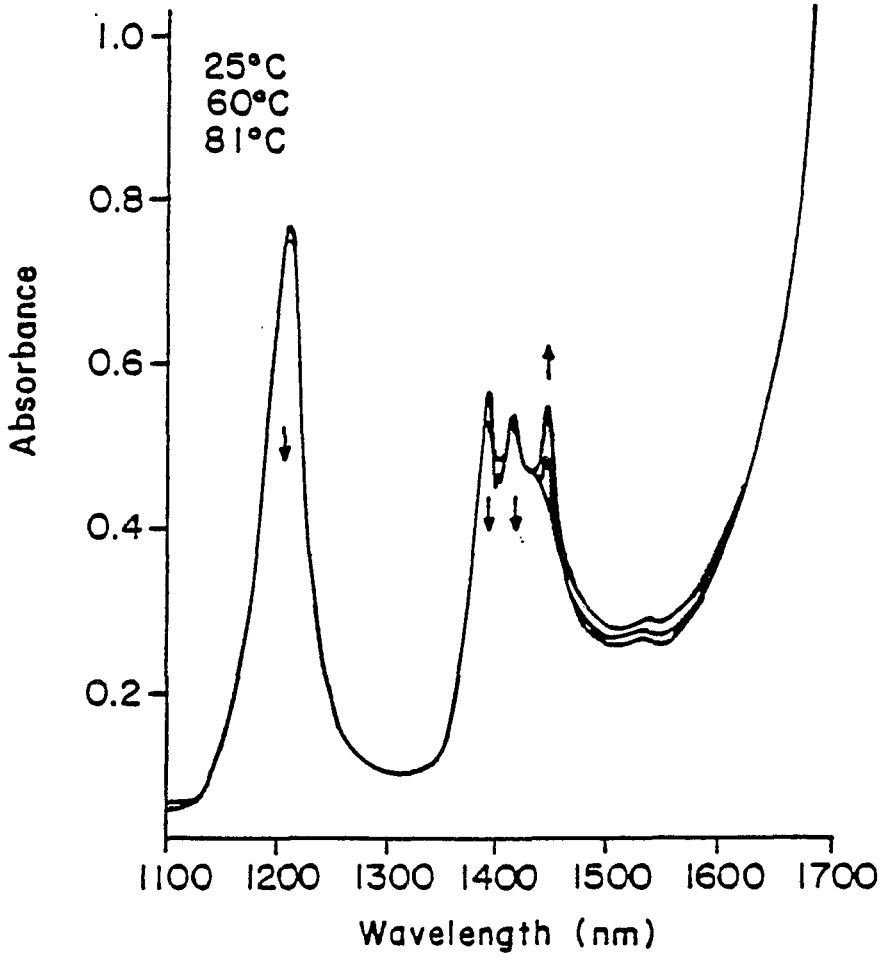
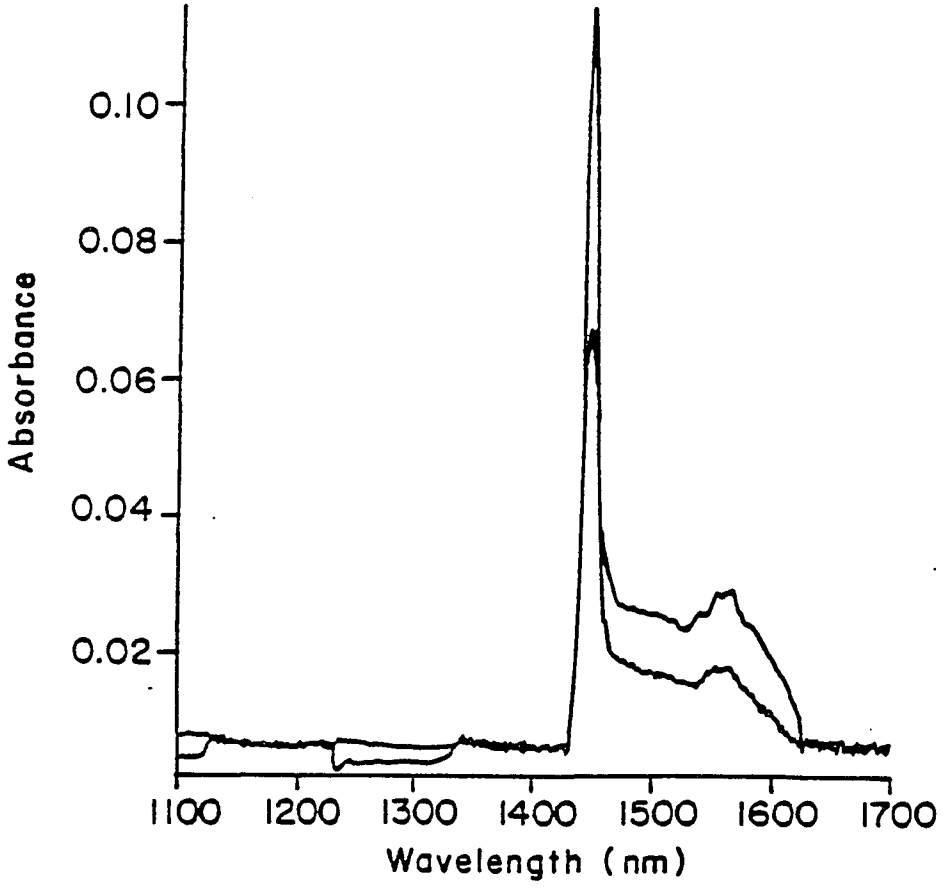


Figure 12. Difference spectra for neat C18 dimer (Emery) at 81°C (top) and 60°C (bottom). The bands attributable to the CH vibrations disappear from the spectra. Only the bands attributable to the OH stretching vibration become clear.



which clearly increases with increasing temperature. This band at 1445 nm corresponds to the free hydroxy group of the fatty acid carboxyl groups. A temperature dependence study of the absorbance at 1445 nm showed two break points at 23°C and 67°C. Break points were reported for OA as well [45]. (1) The first structure, for temperatures from the melting point to 30°C, was attributed to clusters in which the dimer molecules are aligned alternately to form a quasi-smectic liquid-crystal structure. (2) The second one, in the temperature range 30 and 55°C, is attributed to clusters having a less-ordered structure. (3) The third one (above 55°C) appears to be an isotropic liquid. It was concluded that the structure changes arise from the dissociation of the dimeric acid into monomeric species. For the C18 dimer, these break points, which are shown to be at different temperatures than those found with OA, can also be a result of three kinds of structure of the dimer acid, depending on the temperature.

The spectra of clear solutions of C18 dimer (Emery) in carbon tetrachloride, ranging in concentrations from 0.377 to 3.278 mol/L and from 0.520 to 0.127 mol/L, respectively are shown in Figures 13 and 14. In these figures the absorbance at 1445 nm increases clearly with decreasing concentration. This suggests that the hydroxy group of the carboxyl groups is not hydrogen-bonded and that the hydrogen-bonding between the carboxyl and carboxylate groups of the dimers is intermolecular rather than

Figure 13. NIR spectra of the C18 dimer (Emery) in CCl_4 at high concentrations: 3.278 mol/L, 2.375 mol/L, 1.354 mol/L, 0.682 mol/L and 0.377 mol/L. The pathlength of the cell was 1 cm; the temperature was 30°C.

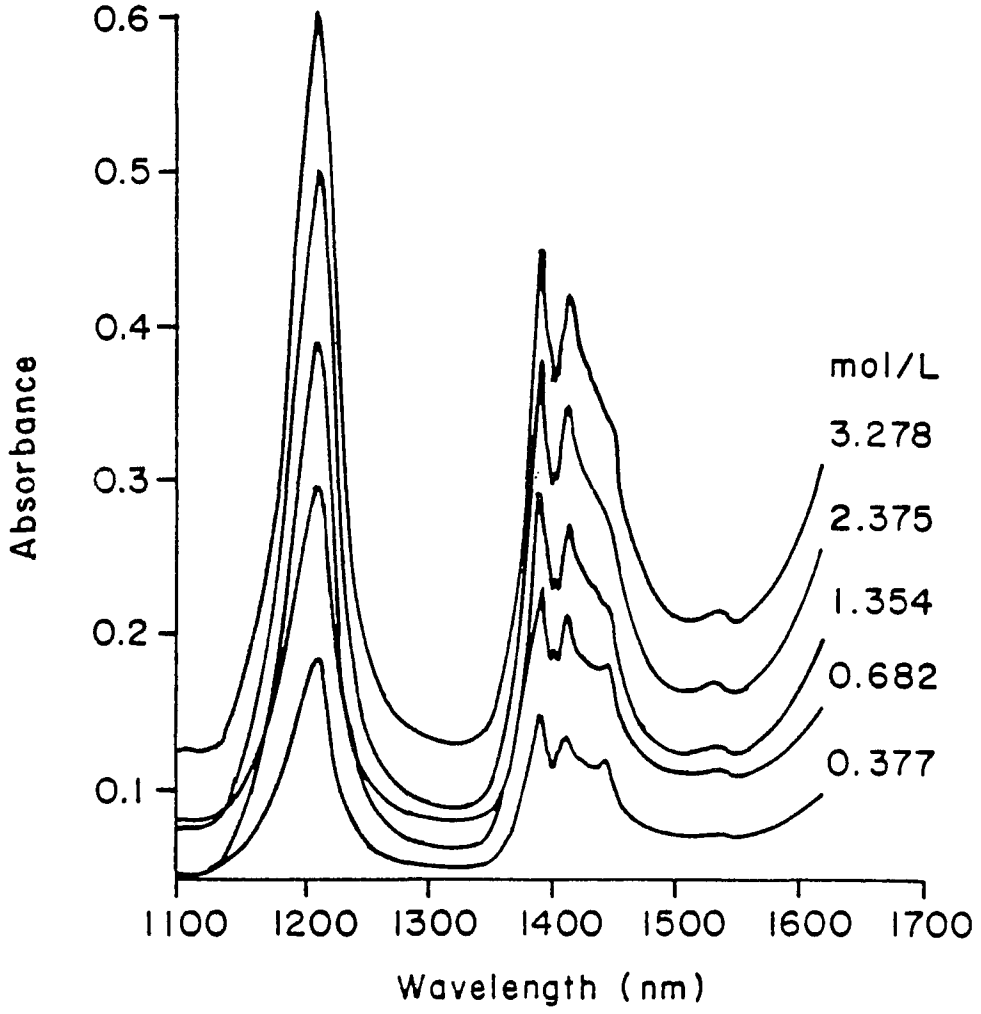
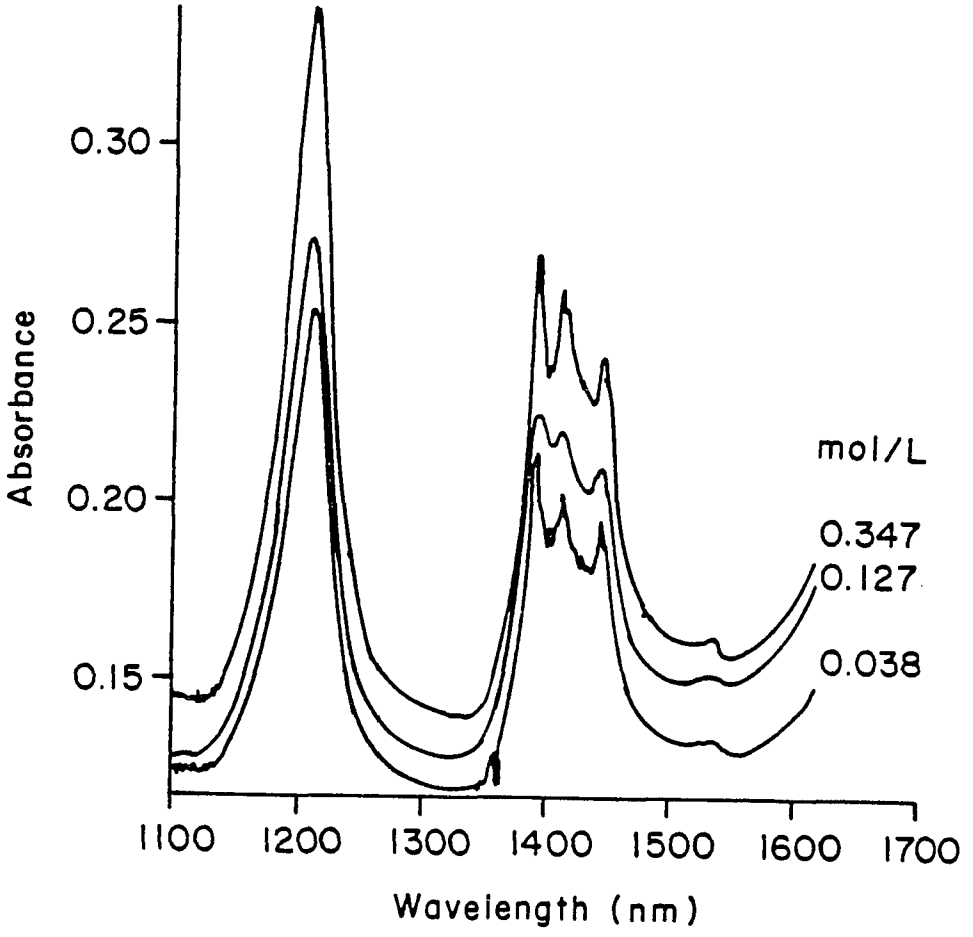


Figure 14. NIR spectra of the C18 dimer (Emery) in CCl_4 at low concentrations: 0.038 mol/L, 0.127 mol/L and 0.347 mol/L. The pathlength of the cell was 3 cm; the temperature was 30°C. It should be noticed that the 1445-nm band increases especially at low concentrations.



intramolecular because intramolecular hydrogen bonding should not be concentration dependent.

Light microscopy

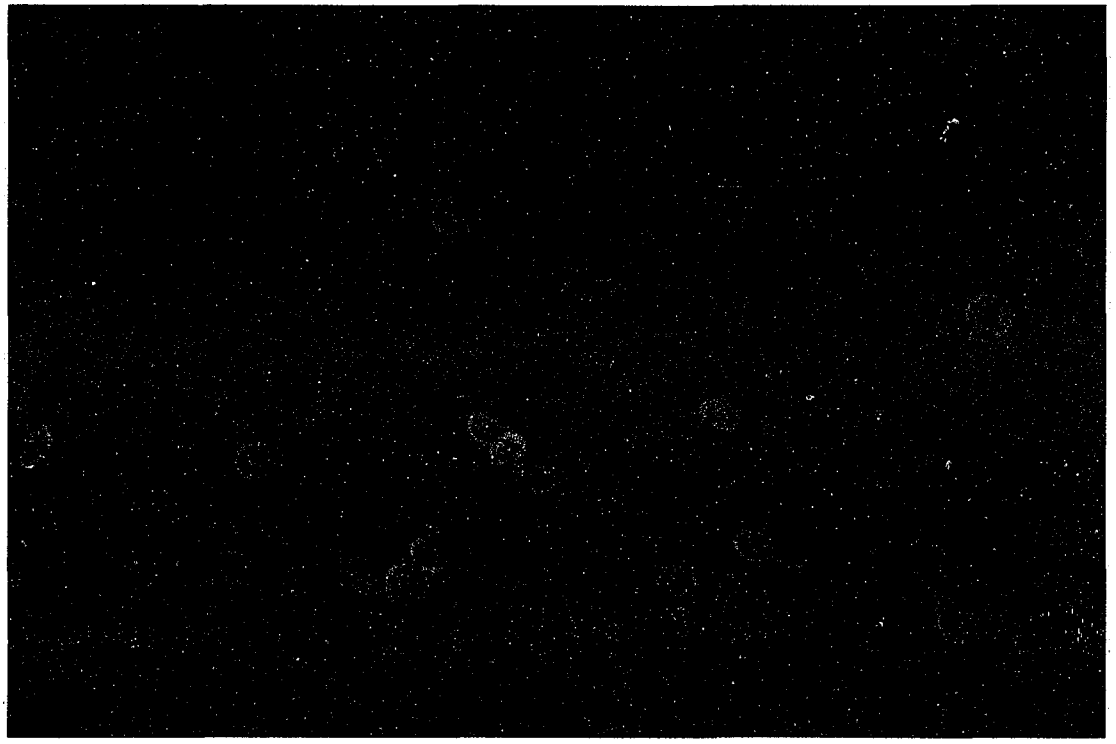
Figures 15 (a-e) shows rRBCs that were suspended in 3.8% citrate (control), and rRBCs which were mixed with OA, OA Δ10D, C18 dimer (Emery) and C18 trimer (Emery). The concentration of the samples were identical. As seen in Figure 15 (c-e), heated OA, C18 dimer and C18 trimer give similar agglutination patterns. Mixtures of chains and rosettes were observed, but no fusion or lysis. Figure 16 shows that OA alone in PBS appears as vesicles, including large multilamellar vesicles. The vesicles were not uniform in size and only some showed multilamellar structure. Vesicles were seen for OA Δ10D, C18 dimer (Emery) and C18 trimer (Emery) in PBS but they were very small and difficult to distinguish and thus photograph. Agglutination was found to be time-dependent and to involve cell clumping but not cell fusion.

Gel filtration

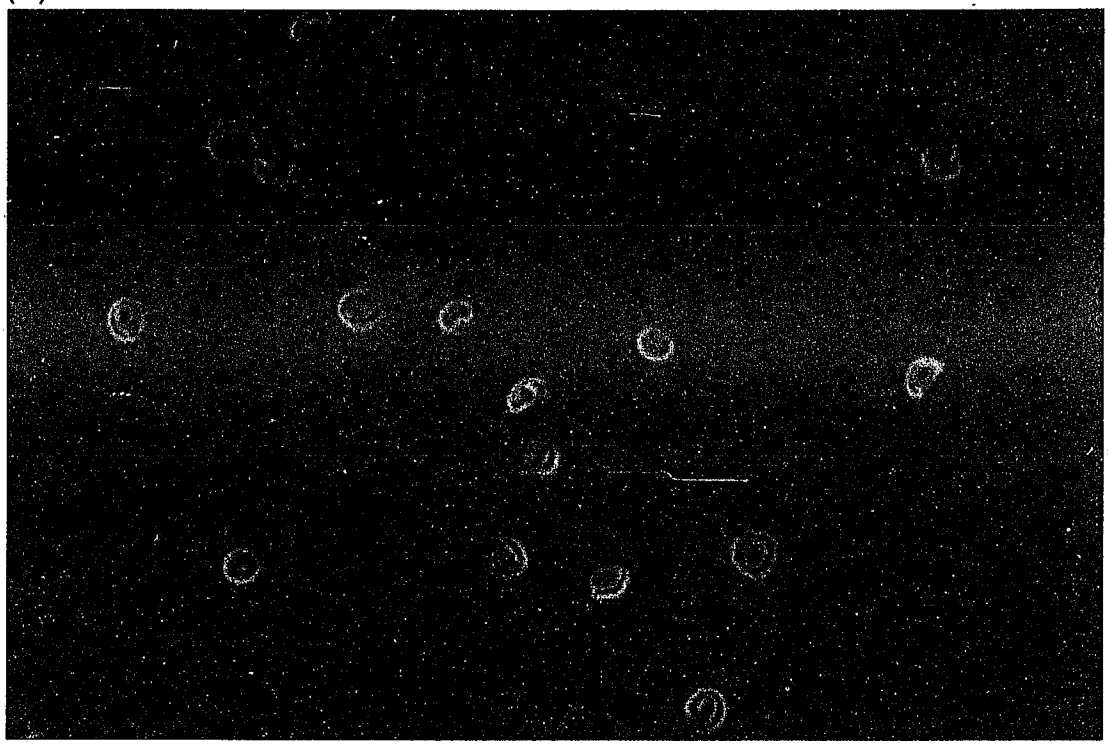
Figure 17 shows that the OA is eluted in tubes 7-10 and 14-21, dimer in tubes 14-19 and 24-39 and trimer in tubes 10-15, 20-34 and 35-58. Blue Dextran eluted in tube 6, thyroglobulin in tube 15 and ferritin in tube 16-17. Figure 17 also shows that OA was retarded least by the column. The C18 dimer (Emery) wasretarded less than the C18 trimer (Emery). This result suggests that the OA dispersion in PBS forms aggregates that are larger than the dispersion of dimers whose aggregates appear to

Figure 15. Light micrographs of rRBC in citrate buffer, pH 7.4. (a) no additions, (b) with OA (1.6 mg/mL), (c) with OA Δ10D (1.6 mg/mL), (d) with C18 dimer (Emery) (1.6 mg/mL), (e) with C18 trimer (Emery) (1.6 mg/mL).

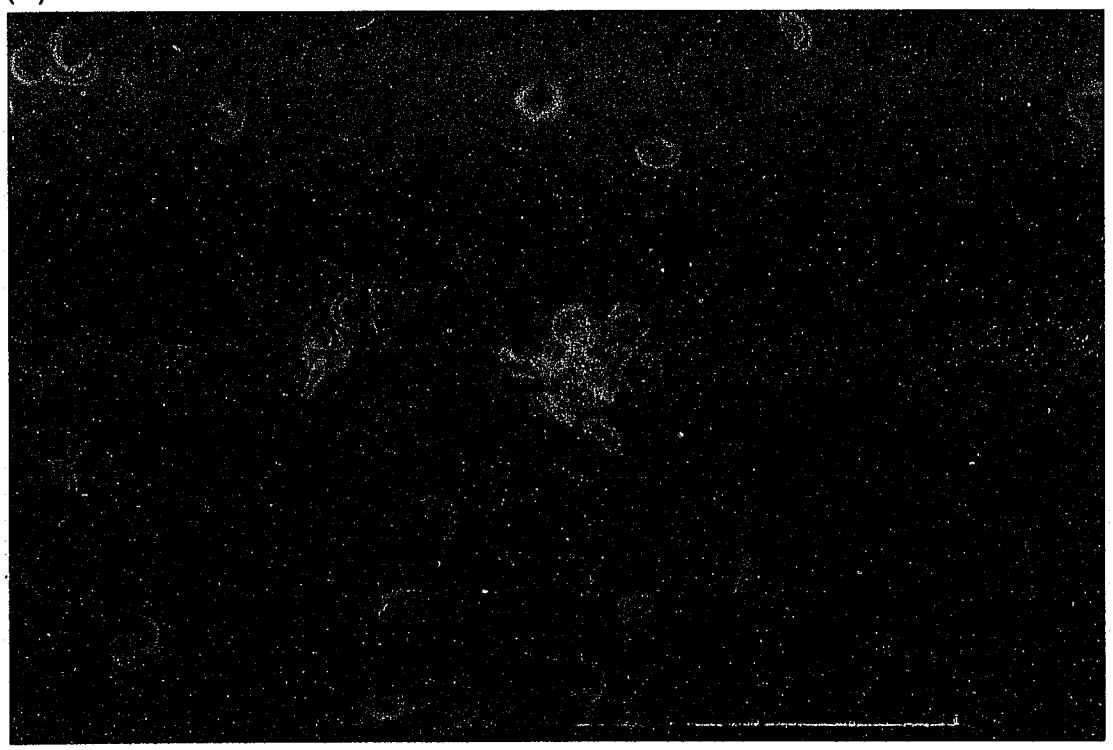
(a)



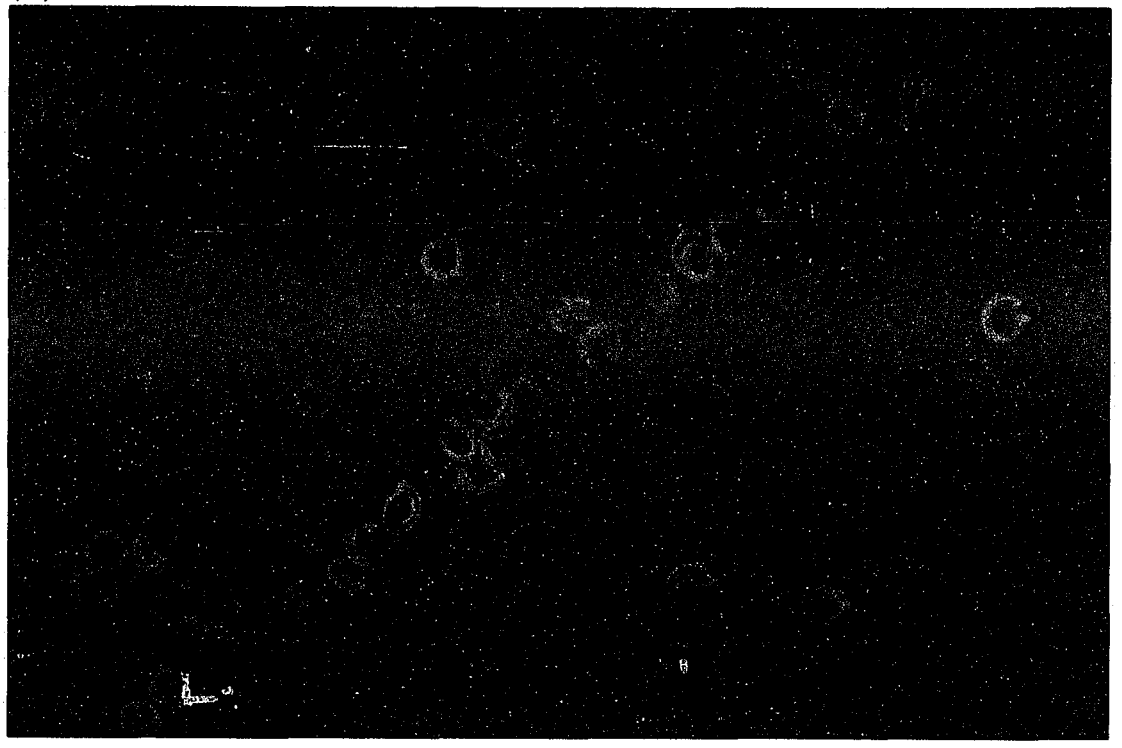
(b)



(c)



(d)



(e)

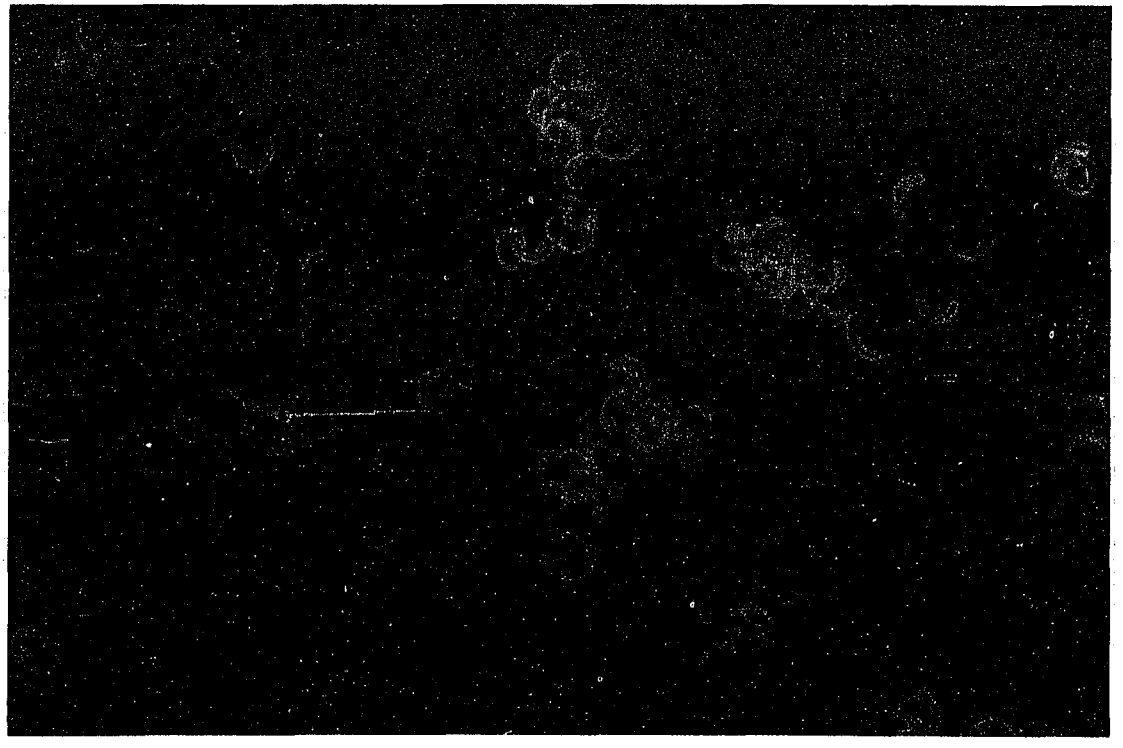


Figure 16. OA (1.0 mg/mL) multilamellar vesicles.

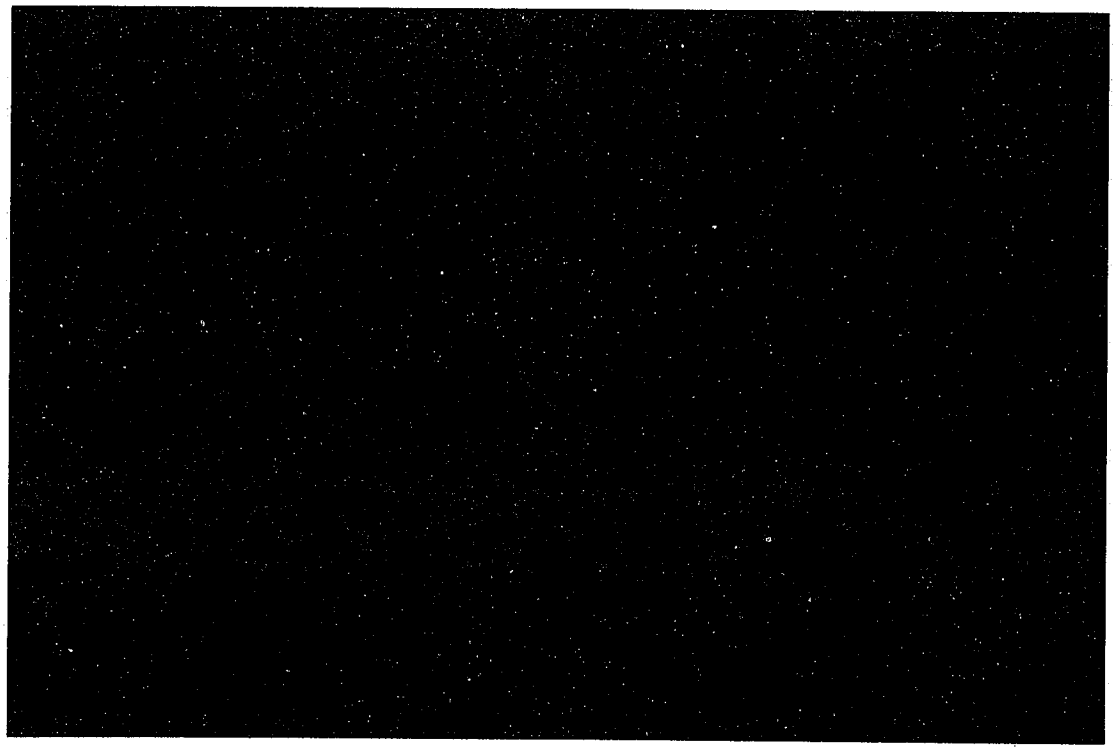
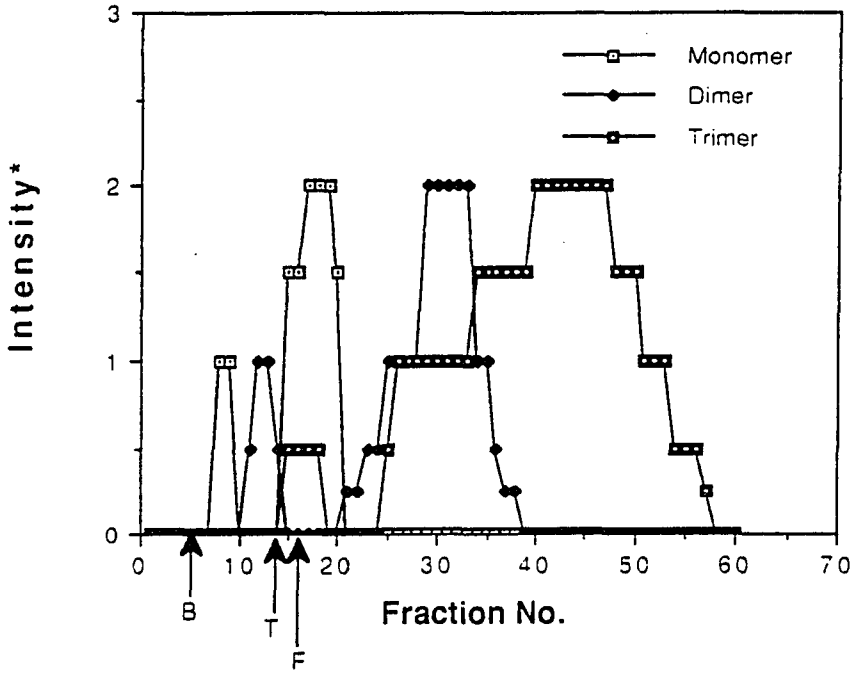


Figure 17. Gel filtration of OA, C18 dimer (Emery) and C18 trimer (Emery) on Sepharose CL-2B. The fractions were analyzed on silica gel TLC and visualized by spraying with 20% $(NH_4)_2SO_4$ and heating to 180°C for 1hr.



B: Blue Dextran (2000 kD)
T: Thyroglobulin (669 kD)
F: Ferritin (440 kD)

*Arbitrary units to indicate darkness of spots on TLC.

be larger than those for trimer. The spherical dimensions of the OA aggregates are shown to be at the same range as the globular dimensions of thyroglobulin and ferritin as well as the monomer parts of the dimer and trimer. The dimer part of the dimer and the dimer part of the trimer are shown to form smaller aggregates and even smaller aggregates with the trimer. This distribution of sizes are also confirmed by our preliminary electron microscopy studies which show mostly large multilamellar vesicles (MLVs) for OA and small unilamellar vesicles (SUVs) for dimers and trimers.

DISCUSSION and CONCLUSIONS

There are many studies about oxidation of oleic acid and other fatty acids. But *none have mentioned the hemagglutinating properties of their oxidation or thermal degradation products except for* Stone [31] and Tsivion and Sharon [32] who attributed the agglutination properties they observed to oleic acid rather than to its degradation or oxidation products.

We have shown that OA lyses red cells, but its thermal oxidation products agglutinate them. TLC and MS evidence suggested that oligomers are formed and that the highest titers for heated oleic acid are associated with dimeric, oligomeric, and polymeric acid fractions. HPLC and SGC did not give as good resolution as the TLC but the highest activity was again associated with the oligomeric and polymeric acid fractions. Although only a small amount of esters were detected by ^{13}C NMR spectroscopy, other relevant carbonyl compounds and epoxy compounds were tested, but the hemagglutination activity cannot be associated with these molecules since they showed mostly lysis or very low activity.

The *structural requirements* for developing activity on heating in air are the following: unsaturation or a hydroxy group and a long hydrocarbon chain. Oligomers of C18 and C22 fatty acids are good agglutinins before heating. We assumed that

thermal oxidation of the unsaturated fatty acid causes the formation of oligomers. After oligomeric formation the unsaturation does not play any role in the hemagglutination process since saturated oligomers are active with the same specific activity as the unsaturated oligomers.

The sufficiency of oligomeric structure for agglutinin activity was confirmed by the hemagglutination tests with a series of oleic acid-derived oligomers. OA dimer (a mixture of 3% monomer, 94% dimer, 3% trimer and traces of tetramers containing cyclic and acyclic structures) and trimer-polymer (a mixture of 2% monomer, 18% dimer, 60% trimer and 20% higher oligomers, containing cyclic and acyclic structures) were generously provided by the Emery Group (Henkel Corporation) and these were tested, as was a sample of "hydrogenated dimer" (a mixture of 54% cycloaliphatic, 37% linear and 9% aromatic C36 acids which had been hydrogenated) generously provided by Unichema International. The commercial dimer products and trimer products were resolved by TLC into monomer, dimer and trimer-polymer fractions. The separate bands were titered against rRBC. Dimer and trimer-polymer fractions are active before heating. A dimer of C22 acid from Unichema which is 95% C44 and largely acyclic gives a high titer without heating. The cyclic, long-chain dicarboxylic acid “C21 diacid”, 5(6)-carboxy-4-hexyl-2-cyclohexene-1-octanoic acid, a gift of Westvaco Corporation, which gives a very low titer and no lysis

before heating, becomes active after 10 days of heating. One possible reason for this change is that the extensive heating might cause the molecule to break into two unsaturated molecules which then can form a dimer or since it is unsaturated, the diacid itself may dimerize further.

The synthesis of oleic dimer by heating methyl oleate with di-tert-butyl peroxide to produce the active, acyclic "dehydro" dimer first characterized by Paschke et al. [56] as containing two double bonds (cis and/or trans) and carbon to carbon linkages at the 8, 9, 10 and 11 carbons gave us the opportunity to test a dimer with a more well-defined structure. The dimer fraction was purified by TLC on silica gel. Chemical ionization (CI) and electron impact (EI) mass spectroscopy confirmed the structure proposed for the dimer by Paschke et al. [56], who postulated a free radical mechanism for its formation. The free radical species couple randomly to form a dimer, resulting in 10 species of dimer. Oleate segments are joined equally at positions 8, 9, 10, and 11.

$$CH_3(CH_2)_6\text{-}\underset{\vdots}{CH}\text{-}CH{=}CH\text{-}\underset{\vdots}{CH}\text{-}(CH_2)_6COOCH_3$$
$$CH_3(CH_2)_6\text{-}CH\text{-}CH{=}CH\text{-}CH\text{-}(CH_2)_6COOCH_3$$

^{13}C and ^{1}H NMR spectroscopy also confirm the structures including the formation of approximately equal amounts of cis and trans double bonds. A small percentage of higher polymers formed during the synthesis of the linear dimers was isolated by

TLC. Mass spectroscopy indicated these were acyclic structures, mainly trimer and tetramer, resulting from *addition* of oleate to the dimer. Small amounts of mono and dihydroxy dimer dimethyl ester were also isolated by TLC for dioleate. The synthesized linear dimers and the commercial mixtures of linear and cyclic C18, C22 dimers and C18 trimers had the same titers. The trimer-oligomer fractions showed consistently higher titers. The specific activities for the hydroxy derivatives are a little lower than for the hydrocarbon acyclic oligomers. Dimerization or polymerization alone appears to be sufficient for agglutination activity

The dioleic dimethyl ester was hydrogenated to distearic dimethyl ester and the presence of the two double bonds was confirmed by the uptake of four hydrogen atoms per mole. ^{13}C NMR spectra of dimethyl dioleate and dimethyl distearate combined with the molecular masses indicate that the dimer is acyclic and the fragmentation patterns confirm the linkage sites. In the distearate, the signals at 27.4 ppm and 32.6 ppm (allylic methylenes, cis and trans, respectively) and at 130-134 ppm (olefinic carbons), which are found in the dioleate, are missing. The appearance of trans double bonds suggests that cis-trans equilibration is fairly complete during the life times of the free radical intermediates.

The species specificities of different active heated fatty acids suggest that the hemagglutination is specific for RBC

surface structure and fatty acid oligomer. Another possibility is that this RBC selectivity for agglutination might be due to RBC topography; an area on the RBC surface which allows one chain of the oligomer access into the bilayer of one RBC and the other chain into another RBC connecting the two.

In an attempt to characterize the nature of fatty acid-RBC interaction, sialic acid was removed from the RBC surface by sialidase (SA) treatment, thus lowering surface negative charge. This treatment increased the titer for all agglutinins tested, which may result from a decreased electrostatic repulsion between the cells and between the cells and fatty acid carboxylate groups. It appears that sialic acid residues are not involved in this process. Removal of Ca^{2+} with EGTA did not affect the titer but adding Ca^{2+} (1 mM) or Mg^{2+} (1 mM) lowered the specific titer. If cations were masking negative charges on the red cell surface, they should have the same effect as sialidase. Since they have the opposite effect, it might be supposed that the divalent cations are binding to the fatty acids carboxyl groups and thus altering some role which they may play in the agglutination process. One possibility is that if the divalent cations are binding to the fatty acid carboxyl groups they may restrict some favorable conformation of the oligomers that is ideal for agglutination or destroy an aggregate which is involved. This restriction might be due to the size (radius) of the cations. The basis for species specificity may be net surface

charge or another factor not obvious at this time. The specific titer of dimer against rat RBC, which increases with pH in the range 6-9, suggests that the active form may be the salt form or the acid salt form. However, since red cells are unstable above pH 9, titers can only be measured in the pH range where an appreciable amount of COOH is present. In saline at pH 8, OA would be about 50% protonated [39, 40, 75] in bulk medium. pH titrations show that at pH 9 the dimer is still 25% protonated and at pH 8 it is 50% protonated. At these pH values Cistola et al. [40] reported a two-phase region containing a lamellar fatty acid/soap phase in an aqueous phase between pH 7 and 9, and a three-phase region containing lamellar fatty acid-soap, micellar, and aqueous phases at pH 9.

Light microscopy showed that OA derived hemagglutinins showed no fusion or lysis of rRBC. Large multilamellar vesicles (LMVs) are formed by OA under our chosen conditions of medium and pH where OA and the other acids would be acid salts [39, 40, 75]. Such vesicles are not observed for the fatty acid oligomers which are hemagglutinins; also gel filtration showed that the oligomers appear to form SUVs. This was confirmed by our initial gel filtration experiment on Sepharose, where we observed that 1 mg/mL OA in saline at pH 8.5 is retarded least and trimer is retarded most. This suggests that the agglutinins form smaller aggregates which may account for their biological behavior.

Using low angle X-ray diffraction Hamilton and coworkers [39, 40] have provided evidence for a lamellar structure for OA at pH 7-9, which we confirmed by our photomicrographs. The hydrocarbon side chains could be "branches" of a backbone.

Space-filling models of a linear dimer were made and various conformations were visualized. The long hydrophobic chains of fatty acid dimers and polymers can link red cells by inserting into the membranes. Another possibility is that oligomers or their aggregates may insert entirely into the membrane and so interact with the endogenous red cell components as to induce local phase changes which favor agglutination [76].

Fourier transform near infrared (FTNIR) studies on dimers in organic solvents, using procedures similar to Iwahashi and coworkers [45], suggest that hydrogen-bonding between the carboxyl and carboxylate groups of the dimers is intermolecular rather than intramolecular even though the model is not strictly applicable. Intramolecular hydrogen-bonding is a possibility which would favor structures with both COOH groups on one side of the molecule.

Fatty acids, depending on size and saturation, can affect the permeability of cell membranes and alter the phase transitions of synthetic vesicles in various ways [77]. It is presumed that the hydrocarbon moiety inserts into the membrane and that the carboxyl group interacts with the phospholipid head-group. Hamilton and coworkers [private communication] have found that

C18 dimer (Emery) can insert into preformed phospholipid vesicles and "flip-flop" or equilibrate to both halves of the bilayer within seconds but slower than monomeric oleic acid. The agglutination which we observe may be due to insertion of part or all of the fatty acid oligomers into red cell membranes but in a manner which links red cells rather than perturbing membrane permeability properties [78]. The oligomeric acids may cause local lipid phase changes that facilitate agglutination. Whether the species specificities which have been observed involve discrimination in insertion into membranes or a difference in some later process is not known at this time.

BIBLIOGRAPHY

1. Lehninger, A. L. 1982. Principles of Biochemistry. Worth Publishers, New York, pp. 324-325.
2. Boyd, W. C. and Shapleigh, E. 1954. Science 119, 419.
3. Sharon, N. and Lis, H. 1972. Science 177, 949-959.
4. Goldstein, I. J. and Hayes, C. E. 1978. In: *Advances in Carbohydrate Chemistry and Biochemistry* (Tipson, R. S. and Horton D., eds). Vol. 35, 127-340. Academic Press, New York.
5. Sharon, N. 1977. Lectins. Scientific American 236, 2-12.
6. Stein, E. A. and Cooper, E. L. 1982. In: *Developmental Immunology : Clinical Problems and Aging* (Cooper, E. L. and Brazier, M. A. B., eds.). pp.85-98. Academic Press, New York.
7. Hall, J. L. and Rowlands, D. T., Jr. 1974. Biochemistry 13, 821-827.
8. Hall, J. L. and Rowlands, D. T., Jr. 1974. Biochemistry 13, 828-832.
9. Cohen, E. and Uhlenbruck, G. 1974. Ann. N. Y. Acad. Sci. 234, 23-50.
10. Yeaton, R. W. 1981. Dev. Comp. Immunol. 5, 535-545.
11. Yeaton, R. W. 1982. In *Developmental Immunology: Clinical Problems and Aging* (Cooper, E. L. and Blazier, M. A. B. eds). pp. 73-83. Academic Press, New York.

12. Cooper, E. L., Stein, E. A. and Wajdani, A. 1984. In : *Recognition Proteins, Receptors and Probes* (Cohen, E. ed.). pp. 43-54. Alan, R. Liss, New York.

13. Marchalonis, I. J. and Edelman, G. M. 1968. J. Mol. Biol. 32, 453-565.

14. Finstad, C. L., Litman, G. W., Finstad, L. and Bood, R. A. 1972. J. Immunol. 108, 1704-1711.

15. Acton, R. T., Bennett, J. C., Evans, E. E. and Schrohenloher, R. E. 1969. J. Biol. Chem. 244, 4128-4135.

16. McDade, J. E. and Tripp, M. R. 1967. J. Invertebr. Pathol. 9, 523-530.

17. Acton, R. T., Weinheimer, P. F. and Niedermeier, W. 1973. Comp. Biochem. Physiol. 44B, 185-189.

18. Baldo, B. A. and Uhlenbruck, G. 1975. FEBS Lett. 55, 25-29.

19. Baldo, B. A. and Uhlenbruck, G. 1975. Carbohydr. Res. 40, 143-151.

20. Acton, R. T. and Weinheimer, P. F. 1974. In *Contemporatory Topics in Immunology* (Cooper, E. L., ed.) Vol 4, 271-282. Plenum Press, New York.

21. Pauley, G. B., Granger, G. A. and Krassner, S. N. 1971. J. Invertebr. Pathol. 18, 207-218.

22. Garte, S. J. and Russell, C. S. 1976. Biochim. Biophys. Acta 439, 368-379.

23. Hammarstrom, S. and Kabat, E. A. 1969. Biochemistry 8, 2696-2705.

24. Ishoyama, I., Dietz, E., and Uhlenbruck, G. 1973. Biochem. Physiol. 44B, 529-547.

25. Bretting, H. and Kabat, E. A. 1976. Biochemistry 15, 3228-3236.

26. Bretting, H., Kalthoff, H. and Fer, S. 1978. J. Invertebr. Pathol. 322, 151-157.

27. Fuke, M. T. and Sugai, T. 1972. Biol. Bull. (Woods Hole, MA) 143, 140-149.

28. Russell, C. S., Rodriguez J. and Lai, P.-S. 1983. Comp. Biochem. Physiol. 75A, 57-64.

29. Russell, C. S. and Lai, P.-S. 1986. Ann. N. Y. Acad. Sci. 463, 131-133.

30. Lai, P.-S., So, L.-P. and Russell, C. S. 1989. Comp. Biochem. Physiol. 93B, 859-865.

31. Stone, J. D. 1946. Austr. J. Exp. Biol. and Med. Sci. 24, 197-205.

32. Tsivion, Y. and Sharon, N. 1981. Biochim. Biophys. Acta 642, 336-344.

33. Kawai, Y., Moribayashi, A., and Yano, I. 1982. J. Bacteriol. 153, 907-910.

34. Kawai, Y. and Yano, I. 1983. Eur. J. Biochem. 136, 531-538.

35. Kawai, Y. 1982. FEBS Lett. 153, 131-133.

36. Forbes, J., Zaleswki, P. D. and Valente, L. 1984. Biochim. Biophys. Acta 732, 179-185.

37. Lai, P.-S., So, L.-P. and Russell, C. S. 1989. Comp. Biochem. Physiol. 93B, 859-865.

38. Hiroshi, M. and Yoshihiko, E. 1992. J. Phys. Chem. 96, 10487-10491.

39. Cistola, P. D., Atkinson, D., Hamilton A. J. and Small, D. M. 1986. Biochemistry, 25, 2804-2812.

40. Cistola, P. D., Hamilton, A. J., Jackson, D. and Small, D. M. 1988. Biochemistry, 27, 1881-1888.

41. Haines, T. H. 1983. Proc. Natl. Acad. Sci. USA, 80, 160-164.

42. Westheimer, F. H. and Benfey, O. T. 1956. J. Am. Chem. Soc. 78, 5309-5311.

43. Smith, R. and Tanford, C. 1973. Proc. Natl. Acad. Sci. USA. 70, 289-293.

44. Hamilton, A. J., and Cistola, P. D. 1986. Proc. Natl. Acad. Sci. USA. 83, 82-86.

45. Iwahashi, M., Hachiya, N., Hayashi, Y. and Matsuzawa, H. 1993. J. Phys. Chem. 97, 3129-3133.

46. Goebel, C. G. September 27, 1949. U. S. Patent (to Emery Industries, Inc.). 2,483,761.

47. Goebel, C. G. December 29, 1953. U. S. Patent (to Emery Industries, Inc.). 2,664,429.

48. Myers, L. D., Goebel C. G. and Barrett F. O. October 4, 1960. U. S. Patent (to Emery Industries, Inc.). 3,076,003.

49. Fischer, E. M. November 19, 1968. U. S. Patent (to General Mills, Inc.). 3,412,039.

50. Goebel, C. G. August 13, 1963. U. S. Patent (to Emery Industries, Inc.). 3,100,784.

51. Leonard, E. C., ed. 1975. *The Dimer Acids.* Humko Sheffield Chemical, Memphis, Tenn., pp 1-112.

52. McMahon, D. H. and Crowell, E. P. 1974. J. Am. Oil Chem. Soc. 51, 522-527.

53. Leonard, E. C., ed. 1979. *Dimer Acids.* Humko Sheffield Chemical, reprinted from Kirk-Othmer: Encyclopedia of Chemical Technology, third edition, 768-782.

54. Sen Gupta, A. K. 1961. Fette, Seifen, Anstrichm., 69, 907.

55. Den Otter, M. J. A. M. 1970. Fette, Seifen, Anstrichm., 72, 667.

56. Paschke, R. F., Peterson L. E., Harrison, S. A. and Wheeler, D. H. 1964. J. Am. Oil Chem. Soc. 41, 56-60.

57. Frankel, E. N. 1962. In *Symposium on Foods: Lipids and Their Oxidation* (Schultz, H. W., Day, E. A. and Sinnhuber, R. O., eds.). Avi Publishing Co., Westport, CN. p. 51.

58. Kagan, V. 1988. *Lipid Peroxidation in Biomembranes,* CRC Press, Boca Raton, Florida.

59. Brill, W. F. 1965. J. Am. Chem. Soc. 87, 3286-3287.

60. Walling, C. and Thaller, W. 1961. J. Am. Chem. Soc. 83, 3877-3884.

61. Kochi, J. K. and Krusic, P. J. 1968. J. Am. Chem. Soc. 90, 7157-7159.

62. Esterbauer, H. and Cheeseman, K. H., Eds. 1987. Chem. Phys. Lipids 44, Nos.2-4.

63. Marquez-Ruiz, G., Perez-Camino, M. C. and Dobarganes, M. C. 1992. J. Am. Oil. Chem. Soc. 69, 930-934.

64. Bottino, N. R. 1962. J. Am. Oil. Chem. Soc. 39, 25-31

65. Friedman, L., Horwitz, W., Shue, G. M. and Firestone, D. 1961. J. Nutr. 73, 85-89.

66. Dixon, J. L. and Ginsberg, H. N. 1990. J. Biol. Chem. 286, 5080-5086.

67. Tronchere, H., Terce, F., Record, M., Ribbes, G. and Chap, H. 1991. Biochem. Biophys. Res. Commun. 176, 157-165.

68. Diplock, A. T., Balasubramanian, K. A., Manohar, M., Mathan, V. I. and Ashton D. 1988. Biochim. Biophys. Acta 962, 42-50.

69. Shinomura, T., Asaoka, Y., Oka, M. and Yoshida, K. 1991. Proc. Natl. Acad. Sci. USA 88, 5149-5153.

70. Casabiell, N., Pandiella, A. and Casanueva, F. 1991. Biochem. J. 278, 679-687.

71. Xesus, C., Jose, L. Z., Celia, M. P., Atanasio, P. and Lelipe, F. C. 1993. Experimental Cell Research. 205, 365-373.

72. M. Kates. 1986. *Techniques in Lipidology*, North Holland/American Elsevier, New York. Second Edition.

73. M. Kates. 1972. *Techniques in Lipidology*, North Holland/American Elsevier, New York.

74. Muller, E., Schroder, C. and Schauer, R. 1983. Hoppe-Seyler's Z. Physiol. Chem. 364, 1419-1429.

75. Vorum, H., Brodersen, R., Kragh-Hansen, U. and Pedersen, A. O. 1992. Biochim. Biophys. Acta 1126, 135-142.

76. Ruthven, N. A. H. L. and McElhaney, R. N. 1992. In *The Structure of Biological Membranes.* E. Yeagle, P., ed., CRC Press, Boca Raton, FL, pp. 127-128.

77. Lamba, Om P., Lal, S., Yappert, M. C., Lou, M. F. and Borcham, D. 1991. Biochim. Biophys. Acta 1081, 181-187.

78. Patrikios, S. I., Britton, O'N., Bing, D. K. and Russell, C. S. 1994. Biochim. Biophys. Acta. 1212, 225-234.