Bile Acids and Lipids

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The staging of cholesterol gallstones with respect to nucleation and growth

D. M. SMALL

Crystalline cholesterol monohydrate\(^1\) is a major constituent of gallstones and stones formed from cholesterol dissolved in bile\(^2\), therefore cholesterol monohydrate crystallization is a prerequisite to cholesterol gallstone formation. Five stages leading to the formation of symptomatic cholesterol gallstone disease have been proposed\(^3\).\(^-\).\(^4\), and each stage is a prerequisite for the next (see Figure 1). The first is a genetic–metabolic stage, in which the genetic propensity or metabolic abnormality which can lead to production of supersaturated bile is present, but not yet manifest. The second stage is a chemical stage in which gallbladder bile becomes supersaturated with cholesterol. This stage is diagnosed by measuring the chemical composition of bile and calculating cholesterol saturation\(^5\). The third stage is a physical stage in which the supersaturated bile is nucleated and growth of cholesterol monohydrate crystals is initiated. This stage is diagnosed by observing

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Estimated mean time before progressing to next stage: ~10 yrs or longer, days–months, 5–10 yrs, or never (silent stones)

**Figure 1** The stages of cholesterol gallstone formation\(^3\).\(^4\). The mean estimations of the times during which each stage exists before progressing to the next stage are given below. Thus, on the average about 10 years are required for the formation of stones from supersaturated bile (i.e. between stages 2 and 4), and approximately 10 years between stages 4 and 5. About half the people probably never develop stage 5. Stage 3 is the shortest of all stages, and probably only exists for days or weeks.

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microscopic crystals of cholesterol monohydrate in fresh bile on subjects who do not yet have stones (see Figure 2). In stage four, microscopic crystals grow or agglomerate into macroscopic stones, and in the fifth stage, macroscopic stones cause symptoms by initiating cholecystitis and/or blocking of the cystic or common duct.

An illustration of these stages comes from studies of American Indians, in particular the Pima Indians. About 70% of Pima women will ultimately develop gallstones, thus a large proportion of them have a predisposition to gallstones\textsuperscript{6}. The chemical stage appears at around the time of puberty or by age 15, and by age 18 many young Indian women have supersaturated duodenal bile\textsuperscript{7,8}, but do not yet have stones\textsuperscript{6}. However, by age 25, more than half of these women will have developed gallstones large enough to be found by cholecystography\textsuperscript{4}. This suggests the mean time between the development of stage 2 and 4 is about 10 years. Finally, about half of such patients will ultimately develop symptoms, on average some 10 years after the initial appearance of stones\textsuperscript{2-4}. Thus, the chemical stage can apparently exist for several years before stones are found. Since cholesterol crystals are rarely reported without stones, the duration of the physical stage (nucleation and precipitation of cholesterol crystals) must be a rather short stage leading quickly to the formation of macroscopic stones. The specific details of each of these sequential events are not known, but answers will be obtained by following these events in patients who have undergone stone dissolution with bile acid therapy and are followed by duodenal drainage and ultrasonography, for recurrence of stages 2, 3 and 4.

Certain people without gallstones have supersaturated bile without crystals. In fact, some populations (e.g. American Indians, Scandinavians), have a high prevalence of supersaturated bile in subjects without stones. However, it appears that populations with highly supersaturated bile are at risk for gallstone formation, since the overall prevalence of gallstone disease is
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related to the supersaturation of bile in subjects without gallstones\textsuperscript{9}. The greater the supersaturation of bile, the greater the prevalence of gallstones\textsuperscript{9} (see Figure 3). Many years ago we noted that hepatic bile from monkeys with interrupted enterohepatic circulation could be quite supersaturated\textsuperscript{10}, yet not precipitate its cholesterol readily. Recently it has been noted that highly supersaturated bile from gallstone patients did not precipitate crystals even after 48 h\textsuperscript{11}. This raises the question of how cholesterol precipitates from bile to form crystals and stones. That is, what really happens during the physical stage of gallstone formation (nucleation and growth)?

Crystallization is similar whether it is from an undercooled melt, or a supersaturated solution; nucleation comes first and crystal growth follows\textsuperscript{12,13}.

![Graph](image)

**Figure 3** Percentage saturation of control gallbladder bile (according to Admirand and Small)\textsuperscript{2} vs. estimated prevalence of gallstones in different populations. Control biles having lowest percentage cholesterol saturation appear to come from populations with lowest prevalence of cholesterol gallstones, while populations with supersaturated control biles have very high prevalences of cholesterol gallstones. Several populations with intermediate saturation (i.e. 65–75%) have intermediate prevalences of cholesterol stones as estimated from post mortem, hospital admission and cholecystogram evidence of gallstones (from Small, ref. 9). N.A. = North America.

HOMOGENEOUS NUCLEATION

In either an undercooled or supersaturated liquid, small clusters of molecules are continuously forming and breaking up (see Figure 4, top, from ref. 14). A critical cluster size must be reached so that the rate of breaking up is less than the rate of formation. The critical size depends on the system, but is of the order of a few tens to a few hundred molecules. The probability of forming such an aggregate is greater the greater the supersaturation, or the greater the undercooling. Marked supersaturation and undercooling occur in many systems before spontaneous or homogeneous nucleation occur. In fact, the
ratio between the true freezing point and the temperature of homogeneous nucleation in \(^\circ\)K is equal to about 0.8 for many substances\(^{12}\). Thus, pure water may be undercooled some 50 \(^\circ\)C below its true melting point before it crystallizes. The process of crystallization from supersaturated solutions is similar. Very high degrees of supersaturation are reached (in some cases

![Diagram of Homogeneous Nucleation](image1)

![Diagram of Heterogeneous Nucleation](image2)

**Figure 4** Diagrammatic illustrations of the processes of nucleation and crystal growth. Above, homogeneous nucleation; below, heterogeneous nucleation. On the left, cholesterol molecules in solution above their true saturation point are in constant rapid equilibrium with small clusters whose rate of formation is equal to their rate of disintegration. Proceeding towards the right, as supersaturation becomes higher a level is reached at which a critical aggregate size is formed and the molecules align in a minute crystal. This critical size contains tens to hundreds of molecules; once formed, spontaneous growth of all such nuclei occurs, rapidly producing a large number of small crystals. Growth of cholesterol monohydrate, shown on the right, is most rapid along the two faces in which molecules come together side by side, rather than end to end, thus producing the flat parallelogram-shaped crystals seen in Figure 2. Below illustrates heterogeneous nucleation. A relatively large particle (exogenous nucleus) having a surface which has a similar lattice allows the cholesterol molecules to adsorb, align and crystallize at a concentration lower than the maximum supersaturation level found with homogeneous nucleation shown above. Anti-nucleating agents can disturb the formation of critical-sized nuclei, adsorb to exogenous nuclei, and anti-growth agents can adsorb to small crystals to prevent or retard further growth (from ref. 15, with permission)

many times greater than true saturation) before homogeneous nucleation takes place\(^{1,3}\). The characteristics of homogeneous nucleation are: the absence of contaminating foreign nuclei which permit a very high supersaturation, the spontaneous formation of many critical-sized clusters at the maximum supersaturation, and the rapid precipitation of massive numbers of small crystals (> \(10^6/\text{ml}\)).
HETEROGENEOUS NUCLEATION

Nucleation of a less supersaturated solution can be brought about by adding particular nucleating agents such as a small amount of the same crystalline material or certain foreign substances; this process is called heterogeneous nucleation (see Figure 4, bottom). It is easy to understand nucleation if the nuclei added are only small crystals of the same material. These crystals act as centres of growth. In fact, the number of larger crystals formed is essentially equal to the number of small crystal nuclei added. They are effective nuclei which permit crystallization at very low levels of supersaturation. However, many foreign substances will also act as nuclei. For instance, small amounts of crystalline platinum are very effective nucleating agents for crystallization of tin, virtually preventing undercooling\(^1\). It is well known that dust can nucleate supersaturated salt solutions. However, the situation in which a

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**Figure 5** Heterogeneous nucleation from identical nuclei and variable nuclei. A number of precipitate particles are represented on the vertical axis, and the initial concentration of the precipitating species on the horizontal axis. \(S_a\) represents the true absolute solubility of the precipitating species, and \(S^*\) represents the concentration at which homogeneous nucleation occurs. Heterogeneous nucleation may occur between these two concentrations. If \(n\) identical nuclei are introduced into the solution, then provided the supersaturation reaches the critical level \(S_b\), \(n\) particles of precipitate will form around each nuclei. The number of particles is constant over the nucleating range (b). The nucleation depends upon the degree of supersaturation, but not on the number of particles. If variable nuclei, each with different capacity to nucleate, are added (curve a), then those with the greatest capacity to nucleate will do so at very low supersaturations, i.e. at point \(S_a\), and those which are less efficient at nucleation will nucleate at higher levels of supersaturation; thus the number of particles will vary with the concentration at which the precipitating species is introduced (along curve a). (Adapted from ref. 13, with permission)
single species of identical nuclei is used and that in which variable nuclei (such as dust) are used produce different results (see Figure 5).

The effectiveness of nucleation of foreign substances varies. Some nucleate nearly as effectively as native crystals, that is, at low supersaturation, while others nucleate only at higher levels of supersaturation. In part, the effectiveness depends on the ‘lattice match’. That is, if the molecular surface of the foreign material is highly matched to the crystal of the material to be precipitated, molecules will adsorb at low levels of supersaturation, align along the similar lattice and crystallize at the surface. The greater the lattice mismatch, the higher the level of supersaturation necessary to indicate nucleation. The characteristics of heterogeneous nucleation are: requires particles of considerable size (100–1000 Å dia.), always occurs at supersaturation levels less than that causing homogeneous nucleation, the number of crystals formed is about equal to the number of nuclei (but see Figure 3), and the effectiveness of foreign nuclei is related to their molecular structure.

To complicate the problem there are also molecules which may act as anti-nucleating agents even in small concentrations. In the absence of heterogeneous nucleation these molecules in some way prevent critical-sized nuclei from forming, and thus extend maximum supersaturation. They may also interact with potential nucleating agents binding to the active surface and thus prevent or retard heterogeneous nucleation. Small peptides and proteins are putative anti-nucleating agents for certain inorganic crystallization.

**CRYSTAL GROWTH**

Once nucleation occurs crystal growth follows. The rate at which crystals grow depends on a number of factors within a given system; the following promote growth:

(a) a large area of surface available for growth;
(b) a rapid movement or diffusion of molecules to the surface;
(c) a facile re-orientation at the surface;
(d) a large driving energy for crystallization, that is, a large difference in free energy between the molecule in the dissolved state and in the crystal;
(e) and a high degree of supersaturation.

The cholesterol monohydrate crystal structure as defined by Craven\(^1\) is a crystal in which tilted bilayers of cholesterol molecules are hydrogen bonded to a layer of water (see Figure 6). The molecules pack side to side along their long axes with their hydroxyl groups and in a plane and their isopentyl tails in a parallel plane displaced about one molecular length. The surfaces on which rapid growth occurs are those surfaces in which the molecules come together side by side rather than end to end, such as the face shown in Figure 6. Roughly equally rapid rates of growth occur along the two faces intersecting at angles of 79.2 and 100.8°\(^1\) while growth along the face roughly perpendicular to the molecular axis is many times slower (see Figure 4, above). This gives rise to the flat plate-like parallelogram, cholesterol monohydrate crystals (shown in Figure 2).

As there are nucleating and anti-nucleating agents, there are also agents
which can potentiate or slow growth. Potentiating materials cause defects in the crystal which allow new surface to form. Anti-crystallizing materials interfere with the growing surface and thus slow or prevent growth. Certain substances may adsorb only to one face of a crystal, allowing other more slowly growing faces to grow relatively more rapidly. This gives the crystal a different morphology; for instance, needles instead of plates.

**BILE AND NUCLEATION**

In the context of human bile and cholesterol gallstones, there is no doubt that bile can be highly supersaturated, but not contain microscopically visible
crystals. Much has been made of the metastable (very slow crystallization) and labile (rapid crystallization) zones of crystallization as determined by Carey and me\textsuperscript{5}, as illustrated in Figure 7. However, some cautions must be expressed. Our studies were carried out in bile salt–lecithin–cholesterol model systems and not in native bile. It is certain that these systems were not entirely dust-free and that exogenous nucleating agents were undoubtedly present. It is known that the common source of nuclei is the solvent. In fact, the number of nuclei is roughly proportional to the volume of solvent\textsuperscript{13}. However, the solutes, glassware, adjacent glass, stirrers, etc., may all provide nucleating agents. Thus, nucleation must have occurred at much lower levels of supersaturation than would be expected for homogeneous nucleation. Therefore the metastable and labile zones only apply to our model system\textsuperscript{5} and not necessarily to native bile.

Thus, according to Walton\textsuperscript{13} homogeneous nucleation of cholesterol from ethanol–$\text{H}_2\text{O}$ occurs at a supersaturation ratio of about 13; that is, 13 times greater than true solubility. Thus, I suspect that homogeneous nucleation of

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**Figure 7** The sodium taurocholate–phospholipid–cholesterol system at 10% solids according to Carey and Small\textsuperscript{5}. The micellar zone at equilibrium is shown at the bottom. Just above the metastable zone, which did not nucleate readily after several hours, is shown. Carey and Small implied that homogeneous nucleation occurred above this zone\textsuperscript{5} but, as discussed in the text, it is probable that the zone requiring heterogeneous nucleation extends well above the metastable zone, and that true homogeneous nucleation only occurs at very high levels of supersaturation. While the exact level at which homogeneous nucleation occurs is not known, it is probably higher than the saturation ratio, $S^*$ (concentration at homogeneous nucleation divided by concentration at true saturation) of 3 (i.e. 300% saturated), and could be as high as 13, as appears to be the case for cholesterol crystallization from supersaturated solutions in ethanol–water systems\textsuperscript{13}.
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cholesterol from bile occurs only at very high supersaturation, for example above a saturation ratio of 3 (＞300% saturation). Thus, given the measured levels of supersaturation (<300%), heterogeneous nucleation and conditions favourable for growth must be necessary for cholesterol crystal formation in bile (see Figure 7). Heterogeneous nucleation requires relatively large particles as nuclei. The liver may secrete some protein and glycoproteins which are probably not large enough to act as nuclei. Therefore, such nuclei must form in, or be introduced into, bile. Under certain circumstances particulate material may form in bile as a result of abnormal secretion or precursors and physicochemical changes occurring subsequently in bile. For example, pigments precipitate with calcium as a result of excessive secretion of unconjugated pigments. In the gallbladder, mucus may coagulate to form a lattice for nucleation, or calcium and fatty acid may precipitate to form nuclei of calcium soaps. External particles including bacteria may be introduced from the duodenum, especially after surgical intervention. In fact, many cholesterol monohydrate stones contain potential nucleating agents such as bile acids, bile pigments and bacteria, mucoid material, calcium soaps, and carbonates.

In addition to the concept that normal bile can be highly supersaturated and requires heterogeneous nuclei to initiate the physical stage, other possible explanations for the high supersaturation without microscopically visible crystals in subjects without stones include:

(a) the presence of an anti-nucleating agent(s) in supersaturated bile which prevent nucleation;
(b) the presence of anti-growth agent(s) (i.e. submicroscopic crystals have been nucleated but do not grow because of an anti-growth factor(s)), and
(c) the absence of anti-nucleating or anti-growth agents in the bile of gallstone patients.

In the future nucleating and growth factors must be understood.

Acknowledgments

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References

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Discussion

Dr Kern

I correctly anticipated that Drs Hofmann and Small would not leave me much to say about staging of gallstone disease, and therefore I will not talk about staging in gallstone disease. I would like to concentrate a little bit on what I think are the key problems that we do not know much about in the pathogenesis of gallstones and gallstone disease, and the major directions for research in the future, some of which has already been covered.

I would like to emphasize and stress the importance of real time ultrasound as a major technological advance which will now, I believe, allow us to learn about the natural history of gallstone disease, that is to acquire information that was impossible to acquire by oral cholecystography or other means. This equipment is portable, the resolution that one gets is extremely good and the stones can be detected, as small as 1 mm. The patients can now be followed to determine the rate of growth of those stones, to determine whether they shrink in size, whether they disappear and in short to acquire information about the natural history of the so-called silent gallstones, information that was not available to us before.

We know very little about the factors that are responsible for the increasing incidence of gallstones with age. We are just beginning to learn something about the factors responsible for predominance in females or the males, we still do not know whether there are hereditary factors that are important, we know little about diet except that patients who eat a high calorie, high cholesterol diet are more prone to get gallstones, but again there may be other factors involved in such patients. We know, thanks to Scott Grundy and his colleagues, quite a bit about the pathogenesis of stones in obesity and we are trying to learn something about stones in pregnancy. But I stress the large areas of gaps of our knowledge.

On the subject of supersaturated bile, it is worth mentioning that in every study supersaturated bile is found in the gallbladder after an overnight fast in normal people. We believe that patients with gallstones have bile which is more supersaturated and which is supersaturated for a longer period of time. I think that it is probably true but I wonder if the data in support of that are really adequate.

The hepatic contribution to the pathogenesis of gallstones is the secretion of supersaturated bile, and the contribution of the gallbladder is the nucleation of bile, precipitation of crystals, and retention of crystals. Growth of crystals and growth of stones are brand new areas that we need to learn a great deal more about. I call your attention to the important work that is coming from Dr Carey’s laboratory, which strongly suggests that at least in the prairie dog model the secretion of supersaturated bile itself has an effect on gallbladder function. Once again I have tried to emphasize areas for research in the future that I think we need for a thorough understanding of the pathogenesis and staging of gallstone disease.
In the discussion of the change from the chemical to the physical stage of gallstone disease it is very important to take into account the diurnal variation of the bile composition. I think that most of the morning have a supersaturated bile (with the possible exception of those days when we are eating continuously) but in the rest of the day, there is high variation between individuals and many factors can influence the diurnal variation. The partition between the gallbladder and the intestine for instance, and the ability of the gallbladder to respond to different types of stimuli are examples. I think that this diurnal variation may be of considerable importance to the interval between the chemical and the physical stage in the development of gallstones.

I think that the literature is not as clear as it might be, in my opinion. What we have in the morning emerging from the liver is bile which is close to supersaturated; what is in the gallbladder in the majority of the young population is unsaturated because it was stored there after the evening meal. One thing that Dr Kern did not discuss and I do not know how he feels about it, is whether the healthy gallbladder makes the bile better. Does the healthy gallbladder desaturate bile by absorbing cholesterol? My opinion is that it does, based on the study of Duane and Bennion. So my opinion is that actually one of the steps in developing gallbladder disease, or one of the advanced steps may be cholecystitis so that the gallbladder never improves bile composition.

I would just like to say that I think that the study of Duane and Bennion showing cholesterol absorption from the gallbladder is terribly important, but as far as I know, it has not been confirmed and I think it is an area that needs to be investigated further.

What Duane and Bennion showed was that after a 5-days fast bile was less saturated in, I think, five out of seven individuals. Now whether those two people are going to be the people that get gallstones, and the other five are not, we do not know. We certainly need much more information on that kind of study.

Is there direct evidence that cholesterol can be absorbed by the normal human gallbladder?

I am not aware of any such studies, maybe Dr Small will be.

Neiderhis and Roth looked years ago for that, but they really could not show it. They could show that lecithin was absorbed and fatty acids, and way back people showed that an inflamed gallbladder absorbed bile salts. One thing I would like to point out though is the study by Carey on cholesterol solubility which he did when he was with me. He showed that if you have more concentrated bile, you can get more cholesterol in solution. We do not really know just how concentrated the early morning bile is. In some surgical patients it rises to 25% solids, whereas the bile that is coming out from the liver is much more dilute, The mixture of these two biles in the gallbladder and how they behave is still a mystery.

We must now stop the discussion about absorption of cholesterol from the gallbladder. I take it from what was said here that we do not have hard evidence that the normal gallbladder can or does absorb cholesterol.

I would like to ask a question of Dr Small. The subject of prophylaxis of gallstones I think will be a more important subject at future Falk meetings, because perhaps half of the patients who acquire dissolution may have a recurrence. With regard to nucleation and the rate of precipitation, is it likely that one may be able to accomplish prophylaxis by bringing the bile saturation just down close to the saturation line? And also, are patients who have a sufficient nidus to facilitate precipitation or who still have a little bit of debris after
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gallstone dissolution more likely to get recurrences and should they be selected out as patients who should continue on prophylactic treatment?

Small
The closer to saturation level that you get the better. That is for certain. I think Dr Mazer and Dr Carey may have some interesting information on what urso may do to mucus, which could be a nucleating agent. But I do not have an answer to your other questions.

Dr Carey
All that I can say about the cholesterol saturation of bile in relation to gallbladder mucus production is that the mucus production parallels the relative cholesterol content of the bile. When you desaturate bile, I would presume, you would decrease the gallbladder mucin production. We have shown in the prairie dog that the gallbladder mucin production is increased tenfold when bile reaches supersaturation. I would assume that with urso or with cheno, if you get desaturation, you would probably reduce the amount of mucus produced by the gallbladder.

Dr Ostrow
I hate to toss in another type of gallstone but I still think that one of the biggest problems we face is, how to tell, what kind of a gallstone a patient has. I wonder whether anybody has any comment on better ways of excluding pigment gallstones and cholesterol stones with enough calcium that do not dissolve.

Dr Paumgartner
In relation to this question I wondered whether the improved methods of measuring radio density by computer tomography would help. I was talking to the X-ray people in our hospital about this problem but they were not very optimistic about this approach.

Unidentified discussant
We have conducted a study using CT scanning to detect calcifications or calcium contents which cannot be detected by ordinary X-ray, and we have found about 30% increase in the sensitivity by using CT scanning.

Dr Schoenfield
I would like to raise what I think are two difficult questions relating to the staging of gallstone disease. To my knowledge, patients with cholesterol crystals without gallstones have not been identified. I am not aware of any patients who had cholesterol crystals without the presence of gallstones. I wonder if perhaps you might explain this for us.

The second question relates to a comment Dr Hofmann made regarding the finding of sludge in ultrasonography and the possible interpretation of sludge as cholesterol crystals. I think that there is some controversy about this interpretation. I wonder what the evidence is, that sludge does represent cholesterol crystals.

Dr Carey
Could I comment that in the surgical literature you will find at least two papers in which they have looked at crystals in bile and they have equated that with biliary symptoms and there have been no stones in the gallbladder. As you know there are some people who maintain that bile crystals are an indication for cholecystectomy in the absence of stones.

Dr Schoenfield
I think the papers referred to in the surgical literature are actually poorly controlled anecdotal observations. I am absolutely certain that there will be in the next year excellent data documenting crystals on duodenal drainage without gallstones as determined by cholecystectomy. The person who did it is Dr Richard Strong at the Naval Hospital in San Diego.

Dr Hofmann
I think Dr Schoenfield has raised a very important point. First of all, I cannot quote accurately, but from my memory of Juniper’s paper in Gastroenterology in 1965 the presence of microcrystals was a non-specific finding with a certain number of false positives and false negatives. Microcrystals could be present in the absence of gallstones. But I am open to correction on that.
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Secondly, if we are talking about staging of disease in its relevance to prophylaxis the only way at present that we can demonstrate this is by duodenal intubation and aspiration, or perhaps by ERCP. We are surely not suggesting that this is a practical way of screening a population, so that we can decide who ought to be treated. I think we have got to attain some clinical perspective from this.

Thirdly, about a question of acalculus cholecystitis, I think Dr Noel Williams from Halifax is probably in the audience. He and his colleagues have done an extensive study on bile analysis and the phenomenon of acalculus cholecystitis. Perhaps they might offer a comment, with the chairman's permission.

I wonder if you would kindly comment on your experience of acalculus cholecystitis in the presence of microcrystals in bile or supersaturated bile as a clinical entity warranting treatment.

Dr Williams

You are probably referring to the paper we did some years ago. We were looking at patients who presented with typical biliary colic and had negative cholecystograms. A lot of these patients in fact were given cholecystokinin to see whether the pain was reproduced and we had a nasal duodenal tube there at the same time. And of these patients there were a whole group of them who had cholecystitis (evidenced by, at least, fibrosis plus the usual parameters of inflammation) with subsequent cholecystectomy. Most of the ones who had cholesterolosis had supersaturated bile but no stones.

Dr Paumgartner

And crystals in the bile?

Dr Williams

Crystals in some but not in all.

Dr Kern

To my knowledge, there is no extensive literature on the chemical composition of sludge which is seen by real-time ultrasound. There is a recent paper in the radiological literature (one of the authors is Larry Way). In the majority this sludge was calcium bilirubinate, in the minority cholesterol monohydrate. The number of patients was small and what I meant to say in my talk was that in some instances this sludge will be cholesterol monohydrate.

Dr Heaton

I hope you will forgive me if I come back to this question of the effect of fasting, and particularly of absorption of cholesterol by the gallbladder. I am rather worried by the implication by Alan Hofmann that the demonstration of a fall in saturation of bile with a fast implies absorption of cholesterol. It is true that the only published evidence of this is the 5-day fast of Duane, but we have in press a paper showing that between 15 and 20 hours of fasting there is a significant drop in the cholesterol saturation of gallbladder bile. This, I think, is much too early and quick to be explained by absorption of cholesterol by the gallbladder. Our suggestion is that the gallbladder is in fact contracting and refilling during a fast and is refilling with less saturated bile because during a fast the liver gradually slows down its synthesis and secretion of cholesterol. In animal work HMG-CoA reductase activity does in fact drop at around 15 to 16 hours after fasting, and I would have thought that this was a very reasonable alternative explanation to the absorption of cholesterol.

Dr Hofmann

I have not read your paper in press, but all that I really meant to say is that we need to know the effect of fasting on hepatic bile and on gallbladder bile, and that these are not necessarily the same thing. We need to define these because we have the idea that initially acute interruption of the enterohepatic circulation causes hepatic bile to become more saturated as shown by Dr Small in the monkey, by Dr Jay and McSherry in the monkey and by Dr Grundy and his colleagues in man. But then we may have a subsequent situation in time, indeed as you say, where synthesis goes down and the hepatic bile, despite interruption becomes less saturated.
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Dr Paumgartner  We have to proceed to the next section of this workshop. The most important point that has emerged from this discussion in my opinion is that so far we have paid a lot of attention to cholesterol saturation of bile but did not pay enough attention to nucleation factors. Dr Small has pointed out the difference between homogeneous and heterogeneous nucleation and I learnt from his discussion that we probably have to pay attention to a number of substances which are present in bile and will influence nucleation because we are probably dealing with heterogeneous nucleation. I think we must pay more attention to crystals than to supersaturated bile. When we see crystals we must accept this as an abnormal situation, whereas when we find supersaturated bile this is not always abnormal but occurs under certain conditions such as an overnight fast also in normals.
Interaction of Oleoyl Coenzyme A with Phospholipid Bilayers†

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Abstract: The effect of oleoyl coenzyme A (CoA) on three phospholipid bilayer systems, human red blood cell ghosts, egg yolk lecithin dispersions, and unilamellar lecithin vesicles, was studied. Addition of oleoyl-CoA to sealed, right-side-out, human red blood cell ghosts resulted in a loss of latent NADH-cytochrome c oxidoreductase activity. The turbidity of lecithin dispersions decreased as a result of the addition of oleoyl-CoA in a concentration-dependent manner. This decrease in turbidity was influenced by the mode of addition of oleoyl-CoA to the phosphoryl, and the most pronounced decrease was observed when oleoyl-CoA was dried together with the lecithin prior to resuspension in an aqueous solution. The presence of cholesterol (lecithin:cholesterol molar ratio 2:1) diminished the effect of oleoyl-CoA on the turbidity of the lecithin dispersions. Addition of oleoyl-CoA to unilamellar vesicles, which contained 5,6-carboxylfluorescein, increased the leakage of the dye from the vesicles in a concentration-dependent manner. This effect was diminished when cholesterol was incorporated into the vesicles (lecithin:cholesterol molar ratio 2:1). The interaction of oleoyl-CoA with lecithin was further studied by preparing mixtures where the lipids were dried together prior to sonication and had lecithin:oleoyl-CoA molar ratios of either 100:1 or 10:1. The resulting complexes were characterized by gel filtration and sucrose density gradient ultracentrifugation. Oleoyl-CoA was associated with particles having a size indistinguishable from that of unilamellar vesicles. At the higher oleoyl-CoA concentration, the complex formed was readily detected by density gradient ultracentrifugation because of the increased particle density. Addition of albumin to the mixtures caused dissociation of oleoyl-CoA from the vesicles but did not result in vesicle disruption under the conditions employed. The results show that oleoyl-CoA forms stable complexes with phospholipid bilayers and suggest that such complexes modify the permeability of the bilayer system.

Long-chain acyl-CoA molecules have been proposed to have a regulatory role in intracellular metabolism. Support for such an effect is based primarily on the in vitro effect of either palmitoyl- or oleoyl-CoA on the activity of several enzymes localized in the cytosol (Kawaguchi & Bloch, 1976; Edgar & Bell, 1979). The physiological significance of such regulation is controversial. Since long-chain acyl-CoA forms micelles at concentrations close to that required for the inhibition of enzyme activity (Zahler et al., 1968), suggesting that the inhibition observed in vitro may be due to nonspecific detergent effects. Recent studies have shown inhibition of several enzymes by palmitoyl-CoA at concentrations below its putative critical micellar concentration (Edgar & Bell, 1979) and where the effect of the CoA derivative was distinguished from that of other detergents (Wititsuwannakul & Kim, 1977; Hsu & Powell, 1975; Kawaguchi & Bloch, 1974), suggesting that acyl-CoA may have a regulatory role in vivo.

An understanding of what actually occurs in vivo is complicated by observations showing that intracellular proteins exist that bind long-chain acyl-CoA (Mishkin & Turcotte, 1974; Jamdar, 1979) and may prevent micelle formation. Further, a recent report indicated that the critical micellar concentration of palmitoyl-CoA was above 30 μM and the aggregation number was between 20 and 40 (Powell et al., 1981). These values are different than the previously reported values of 3–4 μM and 1000 (Zahler et al., 1968). Finally, there is no direct evidence on the state of long-chain acyl-CoA monomers, or micelles, bound to proteins or membranes within the cell. Most previous studies on acyl-CoA-enzyme interactions were performed with soluble enzymes often found in the cytosol. It is now known that many of the enzymes that utilize long-chain acyl-CoA as substrates are membrane bound and are localized on the outer (cytoplasmic) side of sealed microsomal vesicles (Bell et al., 1981). Therefore, acyl-CoA must interact with the membrane prior to or during its metabolism. Previous studies showed an association between palmitoyl-CoA and liver microsomes (Lamb & Fallon, 1972). Recent studies by Polokoff & Bell (1978) suggested that at physiological concentrations, palmitoyl-CoA can penetrate rat liver microsomes to become accessible to an ethanol acyltransferase, shown to be located on the inner (luminal) side of the microsomes. That study and those of Jamdar (1979) and Lichtenstein & Brecher (1980) have shown that acyl-CoA can alter the permeability of rat liver microsomes as reflected by loss of latent mannose-6-phosphate activity.

The precise nature of the interaction between long-chain acyl-CoA and biological membranes is not understood. This study describes the interaction of oleoyl-CoA with human red

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†Abbreviations: 5,6-CF, 5,6-carboxylfluorescein; CoA, coenzyme A; NADH, reduced nicotinamide adenine dinucleotide; Tris, trishydroxymethylaminomethane; Hepes, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid.
blood cell ghosts, multimellar liposomes, and unilamellar vesicles.

Experimental Procedures

Materials. [1-14C]Oleoyl-CoA (sp act. 59 mCi/mmol), [7-3H]cholesterol (sp act. 11 Ci/mmol), and dipalmitoylphosphatidyl[1-myristoyl]-3H)choline (sp act. 27 Ci/mmol) were obtained from the New England Nuclear Corp. (Boston, MA). [2-palmitoyl-9,10-3H]Dipalmitoylphosphatidylcholine was purchased from Applied Science, Inc. (State College, PA). Purity of the radiolabeled phosphatidylcholine and oleoyl-CoA was assessed by thin-layer chromatography by using chloroform–methanol–water (65:25:4 v/v/v) or 1-butanol–acetic acid–water (5:2:3), respectively, as developing solvents. In each case, greater than 95% of the radioactivity migrated as a single spot corresponding to a nonradiolabeled standard.

Egg yolk lecithin was purchased from Lipid Products (Nutley, United Kingdom), cholesterol from Applied Science Laboratories, Inc. (State College, PA), and 5,6-carboxyfluorescein (5,6-CF) (catalog no. 9953) from Eastman Kodak Co. (Rochester, NY). Oleoyl-CoA, acetyl-CoA, NADH, cytochrome c, and bovine serum albumin (crystallized, lyophilized, essentially fatty acid free, catalog no. A-7511) were all obtained from Sigma Chemical Co. (St. Louis, MO). Water was doubly distilled in glass.

Preparation of Lecithin–Oleoyl-CoA Dispersions. Egg yolk lecithin liposomes were prepared by dissolving the designated amount of phospholipid in chloroform–methanol (2:1 v/v) in a bath heater. The sample was evaporated under a stream of nitrogen and then pumped under vacuum with a freeze-drying apparatus for 1 h to remove residual organic solvent. The sample was then resuspended in a 0.02% sodium azide solution by vigorous stirring on a vortex-type apparatus for 2 min. Typically, 6 mg of lipid was dissolved in 25 × 75 mm test tubes and then resuspended in 2 mL of aqueous solution. Preparations containing cholesterol (lecithin:cholesterol molar ratio 2:1) were prepared similarly except that the appropriate amount of cholesterol was dissolved with the lecithin in the organic solvent prior to evaporation under nitrogen.

Oleoyl-CoA was added to the lecithin by three different procedures: (1) Oleoyl-CoA, dissolved in a small volume of water, was added to the chloroform–methanol (2:1 v/v) solution containing the egg yolk lecithin (and cholesterol, when designated); the resulting mixture was immediately evaporated under nitrogen, dried further in the freeze-drying apparatus, and finally resuspended in aqueous solution. (2) Oleoyl-CoA was included in the aqueous solution used to resuspend the dried lipids. (3) Oleoyl-CoA was added directly to the aqueous preparation subsequent to resuspension of the lipid from a concentrated stock solution containing 4.6 mM oleoyl-CoA in 0.01 M sodium acetate buffer, pH 6.0.

Preparation of Unilamellar Vesicles Containing 5,6-CF. Vesicles were prepared according to minor modifications of previously described procedures (Weinstein et al., 1977; Szoka et al., 1979). Egg yolk lecithin (6 or 60 mg) was dissolved in chloroform–methanol (2:1 v/v); the solution was dried as described above and then resuspended in 6 mL of a solution containing 0.25 M 5,6-CF, 0.1 M NaCl, 0.02% sodium azide, and 0.01 M Tris, pH 7.4. The resulting suspension was sonicated continuously in a Branson W-350 apparatus under a stream of nitrogen for 10 min (Brecr et al., 1977) and then centrifuged at 4 °C at 14000 rpm for 60 min. The upper 4 mL of the centrifuged preparation was removed and used for subsequent studies. All subsequent manipulations of the 5,6-CF-containing vesicles were done at 4 °C to minimize leakage.

Vesicles containing cholesterol were prepared by adding cholesterol (1.5 or 15 mg) to the lecithin dissolved in organic solvent so that the molar ratio of lecithin to cholesterol was 2:1. Following removal of the organic solvent, the dried lipids were resuspended in the 5,6-CF solution as described above and sonicated for 1 h in a Branson W-350 sonifier with a 50% pulse setting under a stream of nitrogen. The resulting suspension was then centrifuged at 14000 rpm for 1 h, and the upper 4 mL was removed and used for subsequent treatment.

The vesicle preparations containing entrapped 5,6-CF were applied to a Sephadex G-50 column (0.9 × 20 cm), and the vesicles eluting at the void volume were collected (Guo et al., 1980). The column was preequilibrated and eluted with a solution containing 0.1 M NaCl, 0.02% sodium azide, and 0.01 M Tris, pH 7.4. The 5,6-CF-containing vesicles were stored at 4 °C in dialysis tubing, which was suspended in the elution buffer, and were used within 3 days of preparation. In separate experiments, it was established that the elution profile of the vesicles containing 5,6-CF was virtually identical with that of vesicles lacking the dye with respect to the eluted lecithin.

Vesicle preparations lacking 5,6-CF were made exactly as described above except that 5,6-CF was omitted from the aqueous solution used to resuspend the dried lipids and the filtration through Sephadex G-50 was not performed. For the experiments shown in Figures 6 and 7, all lipids were dried together prior to sonication. Labeled lecithin, cholesterol, or oleoyl-CoA was routinely mixed with the corresponding unlabeled lipid to monitor recovery and to calculate molar ratios based on the initial specific activities.

Measurement of Turbidity of Lecithin–Oleoyl-CoA Preparations. Turbidity was determined by measuring the absorbance at 640 nm in a Coleman spectrophotometer. Initial measurements were made 5 min following the addition of oleoyl-CoA. Tubes were then shaken continuously for the designated time at ambient temperature (22–24 °C) with a wrist-action shaker moving at about 50 oscillations/min.

Measurement of 5,6-CF Release from Unilamellar Vesicles. Release of 5,6-CF was measured by fluorescence with a Perkin-Elmer fluorescence spectrophotometer (MP2-A) at excitation maximum and emission maximum of 490 and 520 nm, respectively (Weinstein et al., 1977). Oleoyl-CoA was added from a concentrated stock solution (4.6 mM) to cuvettes containing 3 mL of a solution with the designated amount of vesicles, and fluorescence was read at selected time intervals. Total fluorescence for each sample was determined at the conclusion of each experiment by adding Triton X-100 to a final concentration of 0.1%. A control sample for monitoring leakage was included in all experiments for all vesicle preparations. All measurements were made at 25 °C. The percentage of 5,6-CF released was calculated by subtracting the fluorescence of the blank sample at the designated time interval from the total fluorescence of the experimental sample, dividing this figure by the total fluorescence in the cuvette as determined by the addition of 0.1% Triton X-100, and multiplying by 100.

Measurement of NADH–Cytochrome c Oxidoreductase in Human Red Blood Cell Ghosts. Human red cell ghosts were prepared by using the procedures outlined by Hanahan & Ekholm (1974). The membranes were sealed (right side out) by incubation in 150 mM sodium chloride and 5 mM sodium phosphate, pH 8.0, for 40 min at 37 °C (Steck & Kant, 1974). NADH–cytochrome c oxidoreductase activity was determined by incubating the designated amount of membrane protein, 0.2 mM β-NADH, 0.25 mg of cytochrome c, 150 mM sodium chloride, and 5 mM sodium phosphate, pH 8.0, in a total
volume of 0.5 mL and monitoring absorbance at 550 nm (Steck & Kant, 1974).

**Gel Filtration.** Samples (0.5 mL) were applied to either Sepharose 4B or Bio-Gel A 15-m (2.6 × 28 cm) columns, which were preequilibrated with a solution containing 1 M NaCl, 0.02% sodium azide, and 0.01 M Tris, pH 7.4, as indicated in the figure legends. Samples were eluted with the preequilibration buffer at a flow rate of 26–28 mL/h. The void volume and total volume for all columns were determined with dextran blue 2000 and tritiated water, respectively. All column chromatography was performed at ambient temperatures (22–24 °C). Eluted fractions were analyzed for radioactivity with Liquiscint (National Diagnostics) as the scintillation cocktail. Double isotope counting conditions were established such that 14C was counted at 68% efficiency with no crossover of 3H into the 14C channel. Tritium was counted at 27% efficiency with a 14% crossover of 14C into the 3H channel. Data are expressed as the dpm for each of the eluted fractions after correction for crossover.

**Sucrose Density Gradient Ultracentrifugation.** Samples (200 µL) were layered onto 3.7 mL of an 8–23% continuous sucrose gradient containing 0.1 M NaCl and 10 mM Hepes, pH 7.4. Centrifugation was routinely performed for 16 h at 4 °C with a Beckman SW 60 Ti rotor spun at 31000 g. Fractions (200 µL) were collected from the bottom of the tubes and analyzed for radioactivity or absorbance determined at 280 nm. Sucrose concentrations of the fractions were determined with a hand refractometer (Bausch & Lomb; range 0–60% sucrose).

**Analytical Procedures.** Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Lipid phosphorus was measured by the method of Bartlett (1959) with a factor of 25 used to estimate phospholipid. Cholesterol was determined by the method of Rudel & Morris (1973). Lipid extracts were obtained by the method of Folch et al. (1957). Thin-layer chromatography was performed on precoated silica gel G plates (Applied Science, Inc., State College, PA). For resolution of major lipid classes, hexane–diethyl ether–acetate (70:30:1 v/v/v) was used as the developing solvent. Individual phospholipid classes were separated with chloroform–methanol–water (65:25:4 v/v/v) as the developing solvent.

**Results and Discussion**

Effect of Oleoyl-CoA on Human Red Cell Ghosts. Recently, it was shown that palmitoyl- or oleoyl-CoA altered the permeability of rat liver microsomes, as reflected by the loss of latent mannose-6-phosphatase activity (Polokoff & Bell, 1978; Janard, 1979; Lichtenstein & Brecher, 1980). To determine if analogous effects could be demonstrated for another biological membrane, we investigated the effect of oleoyl-CoA on sealed, right-side-out human red blood cell ghosts by monitoring the latency of an enzyme located on the inner surface of the membranes. Figure 1 shows the effect of oleoyl-CoA on the activity of NADH-cytochrome c oxidoreductase. As depicted in Figure 1A, activity was low in the untreated (control) ghosts, indicative of sealed, right-side-out ghosts that are not permeable to either cytochrome c or NADH. Addition of saponin, an agent known to interact with cholesterol in the membranes and to produce “leaky” ghosts, resulted in increased enzymatic activity. Oleoyl-CoA (160 µM) also increased the permeability of the ghosts since latent oxidoreductase activity was expressed. This observation was specific for oleoyl-CoA since neither acetyl-CoA nor oleic acid decreased latency at equivalent concentrations to that of oleoyl-CoA (data not shown). As shown in Figure 1B, the...
ability of oleyl-CoA to affect latency was dependent on the ratio of membrane protein to oleyl-CoA and the oleyl-CoA concentration within the range investigated. In order to establish that the loss of latency was not attributable to detergent effects of oleic acid or lyssolecithin inadvertently generated in the system, we established in separate experiments that labeled [1,14C]oleyl-CoA and dipalmitylphosphatidyl[methyl-3H]-
choline were not degraded enzymatically by the ghosts during the 50-min incubation time used for the assay.

The data suggest that the loss of latency for NADH-cytochrome c oxidoreductase activity caused by oleyl-CoA can be attributed to increased permeability of the ghosts to the substrate so that contact with the enzyme, located on the inner surface of the ghosts, can be made. These data extend our previous studies, reporting an analogous effect of oleyl-CoA on the latency of mannose-6-phosphatase, located on the luminal surface of sealed microsomal vesicles from rat liver (Lichtenstein & Brecher, 1980). It is interesting to note that oleyl-CoA produced similar effects in membrane systems with different cholesterol:phospholipid molar ratios (microsomes, 0.2; red cell ghosts, 0.8).

**Effect of Oleyl-CoA Added to Lecithin Dispersions.** A possible explanation for the effects of oleyl-CoA on membrane-associated enzymes could involve a direct interaction with either the lipid or protein components. Although numerous studies have shown interactions of long-chain acyl-CoA molecules with proteins (mostly soluble enzymes), there is very little information on acyl-CoA-phospholipid interactions. Therefore, we performed studies to determine the effect of oleyl-CoA on several model systems containing phospholipid bilayers.

The effect of oleyl-CoA on the turbidity of egg yolk phosphatidylcholine dispersions is shown in Figure 2. The molar ratio of lecithin to oleyl-CoA was approximately 137:1, and the actual concentration of phosphatidylcholine was 3.8 mM. At that concentration, the turbidity of the suspension could be measured directly in a spectrophotometer at an absorbancy of 640 nm. When the lecithin suspension was prepared in the absence of oleyl-CoA, the turbidity remained essentially unchanged, even after 8 days of gentle agitation at ambient temperature (22–25 °C). Addition of oleyl-CoA to the lecithin caused a decrease in turbidity, but the rate at which this decrease occurred was dependent on the procedure by which oleyl-CoA was added. When oleyl-CoA was added subsequently to the resuspension of the lecithin, there was a gradual decrease in turbidity with time. If the oleyl-CoA was present in the aqueous solution used to resuspend the dried lecithin, the decrease in turbidity was more rapid, and changes in turbidity were not seen after the fifth day. If a comparable amount of oleyl-CoA was mixed with the phospholipid and both lipids were dried together prior to resuspension in the aqueous solution, a rapid decrease in turbidity was observed within 5 min of resuspension, followed by a slight but detectable decrease during the next 2 days. The greater effectiveness of oleyl-CoA in decreasing turbidity when it was codried with the phospholipid prior to resuspension in water was also observed when the absolute concentration of both lipids was increased or decreased 5-fold but the molar ratios were maintained at 137:1 (data not shown).

The influence of oleyl-CoA concentration on the turbidity of lecithin dispersions prepared in the absence or presence of cholesterol (molar ratio 2:1) is shown in Figure 3. When measurements were made 0.5 h after oleyl-CoA was added, turbidity was decreased in proportion to the amount of oleyl-CoA present, regardless of the mode of addition. Consistent with the data in Figure 2, when oleyl-CoA was codried with the phospholipid prior to resuspension, the decreased turbidity observed at the early time interval was more pronounced than if oleyl-CoA was added after the lecithin was resuspended. When cholesterol was present with the lecithin, turbidity also was decreased in the presence of oleyl-CoA, but the decrease was consistently less than that observed for the preparations lacking cholesterol. After 8 days of incubation, differences in turbidity caused by the mode of addition of oleyl-CoA were not marked, but cholesterol-containing preparations still were slightly more turbid than those lacking cholesterol.

To assess the extent of hydrolysis of either oleyl-CoA or phosphatidylcholine during the incubation, we prepared mixtures of oleyl-CoA and phosphatidylcholine containing [1-
The effects of oleoyl-CoA on lecithin dispersions are analogous to those reported for bile salt–lecithin systems. When egg yolk lecithin and bile salts are mixed together in the appropriate proportions, clear solutions occur almost immediately following hydration (Small et al., 1966; Small, 1971). However, if a micellar solution of sodium cholate is added to a suspension of multilamellar egg yolk lecithin liposomes, the clearing takes several hours. Presumably the reason for this is that the bile salt must penetrate the bilayer and penetrate layers of lecithin off into bimolecular aggregates. In contrast, if the bile salt is incorporated into the dried system, then water penetrates easily, forming small bimolecular aggregates and a “clear” micellar solution (Small, 1971).

By analogy, when an aqueous solution of oleoyl-CoA is added to a suspension of multilamellar liposomes, the conversion of these large particles (which appear as a turbid suspension) to small, presumably unilamellar vesicles depends on the ability of oleoyl-CoA to penetrate and gradually disrupt the outermost leaflets of the liposomes, a process that takes days to accomplish. If an aqueous solution of oleoyl-CoA is added to dried egg yolk lecithin, which is predominantly in the L_p form (Tardieu et al., 1973; Lomitas et al., 1974), then both swelling of lecithin and penetration of oleoyl-CoA into the outer leaflet interfacing with the aqueous system occur simultaneously. The liposomes formed by this process are presumably smaller than those formed by lecithin alone, as indicated by the lesser turbidity at the beginning of the experiment as shown in Figure 2. However, the process is probably similar to disruption of pure liposomes and takes several days to complete. When oleoyl-CoA is dried together with lecithin, it is probably dispersed in the dried lecithin. Although the exact lattice of this dry mixture is not known, the oleoyl-CoA is presumably incorporated into the bilayered L_p structure. The large and highly charged polar groups may allow water to penetrate rapidly (as in the case of the coidrried bile salt–lecithin system), which produces small particles having low turbidity.

Additional studies were performed to characterize the particles formed when oleoyl-CoA was coidrried with lecithin. Figure 4 shows the elution profile of a coidrried mixture containing [PH]lecithin and [14C]oleoyl-CoA that was mixed with water 1 h prior to application onto the Bio-Gel A 15-m column. The mixture was clear before chromatography, and the molar ratio of phospholipid to oleoyl-CoA was 12:1, respectively. Lecithin eluted in two distinct peaks: the larger peak (83% of the lecithin) corresponded to the void volume, and the smaller peak eluted at a position corresponding in size to a particle somewhat smaller than unilamellar lecithin vesicles prepared by sonication. Some oleoyl-CoA coeluted with each of the lecithin peaks, but most oleoyl-CoA (67%) eluted at the total volume. Oleoyl-CoA alone, when applied to the column, eluted as a single peak at the total volume. Recovery of labeled lecithin and oleoyl-CoA after gel filtration was 79% and 85%, respectively. These recovery values were representative of several other experiments performed in a similar manner. The molar ratio of lecithin to oleoyl-CoA in the peaks corresponding to the void volume and the fraction eluting at 115 mL was 83:1 and 13:1, respectively.

The data in Figure 4 suggest that several particles of different size are formed from the coidrried mixtures. It is important to note that if phospholipid dispersions containing oleoyl-CoA (multilamellar liposomes) are applied to the column, the lecithin absorbs to the gel and does not elute at all. Therefore, in order to focus on the effects of oleoyl-CoA on a single, well-characterized population of particles, we initiated experiments with unilamellar vesicles prepared by sonication.

Effect of Oleoyl-CoA on Unilamellar Lecithin Vesicles. Leakage of 5,6-CF from unilamellar vesicles has been used to demonstrate the relative stability or permeability of these particles in the presence of various perturbing agents (Guo et al., 1980; Kirby et al., 1980). Vesicles containing 5,6-CF were prepared by sonication in either the absence or presence of cholesterol. Figure 5A shows the rate of 5,6-CF release from both types of vesicles incubated with or without 320 μM oleoyl-CoA. Leakage of dye occurred in the absence of oleoyl-CoA and was more pronounced in vesicles prepared without cholesterol. Addition of oleoyl-CoA increased the leakage rate in both vesicle preparations, but the cholesterol-containing vesicles clearly were more resistant to the effect of oleoyl-CoA, since 5,6-CF release occurred at a slower rate.

The influence of different concentrations of oleoyl-CoA on release of 5,6-CF from vesicles prepared with or without cholesterol is shown in parts B (30 min after addition) and C (6 h after addition) of Figure 5. The data in parts B and
FIGURE 5: Effect of oleyl-CoA on the release of 5,6-CF from unilamellar vesicles prepared in the absence and presence of cholesterol. Vesicles were formed by sonication as described under Experimental Procedures and were diluted prior to the addition of oleyl-CoA so that the total amount of 5,6-CF in each cuvette would not exceed 100 

mU when fluorescence was measured at excitation maximum and emission maximum of 490 and 510 nm, respectively. This resulted in a final lecithin concentration of 84.7 

mU. Unilamellar vesicles containing lecithin alone (E, A); vesicles containing lecithin:cholesterol in a molar ratio of 2:1 (E, A). (A) Time course of the leakage of 5,6-CF from vesicles incubated in the absence of oleyl-CoA (A, A); vesicles incubated in the presence of 320 

mU oleyl-CoA (E, O). (B) Effect of oleyl-CoA on the release of 5,6-CF 30 min after addition of oleyl-CoA. (C) Effect of oleyl-CoA on release of 5,6-CF 6 h after addition of oleyl-CoA.

C of Figure 5 are corrected for leakage occurring in the absence of oleyl-CoA with paired controls. After a 30-min incubation, clear-cut differences were observed between the two vesicle preparations. Leakage of 5,6-CF from the lecithin vesicles was produced by oleyl-CoA in a concentration-dependent manner up to 120 

mU, and at higher concentrations no additional leakage was observed. In contrast, no detectable leakage of 5,6-CF due to oleyl-CoA occurred from the cholesterol-containing vesicles.

Following a 6-h incubation period, leakage occurred from both vesicle preparations at all concentrations of oleyl-CoA tested, but values for the cholesterol-containing vesicles were consistently 80–90% of those measured for vesicles prepared without cholesterol. Since leakage in the presence of a relatively high concentration of oleyl-CoA was not complete, at least when compared to dye release in the presence of Triton X-100, it was possible that oleyl-CoA mediated its effect by interacting with, but not disrupting, the vesicles.

Complex Formation of Oleyl-CoA with Unilamellar Lecithin Vesicles. To determine directly if complexes between oleyl-CoA and unilamellar vesicles could be demonstrated, we prepared sonicated mixtures of oleyl-CoA and lecithin and characterized them by gel filtration and sucrose gradient ultracentrifugation.

Figure 6A shows the Sepharose 4B elution profile of a suspension containing lecithin (127 mM) and oleyl-CoA (127 

mU) that was previously sonicated to form unilamellar vesicles. Both lipids were present during sonication, and tracer amounts of dipalmitoylphosphatidyl[methyl-3H]choline and [1-14C]-oleoyl-CoA were included. The labeled lecithin eluted as a single peak approximately midway between the void and total volume of the column, which corresponded exactly to the position where unilamellar vesicles prepared in the absence of oleyl-CoA normally eluted. Approximately 80% of the labeled oleyl-CoA eluted with the vesicles, and the remainder eluted slightly before the total volume. Calculated from the specific activities in the peak vesicle region, the molar ratio of lecithin to oleyl-CoA averaged 137:1. When solutions containing oleyl-CoA alone (4 or 120 mU) were chromatographed, a single peak, also eluting slightly before the total volume, was observed. Including 4 mU oleyl-CoA in the
elution buffer did not alter the elution profile of oleyl-CoA.

- The elution profile of a sonicated mixture of lecithin-cholesterol-oleoyl-CoA (molar ratio 100:50:1; lecithin concentration 12.7 mM) is shown in Figure 6B. The lecithin eluted as a larger particle than vesicles that lacked cholesterol, and the peak was broader, suggesting a more heterogeneous particle population. The molar ratio of lecithin:oleoyl-CoA at the peak was 142:1. The labeled oleyl-CoA eluted predominantly with the cholesterol-containing vesicles, although about 20% of the total oleyl-CoA again appeared slightly before the total volume.

The elution profile of a sonicated mixture of lecithin (12.7 mM) and oleyl-CoA at a molar ratio of 10:1 is shown in Figure 6C. All the lecithin eluted as vesicles, but the oleyl-CoA was about equally distributed between the vesicle region and a peak appearing slightly before the total volume. The molar ratio of lecithin to oleyl-CoA in the peak region averaged 13:1.

When albumin was added to each of the sonicated preparations described above in equimolar amounts relative to the oleyl-CoA, incubated for 1 h, and subsequently analyzed by Sepharose 4B chromatography (Figure 6D–F), between 70% and 90% of the labeled oleyl-CoA was associated with the albumin, whereas the elution profile of the lecithin was essentially unchanged from that of the comparable experiments shown in Figure 6A–C, respectively. The data indicate that oleyl-CoA was associated with lecithin vesicles after sonication, but this association was affected by the addition of albumin, which is known to bind equimolar amounts of oleyl-CoA (Lamb & Fallon, 1972; Lichtenstein & Brecher, 1980). Extending the preincubation period of the lecithin-oleoyl-CoA mixtures with the albumin did not alter the elution patterns (data not shown).

The sonicated mixtures of lecithin and oleyl-CoA were further characterized by sucrose density gradient ultracentrifugation. When lecithin (12.7 mM) and oleyl-CoA (molar ratio 100:1) were applied onto 8–23% sucrose gradients, both labeled lipids remained at the top of the gradient (Figure 7A). If albumin was added to the mixture in amounts equimolar to the oleyl-CoA present and then subjected to sucrose gradient ultracentrifugation, approximately 70% of the oleyl-CoA coesedimented with the albumin, whereas the lecithin again remained at the top (Figure 7C). This was consistent with the observations shown in Figure 6D, indicating that albumin could remove oleyl-CoA from the vesicles.

Of particular interest was the sedimentation profile of sonicated mixtures of lecithin (12.7 mM) and oleyl-CoA at a molar ratio of 10:1 (Figure 7B). In this case, both the lecithin and oleyl-CoA migrated into the gradient, indicating the presence of a particle more dense than that existing when the molar ratio was 100:1. If albumin was added to this mixture, oleyl-CoA again sedimented with albumin, whereas the lecithin was localized at the top of the gradient (Figure 7D). Sedimentation profiles almost identical with those shown in Figure 7 were observed when lecithin vesicles were prepared containing cholesterol (33 mol%).

The complex formed from a mixture containing a lecithin:oleoyl-CoA molar ratio of 10:1 sedimented into the gradient at a position corresponding to a sucrose concentration of about 16%, and the average molar ratio of lecithin and oleyl-CoA in this complex was 13:1. In additional experiments, we established that this complex did not sediment further when the centrifugation time was extended to 24 h, suggesting that sedimentation equilibrium was achieved. Thus, the buoyant density of the hydrated, solvated lecithin-oleo-

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**Figure 7:** Sucrose gradient ultracentrifugation of mixtures containing unilamellar vesicles and oleyl-CoA. Double-labeled samples of lecithin and oleyl-CoA were prepared exactly as outlined in the legend of Figure 6. Aliquots (0.2 mL) of the designated mixture were applied to the top of an 8–23% continuous sucrose gradient, spun at 31000g for 16 h at 4 °C, and analyzed as described under Experimental Procedures. (A) Lecithin:oleyl-CoA molar ratio 100:1; (B) lecithin:oleoyl-CoA molar ratio 10:1; (C) lecithin:oleoyl-CoA:albumin molar ratio 100:1:1; (D) lecithin:oleoyl-CoA:albumin molar ratio 10:1:1.

Oleyl-CoA complex has the density of 16% sucrose, that is, 1.063 g/mL. Taking the anhydrous density of egg yolk lecithin at 1.015 g/mL (Elworthy, 1959; Small, 1967) and the appropriate weight fraction of lecithin and oleyl-CoA in a complex with a molar ratio of 13:1, one can calculate an anhydrous density for oleyl-CoA of about 1.46 g/mL. This is in reasonable agreement with an independent calculation of the anhydrous density of oleyl-CoA based on the densities of its components (1.41 g/mL).

Additional experiments employing 5,6-CF were performed with vesicles containing both oleyl-CoA and lecithin. Following sonication of a codried dispersion of lecithin and oleyl-CoA (molar ratio 10:1) in the presence of 0.25 M 5,6-CF and subsequent passage through Sephadex G-50 columns, much of the dye had leaked out of the vesicle. Furthermore, storage overnight in dialysis tubing at 2 °C also resulted in significant additional leakage. These findings were consistent with increased vesicle permeability when oleyl-CoA was contained in the vesicles.

Our data indicated that the complex was stable during the relatively gentle procedures involved in gel filtration and ultracentrifugation. Although the precise orientation of oleyl-CoA and vesicle cannot be determined by the techniques used in this study, it seems unlikely that the large and highly charged polar nucleotide would be localized within the bilayer.
We would expect that the acyl chain would be incorporated within the bilayer and the remainder of the molecule would be exposed to the aqueous space. In the situations in which aqueous oleoyl-CoA is added to either an aqueous suspension of multilamellar vesicles or dried egg yolk lecithin, it is reasonable to assume that most of the oleoyl-CoA will be present on the outside leaflet of the unilamellar vesicle, since its presence on the inside would require either total disruption of the bilayer or a flip-flop mechanism through the bilayer, which seems a rather unlikely situation with such a large polar group. However, when water is added to a codried mixture of lecithin and oleoyl-CoA, the possibilities of having oleoyl-CoA within the aqueous space of the vesicle cannot be logically ruled out.

The addition of albumin to unilamellar vesicles at a molar ratio equivalent to that of oleoyl-CoA effectively removed oleoyl-CoA from the vesicle and resulted in an oleoyl-CoA–albumin complex. Under these conditions the addition of albumin did not disrupt the vesicles since the phospholipid remained in a particle having the size and density of the vesicles. Thus, albumin either easily removed the oleoyl-CoA from the outside of the vesicle or removed the oleoyl-CoA and then allowed vesicles to re-form. We think the most reasonable orientation for oleoyl-CoA in these systems is on the outside of the vesicle.

Addition of cholesterol to multilamellar bilayer systems has been shown to alter the properties of the system by several criteria, including resistance to disruption by detergents and decreased permeability to glucose (Demel et al., 1969), ions (Scarpa & de Gier, 1971), and dye (Guo et al., 1980). Our studies show that inclusion of cholesterol with lecithin at a molar ratio of 0.5:1 decreased the rate at which oleoyl-CoA disrupted multilamellar liposomes and reduced the effect of oleoyl-CoA on leakage of dye from unilamellar vesicles. Vesicles containing cholesterol could be characterized by gel filtration and sucrose density gradient ultracentrifugation before and after incubation with oleoyl-CoA, and the data indicated that oleoyl-CoA did associate with the vesicles in a manner similar to that observed with vesicles lacking cholesterol. Thus, the apparent stabilizing effect of cholesterol is not caused by reducing the binding of oleoyl-CoA. In contrast, other studies have shown that apoprotein binding to vesicles is reduced by incorporation of cholesterol (Pownall et al., 1979; Tall & Lang, 1976).

In this study, we demonstrated an effect of oleoyl-CoA on a cellular membrane enriched in cholesterol. The loss of latency of NADH–cytochrome c oxidoreductase activity produced by oleoyl-CoA addition to sealed right-side-out human red blood cell ghosts can be attributed to increased permeability of the ghosts to the substrate so that contact with the enzyme, located on the inner surface of the ghost, can be made. In a previous study, we reported an analogous effect of oleoyl-CoA on the latency of mannose-6-phosphatase activity, located on the luminal (inner) surface of microsomal vesicles from rat liver (Lichtenstein & Brecher, 1980). The presence of protein and varied phospholipid composition clearly distinguishes these biological membranes from the model systems, yet it is interesting to note that oleoyl-CoA produced similar effects in membrane systems with different cholesterol:phospholipid molar ratios (microsomes, 0.2; red ghost cells, 0.8).

Recently, it was suggested that the properties of cardiac cells can be altered by the intracellular accumulation of polar lipids such as long-chain acyl-CoA, and such effects may occur during coronary artery occlusion (Katz & Messineo, 1981).

Intracellular concentrations of long-chain acyl-CoA were reported to vary between 110 and 152 μM in rat liver (Blanar et al., 1978). It is not clear if intracellular acyl-CoA exists transiently as monomers, as micelles, bound to soluble proteins, or associated with membranes. The possibility that localized effects of acyl-CoA derivatives on intracellular membranes can modify membrane function, independent of direct effects on soluble or membrane-bound enzymes, remains to be established.

Acknowledgments
The expert technical assistance of Paul Genest is gratefully acknowledged. We also thank Dr. Elizabeth Simons for her advice and help with the fluorometric analysis.

References
Cholesterol Biosynthesis and Modulation of Membrane Cholesterol and Lipid Dynamics in Rat Intestinal Microvillus Membranes

Thomas A. Brasitus and David Schachter

ABSTRACT: Experiments were performed to test the hypothesis that cholesterol biosynthesis in the rat ileal enterocyte, the major absorptive cell lining the distal epithelium of the small intestine, can modulate the cholesterol content and the motional freedom of the plasma membrane lipids. Decreased sterol biosynthesis in vivo was elicited by feeding sodium taurocholate or by fasting the rats, whereas increased synthesis was induced by biliary ligation or feeding cholestyramine, a bile salt-binding resin; these effects were monitored by assay of mucosal 3,5-diacetyl-3-methylglutaryl coenzyme A reductase. After each procedure, isolated microvillus membranes were examined to determine the lipid composition and the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene. The results demonstrate that variations in cholesterol biosynthesis in vivo can modulate the cholesterol content and the motional freedom of the lipids of the microvillus membrane; similar effects were not observed on the basolateral membrane. The observations suggest that the normal pattern of decreased lipid motional freedom in microvillus membranes of the distal compared to the proximal small intestine of the rat results from higher rates of cholesterol biosynthesis in the distal mucosa.

There is considerable evidence that many functions of biological membranes are influenced by the composition and physical state of the membrane lipids (Lee, 1975; Melchior & Stein, 1976; Sandermann, 1978). The functional significance of the lipids is further indicated by observations that membranes which differ in function also differ in lipid composition and lipid motional freedom or "fluidity". Such differentiation is illustrated clearly by comparisons of the luminal (microvillus) and centrumal (basolateral) portions of the plasma membrane of the rat enterocyte, the major absorptive cell of the small intestinal mucosa. These antipodal membranes, which regulate the exchange of substances between organism and environment, differ in ultrastructure (Blom & Fawcett, 1968; Oda, 1976), enzyme and transport activities (Douglas et al., 1972; Lewis et al., 1975; Murrer et al., 1974, 1976), electrophysiological properties (Rose & Schultz, 1971; Okada et al., 1977), and protein components (Fujita et al., 1973). Correspondingly, the membranes differ in lipid composition (Forstner et al., 1968; Douglas et al., 1972; Kawai et al., 1974; Lewis et al., 1975; Brasitus & Schachter, 1980), and lipid molecules of the basolateral as compared to the microvillus membrane have considerably greater motional freedom (Brasitus et al., 1980; Brasitus & Schachter, 1980; Gray et al., 1981).

Although the evidence suggests the existence of regulatory mechanisms which maintain the lipid composition and fluidity characteristic of specific membrane organelles, the precise nature of these mechanisms is largely unknown. Accordingly, the present studies were initiated to examine the role of membrane cholesterol, a component which decreases the motional freedom of bilayer lipids above their transition temperatures (Oldfield & Chapman, 1971). Specifically, we explored the hypothesis that the cholesterol content of rat enterocyte plasma membranes is modulated by the rate of cholesterol biosynthesis in the cell. Several lines of evidence point to this possibility. Studies of the steady-state fluorescence polarization of lipid-soluble fluorophores indicate that the lipid fluidity of rat microvillus membranes is least in the distal (ileal) segment of the intestine and increases in the proximal portion (Schachter et al., 1976; Schachter & Shinitsky, 1977). Corresponding to this distribution, it was found that both the rate of incorporation of precursors into cholesterol and the

1 The term "lipid fluidity" as applied to model bilayers and natural membranes is used throughout this report to express the relative motional freedom of the lipid molecules or subunits thereof. When assessed by the estimation of steady-state fluorescence anisotropy of the fluorophore 1,6-diphenyl-1,3,5-hexatriene, changes in the fluorescence anisotropy may be due to alterations in the correlation time and/or maximal hindered anisotropy of the probe. As described previously (Brasitus & Schachter, 1980), we use the terms "lipid fluidity" or "motional freedom" to designate both kinds of alterations.

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The effect of elevated biliary tract pressure on biliary lipid metabolism and bile flow in nonhuman primates

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The effects of low (control), moderate, and high biliary tract pressures on biliary lipid metabolism and bile flow were studied in six rhesus monkeys and two baboons. Moderate pressures did not influence bile flow or biliary lipid metabolism. High biliary tract pressures produced significant reductions in bile flow and the secretion rates of bile salts, phospholipids, and cholesterol, but cholesterol saturation in bile improved. High pressure also completely inhibited bile salt synthesis, and the bile salt pool size decreased slightly due to continuing small losses in urine and bile samples. During the high pressure period bile salts accumulated in the liver and peripheral tissues. After pressure had been returned to control levels, bile flow and biliary lipid secretion rates returned to normal levels. Bile salt secretion recovered more slowly than secretion of the other lipids and bile became more saturated with cholesterol than in the control period. Bile salt synthesis resumed approximately 10 hr after pressure had been returned to control levels. The high biliary tract pressures induced in these studies are now known to occur in about 25% of normal human subjects. (J Lab Clin Med 99:342, 1982.)

Abbreviation: disintegrations per minute (DPM)

The effects of elevated biliary tract pressures on biliary lipid metabolism have been incompletely studied. Those studies which are available have usually been performed immediately after surgery or anesthesia,1-4 without preservation of the enterohepatic circulation of bile salts,5-7 and have focused on the effect of high pressure on bile flow.1-7 Bile flow was reduced in some of these studies,5-6 but in others it remained unchanged.5-4

Recent endoscopic studies have shown that about 25% of normal individuals,6-9 patients with cholelithiasis, or persons who have had cholecystectomy* have resting common bile duct pressures of greater than 15 mm Hg (20 cm saline). This unexpected finding increases the need for a description of the effects of elevated biliary tract pressures on biliary lipid metabolism, particularly since gallstones have been produced in two animal models of partial biliary tract obstruction10,11 and because complete obstruction of bile flow increases cholesterol saturation of bile in primates.12

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Effect of pressure on bile lipids and flow

Fig. 1. Model for inducing and measuring biliary tract pressure while collecting bile samples and urine at 10% interruption of the enterohepatic circulation. Pressure in biliary tract was altered by raising or lowering the calibrated glass cylinder within the pipette until the biliary tract pressure, as shown in pressure recording, was at the desired level.

The present experiment was performed to determine the effects of elevated biliary tract pressures on bile flow, biliary lipid secretion, and bile acid metabolism. It seemed possible that alterations in flow and secretion of lipids might result in changes in bile cholesterol saturation either during or after high pressure. We found that elevated pressures reduced flow and lipid secretion rates, inhibited bile acid synthesis, and produced changes in cholesterol saturation of bile.

Methods

Experimental model. Six female rhesus monkeys (animals a to f, weighing 4 to 6 kg) and two baboons (animals G and H weighing 12 to 16 kg) were used in the study. Animals were given primate feed, 40 gm/kg/day, and water ad libitum and were adapted to restraining chairs. The primate model used has been described elsewhere and Fig. 1 summarizes the modifications made to suit this experimental protocol. Briefly, a laparotomy was performed and the supraduodenal common bile duct was divided and closed. A T tube was placed in the common bile duct and two limbs of the tube were exteriorized. The gallbladder was defunctioned by aspiration of contents and division and ligation of the cystic duct. A tube-duodenostomy was performed. Postoperatively, biliary tract pressures were measured through one limb of the T tube by a pressure transducer and recorder (Hewlett-Packard Co., Palo Alto, Calif.). Bile flowed through the other exteriorized limb into a stream-splitter, which collected 10% of the bile for analysis and returned the remainder to the animal through the tube-duodenostomy. Biliary tract pressure was regulated by raising or lowering the end of the bile-collecting limb of the T tube, with a vertically positioned glass cylinder and pipette interposed between the T tube and the stream-splitter (Fig. 1).

Experimental procedure (Fig. 2). Animals were permitted to recover from surgery. The study was performed 18 to 21 days after operation, i.e., when food intake and bile salt pool size had returned to normal levels. From day 7 until the experiments were completed, the animals were maintained at...
10% interruption of the enterohepatic circulation of bile salts, by using the stream-splitter, except where stated below.

Two days prior to the pressure study, the bile acid pool was "washed out" and measured,$^{14}$ labeled with $^{14}C$-cholic acid (4 to 5 $\mu$Ci in rhesus monkeys and 11 to 15 $\mu$Ci in baboons; New England Nuclear Corp., Boston, Mass.) and returned by the duodenostomy tube over 2 hr. The pool was not labeled in animal a.

Three pressure levels were induced on the experimental day: low and moderate pressures (4 to 8 and 10 to 14 cm saline) for approximately 6 hr each and then high pressures (17 to 22 cm saline) for 24 hr. In three animals, f, G, and H, only control and high pressures were studied. Then pressures were lowered to 4 to 8 cm saline. The period that followed is called the recovery period, and during this time the animals were treated differently. Animals a to c were maintained on the stream-splitter at 10% interruption of the enterohepatic circulation in order to study recovery period bile flow and secretion rates. In animals d, e, and G (two monkeys, one baboon) all bile was collected (100% interruption) and the change of bile flow and tissue radioactivity was noted. In all the animals tissues were removed at 24 hr after the last pressure had been imposed. In animal a, the liver was removed but the bile duct was ligated and the gall bladder left in situ. In animal G, the total bile collection was not possible during the study and animal G was sacrificed without relief of obstruction. The high pressure level was considered to be at 17 Kcal/kg/24 hr and was damaging to the animal. Only three animal had been previously studied (animal f, G, and H) within the study.

Chemical determinations were made using the method of Silbergeld et al.$^{15}$ Tissue samples were prepared and a-Pen II (Packard Instrument Co.) was used for the radioactivity. Chrom Q as a solid absorbent was used. Known amounts of activity were gas-liquid chromatographed after trifluoroacetylation and hexadeuteroacetylation for tissue extraction.

Gas-liquid chromatography was used to determine the remaining activity. The second acid treatment was used on the initial evaporate. The results were expressed in counts per minute (cpm) and compared with standards run daily.

The radioactivity of the bile was determined on a NEF-922; $\beta$-Counter (Nuclear Electron Inc.) to determine the percentage of radioactivity remaining in the bile after extraction. The specific activity of the bile was found to be 0.25 $\mu$Ci/ml before and after the pressure and with subsequent perfusion with $^{14}$C-labeled bile. All bile samples were injected $^{14}$C-labeled bile, and the thin-layer chromatograms were used to identify the bile salts. The enterohepatic circulation was maintained at 10% interruption of the bile acid pool by using the stream-splitter.
Table I. Effect of elevated biliary tract pressures on relative composition, molar ratios, and percent cholesterol saturation

<table>
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<tr>
<th>Relative composition</th>
<th>Molar ratios</th>
<th>% cholesterol saturation</th>
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<tbody>
<tr>
<td>Bile salt</td>
<td>Phospholipid</td>
<td>Cholesterol</td>
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<tr>
<td>Low pressure</td>
<td>86.48</td>
<td>11.49</td>
</tr>
<tr>
<td>Moderate pressure</td>
<td>86.69</td>
<td>11.91</td>
</tr>
<tr>
<td>High pressure</td>
<td>87.33</td>
<td>11.57</td>
</tr>
</tbody>
</table>

B = bile salt; P = phospholipid; C = cholesterol.
Values are means; S.E.M. in parentheses.
*p < 0.05 vs. control.

interruption in order to measure postobstructive pool size. Three animals were sacrificed by exchange of blood volume under anesthesia (pentobarbital 50 mg, intravenously), and their organs and tissues were weighed and frozen in order to determine the distribution of radioactive bile salts in the body. In animals I and H, this was done at the end of the high pressure period without relieving the obstruction; in animal G, the exchange of blood volume under anesthesia was performed after 10 hr of total bile collection for postobstructive pool size measurement. Animals were fed as usual during the experiment, and uneaten food was weighed. In animal e, an elemental liquid diet (Vivonex, 50 Kcal/kg/24 hr) was fed by duodenostomy and food was withheld. Urine was quantitatively collected in three animals. Biliary tract pressure was monitored continuously on a chart recorder and was always within the stated ranges.

Chemical analyses. The concentrations of cholesterol, phospholipid, and total bile salts in bile were measured by methods previously described. 14 Individual bile salts were measured by gas-liquid chromatography in many of the bile samples. These samples were extracted by a modification of the method of Sandberg et al. 17 The methyl esters and then the trifluoroacetates of the bile salts were prepared and dissolved in acetonitrile, and an aliquot was used for gas-liquid chromatography on a Packard chromatograph (Packard Instrument Co., Inc., Downers Grove, Ill.) at 240° C, with Gas Chrom Q as support and 1% WF-1 (Dow Corning Corp., Midland, Mich.) as the stationary phase. A known amount of 14C-taurocholic acid (Mallincrodt, Inc., St. Louis, Mo.; pure by thin-layer and gas-liquid chromatography) was added to each sample prior to extraction and a small aliquot of the trifluoroacetates was counted (see below) in order to obtain a correction for bile salt loss during extraction.

Gas-liquid chromatography was used to determine bile salt concentration in urine. Urine samples were treated by initial evaporation of one half of the total sample, using a rotary evaporator. The residue was washed with 25 ml of methanol and this solution was filtered and then evaporated. The remaining steps were as for bile. Loss was corrected for by addition of 14C-taurocholic acid prior to initial evaporation. Bile acid sulfates in urine were also assessed in two animals by the method of Stiehl et al. 18

The radioactivity in bile samples was measured by adding 1 ml of Hyamine Hydroxide (cat. no. NEF-921; New England Nuclear), 2 ml of methanol, and 12M diluted Liquifluor (New England Nuclear) to 20 μl of bile in a scintillation vial. Counting was done in a Packard liquid scintillation spectrometer and quenching was corrected by an internal standard (14C-toluene; New England Nuclear). Extracts of bile and urine to be used for gas-liquid chromatography were similarly treated to obtain the correction factor for loss during extraction. The 14C-cholic acid used in these experiments was found by gas-liquid chromatography to be pure bile salt, with more than 95% as cholic acid, and with specific activity of 40 mCi/mmole. In order to ascertain whether the radioactivity in the injected 14C-cholic acid had remained in the cholic acid, its conjugates, or its bile salt derivatives, thin-layer chromatography of selected bile samples was done on silica gel G. Two solvent systems were used for each sample (isooamyl acetate 40: propionic acid 30: n-propanol 20: water 10; and
Fig. 3. Effect of low, moderate, and high biliary tract pressures on bile flow and biliary lipid secretion. Asterisk, Significant change from control value, p < 0.05. The mean and S.E.M. of data are shown.

chloroform 100: methanol 25: acetic acid 8). The spots were scraped, eluted, and counted as described for bile samples above. Ninety-five percent of radioactivity was recovered in cholic acid or its conjugates.

Bile salt radioactivity in the organs and tissues was measured by modification of the method of Hansen and Bush. Each sample was macerated with fine scissors in a scintillation vial, and then 2 ml of NCS (Nuclear Chicago, Chicago, Ill.) was added. Digestion was carried out at 55°C in a water bath until a clear one-phase solution was obtained. From 24 to 48 hr of digestion were usually required. In the final 30 min, 0.5 ml of a saturated solution of benzyl peroxide was added to bleach the solution. After cooling, 17 ml of Aquasol (New England Nuclear) was added to the vial and the pH was adjusted to 6.0 to 7.0 with glacial acetic acid. The vials were refrigerated in the scintillation counter for 24 hr to reduce chemiluminescence. When the blank had reached usual background levels, the samples were counted and quenching was corrected by the addition of a 14C-toluene internal standard (New England Nuclear). To further ensure that this method did not result in counts due to residual chemiluminescence, the tissues of another monkey that had not received any isotope were treated in an identical fashion and no counts above background were detected after cooling.

Physiological measurements. Bile flow (ml/kg/hr) was calculated from the volume of bile collected over each sample period and the percent interruption produced by the stream splitter. The hepatic secretion rates for bile salt, phospholipid, and cholesterol (μmol/kg/hr) in each sample were determined from the bile flow and the concentration of the particular lipid in the sample. The relative composition of the bile lipids was calculated and plotted on triangular co-ordinates as previously described. Percent cholesterol saturation was calculated from the critical tables of Carr. Bile salt synthesis was followed in these experiments by a semilogarithmic plot of the ratio of isotope to bile salt mass, i.e., mCi of cholic acid per millimole of total bile salt vs. time. In a well-mixed one-pool system, e.g., bile salts in the enterohepatic circulation, a straight line with negative slope would indicate steady-state bile salt synthesis. Because the rate of new bile salt synthesis determines the magnitude of the negative slope, a decrease in slope would indicate a decreased rate of total bile salt synthesis.
Flow and biliary lipid secretion. and S.E.M. of data are shown.

and a zero slope cessation of synthesis. Fluctuation of the ratio not adhering to a straight line would indicate divergence from steady state.

In the period just prior to the pressure study, the bile salt pool size was measured by the washout technique previously described. Briefly, the interruption of the enterohepatic circulation was changed from 10% to 100%, samples were collected over short periods, the low point was identified, and the pool size was calculated as described. A different situation pertained after high pressure because bile salts might be widely distributed in the body and might not be secreted in 4 to 6 hr. From initial experiments performed in the first few animals, it was known that almost no synthesis occurred during the high pressure period or in the first 10 hr of the recovery period (see below). Therefore the bile salt pool retained in the animal at 10 hr into the recovery period should equal the control bile salt pool size minus losses of bile acids up to that point. The losses consisted of bile acids excreted in urine, bile acids removed during sampling in the high pressure period, and bile acids washed out in the first 10 hr of the recovery period at 100% interruption of the enterohepatic circulation (animals d, e, G).

Retained pool after 10 hours (μmol/kg) =

Calculated DPM remaining in animal after
10 hr of postobstructive pool washout × Control bile acid pool size
Calculated DPM in animal at the start of the high pressure period

The calculated values were obtained by subtraction of losses of DPM in bile samples and urine from injected DPM. In animal G the retained pool could be determined from radioactivity in organs and tissues, since the specific activity remained constant throughout the high pressure period and for 10 hr of the recovery period. The value obtained by this method, 9 μmol/kg, was very close to the value obtained by the formula, 11 μmol/kg.

Statistical methods. All results were analyzed for significance by the Wilcoxon ranking or Student t test for paired data.
Fig. 5. Relative composition of bile in low and high pressure periods (L, shaded area, and H) and in the recovery period in animals d, e, and G. The dashed line connects the data points in the recovery period and the times are those from start of recovery period; heavy line is Admirand-Small line of maximum cholesterol solubility.

Results

Bile flow, biliary lipid secretion rates, relative composition, lipid ratios, and cholesterol saturation

Moderate and high pressure periods (Fig. 3, Table I). Moderate pressure, 10 to 14 cm saline, caused no significant changes in bile flow, secretion rates of biliary lipids, relative composition, biliary lipid ratios, or percent cholesterol saturation (Fig. 3 and Table I).

High pressures (17 to 22 cm saline) produced significant and substantial reductions in bile flow and secretion rates of bile salt, phospholipid, and cholesterol (Fig. 3).

The reduction in bile flow was evident very shortly after the high pressure level had been attained. Bile flow was less than one-half control rates in seven of eight animals, and bile salt secretion rate decreased to this level in all animals.

Of the three lipids, cholesterol secretion fell most and bile salt secretion least, resulting in a significant decrease in the percent cholesterol (relative composition), percent cholesterol saturation, and significant increases in the bile salt/phospholipid, bile salt/cholesterol, and phospholipid/cholesterol concentration ratios (Table I).

The percent reduction in bile acid secretion rate was greater than the percent reduction in bile flow. Not surprisingly, therefore, there was a significant reduction in bile acid concentration, p < 0.01. Reductions in cholic, chenodeoxycholic, and deoxycholic acid secretion contributed proportionately to the reduction in total bile salt secretion.

Recovery period (Fig. 4). Three animals (a to c) were maintained on 10% interruption after pressures were returned to normal, three (d, e, and G) were placed on 100% interruption of the enterohepatic circulation to determine pool size, and two (f and H) were sacrificed for determination of tissue distribution of radioactive bile salts at that time. The
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Fig. 289.

Fig. 290.

Fig. 291.

Fig. 292.
Table II. Effect of elevated biliary tract pressure on bile salt pool size (µmol/kg)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Control period pool size by washout technique</th>
<th>Recovery period pool size</th>
<th>Change in pool size</th>
<th>Bile salt loss at high pressure (bile samples and urine)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Washout technique</td>
<td>Retained pool</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>d</td>
<td>261</td>
<td>106</td>
<td>137</td>
<td>243</td>
</tr>
<tr>
<td>e</td>
<td>197</td>
<td>90</td>
<td>83</td>
<td>173</td>
</tr>
<tr>
<td>G</td>
<td>228</td>
<td>193</td>
<td>9</td>
<td>202</td>
</tr>
</tbody>
</table>

In animals d and e, the retained pool was calculated by the formula method given in the text. In animal G, the retained pool was measured directly via determination of residual radioactivity in organs and tissues. Note that the small reduction in pool size is approximately equal to the measured losses in bile samples and urine during the high pressure period.

observed by polarizing microscopy. In animal G, bile was still supersaturated after 10 hr of pool washout, when the animal was sacrificed (Fig. 5). Interruption of the enterohpatic circulation in monkeys normally results in only a short (4 to 6 hr) period of supersaturation. Therefore, in all six animals (a to e, G) in which bile was collected in the recovery period there was increased cholesterol saturation of bile.

Bile salt synthesis rate, pool size, urinary losses, and tissue distribution. The pattern of decay of specific activity for animals c and e is shown in Fig. 6. This pattern was similar to all other animals tested (b, d, and G). When log specific activity was plotted against time, a steady negative slope was observed during low and moderate pressures followed by a slope that was close to zero throughout the high pressure period and for several hours of the recovery period. Then specific activity began to fall steadily once again. The data indicate almost complete inhibition of bile salt synthesis at high pressure and for several hours after high pressure was released.

Estimates for the retained bile salt pool at a point 10 hr into the recovery period are shown in Table II. The bile salt pool can be accounted for by a combination of small losses during high pressure, pool washed out during the initial part of recovery, and pool still retained after this period. There would appear to be a small reduction in pool size during 24 hr of high pressure due to losses, which were not made up because of inhibition of synthesis. Bile salt loss from animals d, e, and G through bile sampling during the high pressure period amounted to 24.3 ± 4.0 µmol/kg (S.E.M.). Urinary losses during the same period were 3.9 ± 0.4 µmol/kg, and this included a very small amount of sulfated bile salts. Fecal bile salt losses were not measured in this study, since 4 to 5 days would be required to obtain a steady state in respect to bile salt loss in stool. However, assuming that intestinal bile salt absorption was not impaired, the loss was approximated as follows: bile salt secretion rate × 0.02 (ref. 15), which in the three animals was 4.7 ± 0.7 µmoles/kg for the high pressure period.

The tissue distribution of radioactive bile salts is shown in Table III. Immediately after the 24 hr period of high pressure, the bile salts were widely distributed, with the largest percent found in liver (animals f and H). Ten hours after high pressure was released (animal G), almost all radioactivity was found in liver, splanchic organs, or blood, i.e., in the enterohpatic circulation.

General effects. Some animals refused the full allotment of food during the high pressure period. There was no correlation between the changes in flow, lipid secretion and composition, and the degree of reduction in feeding. Animal e, which received the liquid elemental diet, responded to high pressure in a manner similar to that of the other animals.
Table III. Percent tissue distribution of radioactive bile salts

<table>
<thead>
<tr>
<th>Animal</th>
<th>Liver</th>
<th>Blood</th>
<th>Splanchnic organs</th>
<th>Muscle</th>
<th>Fat</th>
<th>Skin</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>f</td>
<td>31.9</td>
<td>13.3</td>
<td>8.9</td>
<td>16.6</td>
<td>20.1</td>
<td>7.5</td>
<td>4.6</td>
</tr>
<tr>
<td>H</td>
<td>25.6</td>
<td>3.8</td>
<td>10.2</td>
<td>33.8</td>
<td>7.3</td>
<td>8.1</td>
<td>11.1</td>
</tr>
<tr>
<td>G</td>
<td>28.8</td>
<td>8.6</td>
<td>36.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4.8</td>
</tr>
</tbody>
</table>

In animals f and H, tissues were obtained at the end of the high pressure period. In G, tissues were obtained 10 hr into the recovery period.

Discussion

The experiments were performed on conscious primates remote from surgery with 10% interruption of the enterohepatic circulation of bile salts for purposes of bile sampling. This level of interruption has been shown not to alter normal bile salt secretion rate or pool size, since the induced loss is compensated by increased synthesis of bile salts.15

High biliary tract pressure has been reported to reduce bile flow2, 5, 6 or cause no change.4, 4 In this study, a consistent reduction in flow was induced in two species. Unquestionably, diminished net secretion of bile salts contributed to the reduction in bile flow. The fall in bile salt concentration during high pressure suggests that the component of flow dependent on bile salt secretion was more seriously compromised than non–bile salt dependent parts of bile flow.

The reduction in bile salt secretion at high pressure resulted in a redistribution of bile salts in the body. Normally most of the bile salt pool resides in the biliary passages and intestine in these cholecystectomized animals, but at high pressure it was found in most organs and tissues, with the largest percent in the liver. Presumably, as the pool was reabsorbed from the intestine, it was taken up by the liver but could not be re-excreted. It is likely that the accumulation of bile salts in the liver was responsible for the great suppression of bile salt synthesis. Other bile salts were either not taken up by the liver or were regurgitated and passed into the systemic circulation to a variety of organs and tissues. Once high pressure was relieved, bile salts entered the area of the enterohepatic circulation within a few hours. Bile salt synthesis remained suppressed during the initial part of the recovery period, presumably because the concentration of bile salt in the liver was still high. Another possibility is that there was a lag phase between stimulus and onset of synthesis, as has been demonstrated in normal animals.15 The bile salt pool was slightly reduced by high pressure due to continued small losses and inhibition of synthesis. Bile sampling would not contribute reduction in pool size in clinical circumstances, but the urinary losses and small stool losses would. This effect might cause large reductions in the pool size if the partial obstruction were to continue for some days. The results for synthesis, pool size, and distribution of bile salts produced a consistent pattern in keeping with other findings in the study, although only a few of these valuable animals could be used to assess pool size and distribution in the recovery period.

High pressure reduced cholesterol and phospholipid secretion more than bile salt secretion, but on release of high pressure, cholesterol and phospholipid secretion recovered more rapidly than bile salt secretion. It has been shown that biliary obstruction increases hepatic cholesterol synthesis,24 but cholesterol availability alone cannot explain the changes in the recovery period, since bile salts were also present in the liver in excess. Some independence of cholesterol and phospholipid secretion from bile salt secretion has been suggested in experiments in which secretion of the former lipids was plotted against induced bile salt secretion25, 29 and by other studies which demonstrated that certain
agents such as colchicine cause greater depression of cholesterol and phospholipid secretion than bile salt secretion. Such independence of secretion seems to have also occurred in this experiment, but its cause will remain obscure until more is known about the normal physiology of lipid excretion. Whatever the cause, the result has been a decrease in cholesterol saturation at high pressure and the reverse in the recovery period.

Actual supersaturation was produced in the recovery period when the enterohepatic circulation was completely interrupted; when the interruption was maintained at 10%, the bile became more saturated with cholesterol, but supersaturation did not occur. The normal lithogenic index in rhesus monkey bile is low, and it is possible that in other species with higher normal values, such as man, supersaturation would occur under these circumstances.

The fact that inhibition of synthesis starts shortly after the onset of high pressure is made rather more interesting by the recent observation that some apparently normal persons as well as patients with cholelithiasis have resting common bile duct pressures in the range of the high pressure zone of this study. Patients with cholesterol cholelithiasis are believed to have a small bile salt pool due to an abnormal sensitivity to bile salt feedback inhibition. Although they have a bile salt synthesis rate which is normal, it is inappropriately low for the mass of bile salts returning to the liver. The cause of this sensitivity has not been explained. This study has demonstrated that high pressure can affect bile salt synthesis rates over short periods of time, and it seems possible that high pressure could influence synthesis rates and bile salt pool sizes in humans.

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Rapid method for determining cholesteryl ester transitions of apoB-containing lipoproteins

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Summary  A wide variety of cholesteryl ester-rich apoB-containing lipoproteins undergo an order-disorder transition in the cholesteryl ester core at approximately normal body temperature. The transition occurs over several °C with the mid-point being as high as 57°C in some cholesterol-fed animals. The transition midpoint of normal human low density lipoprotein (LDL) appears to vary from as low as 26°C to about body temperature. However, to screen a large population of patients at risk for atherosclerotic cardiovascular disease (ACD), a rapid method for determining the transition temperature of LDL is needed. Since apoB-containing lipoproteins (VLDL and LDL) are readily precipitated from plasma by dextran sulfate and magnesium sulfate, we have studied the thermal properties of this precipitate using differential scanning calorimetry (DSC). The VLDL-LDL precipitate undergoes a reversible thermal transition similar in transition temperature and enthalpy to the cholesteryl ester transition of isolated pure LDL. The transition is seen with the precipitate from VLDL-free plasma, but no transition is seen when VLDL and LDL have been removed. Cholesteryl ester-rich apoB containing lipoproteins were isolated from a variety of sources (man, cholesterol-fed monkeys, and rabbits) and their transition temperatures compared with the apoB-containing lipoprotein precipitates from the same source. The mid-point of individual variations varied over a wide range (17–57°C) and the correlation between the pure lipoprotein and the plasma precipitate was strong (r = 0.98, P < 0.001). Thus, DSC of the plasma apoB precipitate may be used as a rapid method of determining the cholesteryl ester transition of LDL and other apoB-containing lipoproteins.


Supplementary key words  differential scanning calorimetry • VLDL • LDL • dextran sulfate-Mg²⁺ precipitation of apoB-containing lipoproteins • lipid transition • cholesteryl esters • lipoprotein transitions

Serum low density lipoproteins are the major carriers of cholesterol in human plasma. Most of the cholesterol is esterified and resides in the core of the particle with the other neutral lipid, triglyceride (1). When isolated LDL is heated from 0°C to 45°C it undergoes a reversible endothermic transition which has been ascribed to an order—disorder phase transition of the cholesteryl ester (CE) (2). X-ray diffraction studies suggest that the core cholesteryl ester below the transition is organized in a smectic arrangement, whereas above the transition it is in a more disordered state (3).

Recent experiments in subhuman primates have shown strong positive correlations between the severity of atherosclerosis, LDL molecular weight, and CE transition temperature of LDL (4). Therefore, the transition temperature may be physiologically important. If it were above 37°C, the cholesteryl ester of the LDL would be in a more ordered state at body temperature, which could affect its metabolism.

In normal humans and Type II patients fed polyunsaturated diets, the mean transition of isolated LDL temperature was 30.3 ± 2.3°C. However, some subjects had transitions as low as 26°C while others were as high as 38°C (1, 2). Thus, some subjects and perhaps patients at risk for ACD may have transition temperatures above body temperature. Therefore, we wished to screen a large number of normal subjects and high risk patients for the LDL transition temperature. Since determination of LDL transition requires tedious procedures to isolate and purify intact LDL and concentrate it for calorimetry, we tested the hypothesis that apoB-containing lipoproteins, rapidly precipitated from plasma as polyaniometal complexes (5), would show the LDL transition. Thus, we have compared the thermal properties of the pellet formed when the apoB-containing lipoproteins are precipitated from plasma by dextran sulfate and magnesium sulfate (6) with ultracentrifugally isolated LDL from the same source. We have chosen a variety of LDL or cholesteryl ester-rich lipoproteins from both human and animal sources so that the peak transition temperature ranged from 17–57°C. We find the precipitate undergoes a reversible CE transition which closely resembles that for LDL in temperature and enthalpy and believe that this technique may be used to screen large populations for LDL transition temperatures.

METHODS

Plasma was obtained from five fasted healthy young men on a normal American diet, one woman on a high salmon oil diet,1 nine non-human primates (Cercopithecus aethiops, n = 6, Macaca fascicularis, n = 3) fed either monkey chow or a high cholesterol diet, and three rabbits fed a high cholesterol diet. (The non-human primate samples were kindly supplied by Dr. L. Rudel). In humans and rabbits, lipoproteins were separated by sequential ultracentrifugation (7) (VLDL was isolated at 1.006 g/ml and LDL in density range 1.063–1.066 g/ml). Fractions were dialyzed where necessary to remove KBr and were concentrated by vacuum dialysis in

1 This plasma was kindly supplied by Dr. W. Connor.
preparation for calorimetry as described (2). Lipoproteins from non-human primates were isolated by ultracentrifugation and agarose column chromatography as described (4).

ApoB-containing lipoproteins were precipitated from plasma using dextran sulfate and magnesium sulfate (6). Fifty μl of dextran sulfate solution (20 g/l), and 100 μl of 1.1 M magnesium sulfate were added to 1 ml of plasma. After mixing and centrifuging at 1000 rpm for 10 min, the supernatant was removed and the pellet was transferred to a 75-μl DSC pan (Perkin-Elmer, Norwalk, CT) with a long Pasteur pipet. The same combination of reagents was added to 1 ml of subnatant after VLDL or LDL and LDL had been removed by ultracentrifugation at 1.006 or 1.063 g/ml, respectively. A precipitate occurred with the former, but not after both apoB-containing lipoproteins had been removed.

Calorimetric studies were performed on a Perkin-Elmer DSC-2 differential scanning calorimeter at a full range sensitivity of 0.2 M Cal/sec. Samples were hermetically sealed in stainless steel DSC pans (Perkin-Elmer, Norwalk, CT) and heated at 5°/min. A variety of heating and cooling runs were performed between 0°–45° and 0°–100° C and enthalpies (ΔH) were calculated from the areas under the peaks as measured by planimetry, and compared to an indium standard as described previously (1, 2).

To express ΔH in terms of cholesteryl ester content, following calorimetry DSC pans were opened and the lipids were dissolved in at least 40 volumes of chloroform/methanol 2:1 (v:v) and extracted by the procedure of Folch, Lees, and Sloane Stanley (8). The cholesteryl ester composition was determined by thin-layer chromatography (9). Repeated runs on the same LDL sample gave a variation in peak temperature of less than 0.3°C.

**RESULTS**

Fig. 1 shows representative heating and cooling curves of intact LDL and precipitate from the same plasma sample. LDL undergoes a reversible transition over a range of about 12°C. Qualitatively similar behavior was seen with the precipitate, including a small degree of undercooling when cooling from 45°C to 0°C. The width and shape of the transition of pure lipoprotein and precipitate was similar. The mean enthalpy of transition of the precipitate was 0.68 ± 0.07 cal/g CE (mean ± standard deviation) which is not different from the enthalpy found by us earlier for LDL (0.69 ± 0.06 cal/g CE) (2). On heating the precipitate to 100°C, an irreversible transition occurs. After cooling to 0°C and reheating, a reversible transition occurs between 20°C and 37°C that has a larger enthalpy (0.85 ± 0.04 cal/gm CE), similar to that described previously (1, 2).

To identify the source of the transition, precipitates were prepared from whole plasma, VLDL-free plasma, and VLDL, LDL-free plasma. Fig. 2 shows that a comparison of heating curves obtained from LDL, precipitate from whole plasma, and precipitate from the VLDL-free plasma are similar. When dextran sulfate and magnesium sulfate are added to apoB lipoprotein-deficient plasma, no precipitate is formed and no transition occurs in this precipitate-free mixture.

Fig. 1. Differential scanning calorimetry curves of solutions of pure LDL and whole plasma precipitates from the same subject. a), Heating curve LDL from 0° to 45°C; b), cooling of LDL from 45°C to 0°C; c), heating of whole plasma precipitate 0°–45°C; and d), cooling whole plasma precipitate 45°–0°C.

Fig. 2. Differential scanning calorimetry curves of intact LDL, and plasma precipitates, from the same subject. a), Intact LDL; b), whole plasma precipitate; c), VLDL-free plasma precipitate; and d), apoB lipoprotein-free plasma plus dextran and magnesium sulfate (no precipitate formed).
By using plasma from several sources, we were able to obtain a wide range of lipoprotein transition temperatures. LDL from the human subjects had transition temperatures between 17.7°C and 31.0°C and in cholesterol-fed monkeys between 41.5°C and 57.0°C. Rabbits fed a high cholesterol diet develop hypercholesterolemia and a cholesteryl ester-enriched VLDL (β VLDL) that undergoes thermotropic transitions (11). Since β VLDL is an apoB-containing lipoprotein, it precipitates with dextran sulfate and magnesium sulfate. The isolated rabbit VLDL had transition temperatures at or slightly above body temperature and were identical to those for the precipitate formed from the same plasma.

When the lipoprotein transition temperatures were plotted against precipitate transitions from the same subject a very strong correlation was found (r = 0.98, P < 0.001). The line of best fit lies very close to the line of identity (Fig. 3).

DISCUSSION

Low density lipoprotein undergoes a reversible thermotropic transition at approximately body temperature and an irreversible transition at high temperatures. We have previously shown that the lower transition is due to a reorganization of the cholesteryl ester within the core of the particle and the high transition due to particle disruption (1–3). The pellet of precipitated apoB lipoproteins (VLDL and LDL) appear to undergo very similar transitions. Since normal human VLDL does not undergo any thermal transitions between 10 and 50°C (10), the transition seen in the precipitate appears to arise from the precipitated LDL. Several other lines of evidence support this. First, there is a strong correlation between LDL transition and precipitate transition temperatures, including high melting LDL of cholesterol-fed monkeys. Second, when VLDL is removed from plasma, the transition persists in the VLDL-free precipitate, but not when both VLDL and LDL are removed. Finally, the enthalpy of transition of the precipitate is the same as that of LDL reported in the literature both before and after denaturation (2). Thus, the transition seen in the precipitate most probably arises from LDL.

The main advantages of using the precipitate to identify the LDL transition are the ease and speed with which the determination can be made. Previously, several days were required to isolate and concentrate the LDL sample before calorimetric experiments could be performed. Using the precipitation method, isolation and concentration occur simultaneously and the total determination takes less than 1 hr. Thus, it should prove a valuable tool for screening large numbers of subjects. It should be noted that rabbits fed a high cholesterol diet carry almost all their plasma cholesterol in cholesteryl ester-rich β-VLDL (11). This lipoprotein contains apoB and is thus precipitable by dextran sulfate and magnesium sulfate. The precipitate transitions are identical to those for the isolated β-VLDL and thus probably reflect β-VLDL transitions. The technique, therefore, would have application for determining transition temperatures of any apoB-containing lipoprotein that normally undergoes CE transitions.

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Physical studies of d < 1.006 g/ml lymph lipoproteins from rats fed palmitate-rich diets

Susanne Bennett Clark, David Atkinson, James A. Hamilton, Trudy Forte, Betty Russell, Elaine B. Feldman, and Donald M. Small

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Abstract  At body temperature the stable form of triglycerides rich in saturated fatty acids is crystalline. We examined the physical state of triglyceride-rich lymph lipoproteins from rats fed saturated fat, as a function of temperature. When chylomicrons and very low density lipoproteins were collected, isolated, and examined at 37°C, they were liquid as judged by differential scanning calorimetry, x-ray diffraction analysis, and proton nuclear magnetic resonance spectroscopy, and they appeared spherical by electron microscopy. At 23–26°C, triglyceride began to crystallize in the α form, which transformed to the stable β form at lower temperatures. On cooling from 23°C to 17°C, considerable crystallization occurred and the particle density was increased significantly. When lipoproteins were held at 0–7°C, about 75% of the triglyceride crystallized, distorting the lipoprotein shape. Reheating from 0°C to 37°C left 25% of the triglyceride unmelted. Heating to 58°C was necessary to melt all the crystallized triglyceride and to restore the spherical lipoprotein shape. After complete melting of cooled lipoproteins, the liquid state was maintained on recrystallizing to 37°C, with formation of a metastable particle similar to the nascent lipoprotein. Isolation of lipoproteins containing highly saturated triglyceride at temperatures below 23–26°C results in partial crystallization, alters their physical properties, and may affect their metabolism.—Bennett Clark, S., D. Atkinson, J. A. Hamilton, T. Forte, B. Russell, E. B. Feldman, and D. M. Small. Physical studies of d < 1.006 g/ml lymph lipoproteins from rats fed palmitate-rich diets. J. Lipid Res. 1982. 23: 28–41.

Supplementary key words  chylomicrons • lipid phase behavior • scanning calorimetry • x-ray diffraction • nuclear magnetic resonance spectroscopy • electron microscopy

Intact lipoproteins (LP), such as low density lipoproteins (LDL) from normal human plasma (1), show phase transitions during heating and cooling through temperatures encompassing body temperature. Studies from this laboratory established that these phase changes in LDL are due to phase changes in the cholesterol esters that comprise the LP core (1, 2). Cholesterol ester-rich LP from swine (3, 4), monkeys (5), cattle (6), and rabbits (7) also have been shown to undergo similar transitions. It seems probable that the physical properties of all lipid-rich LP are strongly influenced by the properties of their component lipids.

Lipoprotein isolations are typically performed at temperatures between 4–15°C, a temperature range that encompasses the crystallization temperatures of triglycerides (TG) rich in saturated fatty acids (8, 9). We chose to study dietary LP particles secreted in intestinal lymph because their triglyceride-fatty acid (TG-FA) composition can be modified readily by feeding diets of defined FA composition (10–13). Thus, TG-rich lymph LP, which are secreted during ingestion of diets rich in saturated fat, contain significant amounts of saturated fatty acids (14). The physical properties of chylomicrons and very low density lipoproteins (VLDL) formed under these conditions probably are influenced by the temperature at which they are isolated. Recently, Puppione et al. (15) described a novel TG-rich LP obtained from bovine plasma and lymph, which was characterized by flat appearance, asymmetric shape, and anomalous density after isolation at 4–16°C. It was suggested that this LP was an artifact formed by the crystallization of the saturated TG present within the LP. During the course of our dietary studies in rats (12–14), electron micrographs of chylomicrons and VLDL isolated at 0–4°C revealed similar irregular shapes and prompted the present in-depth study of the physical properties of these lipoproteins. Our experiments demonstrate that highly saturated LP-TG secreted by the intestine in native LP are present as metastable, undercooled liquids. Crystallization of TG and concomitant structural changes in the LP are produced on cooling below the TG nucleation temperature.

Abbreviations: LP, lipoprotein; TG, triglyceride; LDL, low density lipoprotein; VLDL, very low density lipoprotein; FA, fatty acid; TLC, thin-layer chromatography; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance.


METHODS

Lipoprotein collection and isolation

Male rats aged 6–7 weeks were fed a semi-synthetic fat-free diet, to which 15% (wt/wt) triglyceride containing 75% palmitate was added (13, 14), for 4 weeks. The major mesenteric lymphatic trunk was cannulated when the rats were in the fed state and food was removed. Twenty-four hr later they received, by gavage, 100 mg of tripalmitin suspended by sonication in 2 ml of Krebs-Ringer phosphate buffer containing 20 mM Na taurocholate (time 0). The animals were maintained in restraint cages in a constant temperature room held at 37°C and were allowed free access to 0.5 N NaCl containing 100 g/l dextrose. Eight hr after tripalmitin gavage, the high palmitate diet was again administered. Lymph was collected continuously, at 37°C, in two batches, i.e., 0 to 8 hr and 8 to 24 hr after gavage. The collection tubes contained EDTA, NaN3, and DTNB to achieve final concentrations of 1 g/l, 1 g/l, and 4 mM, respectively.

Preparative ultracentrifugation of lymph chylomicrons and VLDL was begun on the day of lymph collection and was performed entirely at 37°C. Aliquots of lymph (9–10 ml) were layered with 2–3 ml of a d 1.006 g/ml solution, pH 7.4, containing NaCl (8.5 g/l), NaN3 EDTA (1.0 g/l), and NaN3 (0.5 g/l), and the chylomicrons were floated in a swinging bucket rotor (SW 41) at 3 × 106 g·min. The top (chylomicron) fraction was harvested by tube slicing and the infranatant was spun at 106 g·min to float VLDL. Lipoprotein fractions containing NaN3 were stored at 37°C and at no time were they allowed to cool below 27°C prior to physical examination. For x-ray diffraction experiments, chylomicrons were further concentrated by recentrifugation under the same conditions. Compositional analysis (see below) of aliquots taken immediately after isolation and at the end of the physical studies confirmed that LP degradation by LCAT and lipoprotein lipase were negligible.

Lipid analysis

Aliquots of the four chylomicron and four VLDL fractions were extracted with chloroform–methanol 2:1 (16) and the total lipid contents were determined gravimetrically, using a Cahn balance (Model 25). Neutral and polar lipid classes were separated by thin-layer chromatography (TLC) and were quantitated by charring and densitometry, essentially as previously described (17), except that tripalmitin replaced triolein as the TG standard used to calculate TG mass. Glyceride-glycerol was also estimated spectrophotometrically (18). TG fatty acids were determined by gas–liquid chromatography of the methyl esters (19) after TLC fractionation of the lipid classes and elution of TG.

Differential scanning calorimetry

Aliquots (70 µl) of each lymph chylomicron and VLDL sample, containing 1.3–4.1 mg lipoprotein TG in d 1.006 g/ml saline solution, were placed in weighed DSC pans prewarmed to 37°C. Reference pans contained 70 µl of the saline solution.

To compare the LP with their component lipids, lipid extracts (16) of one chylomicron and one VLDL sample were concentrated and transferred to weighed DSC pans. The solvent was removed under N2 and the pans, containing 2.5 mg of chylomicron-TG and 6.2 mg of VLDL-TG, respectively, were lyophilized overnight. Reference pans were empty.

Lipoproteins and LP lipids were studied in a Perkin-Elmer DSC-2 differential scanning calorimeter at a sensitivity of 0.1–0.2 mcal/sec and heating and cooling rates of 5°C/min, except where otherwise noted. Preliminary experiments using non-denatured LP established that the enthalpy changes (ΔH) and peak temperatures (Tp) of the major melting transitions, and the onset (To) and peak (Tm) crystallization temperatures during cooling, were the same within experimental error when heating and cooling rates were between 1.25–10°C/min.

For each LP sample, the first scan was a heating run from 37°C to 60°C, that is, from the temperature at which the native LP was secreted to just above the melting point of tripalmitin. Several heating and cooling runs were then performed between 37°C and 60°C. Subsequently, the LP samples were cooled several times to temperatures below 27°C, with each cooling run followed by a heating run to 60°C. The LP was then held between 0°C and −3°C for various times and was again heated to 60°C. Finally, the LP was heated to 90°C to denature and disrupt the particles. Cooling and reheating was then repeated on the denatured LP as described above. Major transitions were defined by the parameters illustrated in Fig. 1.

X-ray diffraction

X-ray diffraction measurements were performed on chylomicrons from one 8–24 hr lymph collection (Rat 2). The LP was further concentrated approximately 4-fold by recentrifugation at 37°C and the sample, suspended in the d 1.006 g/ml saline solution, was scaled in a 1-mm I.D. Lindeman glass tube warmed to 37°C (Lindeman Corp., Indianapolis, IN). X-ray diffraction patterns were obtained over the range 1/60–1/2.5 Å−1 using a Jarrell-Ash microfocus x-ray generator and slit-collimated Luzzati-Baro x-ray camera modified to in-
Fig. 1. Example of differential scanning calorimeter tracing defining major parameters obtained when chylomicrons, isolated at 37°C, are cooled and reheated. Cooling: $T_c$, crystallization onset temperature; $T_p$, peak crystallization temperature. Heating: $T_n$, onset of melting phase transition; $T_m$, melting transition half completed; $T_p$, peak of melting transition; $T_o$, end of melting transition; $\Delta H$, integrated area under the curve, equivalent to the enthalpy change within the system.

include a single mirror focusing system. Diffraction patterns were recorded for 10 min using a linear position sensitive counter (P.S.D. 1100, Tennelec, TN) coupled to a computer based analysis system (TN 1710, Tracer Northern, WI). Short spacings were estimated to $\pm 0.1$ Å and long spacings to $\pm 3$ Å, based on sample-detector distance and the accuracy with which the peak maxima could be located.

To compare the diffraction patterns of intact LP and LP lipids, an aliquot of VLDL from the same lymph collection was heat-denatured at 100°C for 10 min and the lipids were separated by high speed centrifugation in capillary tubes. The top, oil phase was transferred to a Lindeman tube and diffraction patterns obtained as above.

Proton magnetic resonance spectroscopy (NMR)

Fourier transform NMR spectra were obtained on a Bruker WP-200 Spectrometer System, operating at a field strength of 47 KGAuss corresponding to proton observation at 200 MHz; the system was equipped with an Aspect 2000 Data System, a 5-mm proton probe, and quadrature detection.

NMR measurements were made on VLDL from the 8–24 hr lymph collection of Rat 2. The sample tube contained 21.1 mg of TG in 0.7 ml of the d 1.006 g/ml saline solution. D$_2$O ($\sim 10\%$) was added as an internal lock and shim signal and gated homonuclear decoupling was employed to suppress the H$_2$O peak. The pulse interval (4 sec) was sufficient to obtain equilibrium intensities for all peaks, based on spin lattice relaxation measurements in similar systems. Subsequently, to obtain NMR spectra as a function of time, the sample was dialyzed against 10 ml of D$_2$O (Wilmad Co., 99.7% D) for 16 hr at room temperature, heated to 60°C, and concentrated 2-fold in a vacuum desiccator at 40°C.

Sample temperature in the NMR probe was regulated to $\pm 1^\circ\text{C}$ using a Bruker BVT-1000 Variable Temperature Unit; liquid nitrogen vapor was used to cool below 26°C. The sample was pre-equilibrated in a water bath at the planned probe temperature ($\pm 2^\circ\text{C}$) before each measurement, and was further equilibrated for 5 min in the probe before pulsing. After data acquisition for 10–15 min, the sample (containing no vortex plug) was ejected from the probe and its temperature was measured immediately ($<1$ min), using an Omega "Trendicator" (Model 410A) equipped with a thin thermocouple. The sample was then pre-equilibrated at the next temperature. This procedure avoided large temperature fluctuations ($>2$–3°C) between NMR experiments.

Proton peak assignments were made according to Finer, Flook, and Hauser (20). Chemical shifts in parts per million (ppm) downfield from tetramethylsilane, using the terminal methyl peak at 0.90 ppm as an internal standard, were (CH$_3$)$_2$: n: 1.30 ppm; CH$_2$CH$_2$: 1.57 ppm; CH$_2$C = C: 2.02 ppm; and CH$_2$CO: 2.23 ppm; these were temperature independent within the accuracy of measurement ($\pm 0.03$ ppm).

Peak areas representing integrated intensities were determined by planimetry on expanded printouts, to an estimated accuracy of $\sim 10\%$ for T $\geq 23^\circ\text{C}$ and $\sim 20\%$ for T $< 23^\circ\text{C}$. The linewidth (Hz) of the bulk CH$_2$ was measured digitally on the NMR display as the width at half the peak height.

Electron microscopy

Lipoprotein samples, dispersed in the d 1.006 g/ml saline solution, were shipped in insulated Dewar flasks to Berkeley, where they were immediately transferred to a 37°C incubator. LP was not dialyzed before negative staining. Electron microscopy of samples at 37°C or higher was carried out by maintaining sample, stain, grids, and forceps at the desired temperature by means of a slide warmer. A small droplet of sample was placed on the grid and allowed to remain there for 30 sec. The grid was then stained and washed with approximately 20 drops of 2% sodium phosphotungstate, pH 7.4, at 37°C or 58°C, which rinsed most of the excess salt and protein material present in the sample. Negatively stained samples were immediately examined in a JEOL 100C electron microscope.

To determine the effects of low temperature on lipoprotein structure, the samples were placed in the refrigerator (4°C) for 24 hr. During negative staining for electron microscopy, grids, forceps, stains, and samples were kept at 4°C.

Analytical ultracentrifugation

Lymph chylomicrons and VLDL, which had not been cooled below 23°C, were examined by turbidometric an-
alytical ultracentrifugation using a Beckman Model E ultracentrifuge equipped with a photoelectric scanning system and multiplexer, as described by Ma, Schumaker, and Knobler (21). TG-rich LP from the 8–24 hr lymph collection of Rat 2 was diluted 76-fold with 0.196 molal NaCl (1.0063 g/ml) containing Na_2 EDTA (400 mg/l), NaN_3 (500 mg/l), and gentamycin (50 mg/l), at 23°C. The LP dispersions were spun in a Beckman AnD rotor at 3000 rpm (VLDL) or 1500 rpm (chylomicrons), first at 23°C and then at 17.5°C, in order to observe any differences in the flotation rates at the two temperatures. Absorbance at 330 nm along the cell was monitored at 8-min intervals.

RESULTS

Lipid composition of lymph chylomicrons and VLDL

Triglycerides comprised 84.5–86.5% of the chylomicron lipid mass in both 0–8 hr and 8–24 hr collection periods in both rats, and phosphatidylcholine comprised 10.4–12.2%. Free and esterified cholesterol, sphingomyelin, and phosphatidylethanolamine were present in small amounts (0.4–1.3%). Fatty acids, partial glycerides, and lysolecithin were not detectable, even after storage for more than 3 months at 37°C. VLDL was similar to chylomicrons except that less TG (71–81%) and more phosphatidylcholine (14.7–23.8%) were present. In both chylomicrons and VLDL, 58–72% of TG fatty acids were palmitate and 6–16% were stearate. For the eight LP samples examined, TG-fatty acids averaged 74 ± 1% saturated (Table 1).

Differential scanning calorimetry

On first heating lymph chylomicrons and VLDL from 37°C to 60°C, no enthalpy changes were observed. Thus, the LP lipids were in a liquid state when secreted. A typical experiment appears in Fig. 2A and all eight lipoprotein samples showed generally similar patterns.

During cooling from 60°C at 5°C/min, the LP samples began to crystallize at around room temperature (T_c, range 17–22°C, Table 2). Little change in the crystallization onset (T_o) or the peak (T_p) temperatures occurred after the LP was denatured by heating to 90°C. In contrast, lipids isolated from LP began to crystallize at temperatures 10°C higher than within the LP, suggesting that the size, shape, or protein component of the LP favored undercooling of the LP lipid.

When LP or denatured LP samples were reheated from −3°C to 60°C, each showed a single major melting transition. The melting onset temperatures (T_o) ranged between 11–32°C, and melting was complete between 45–64°C in individual samples (Table 3). This major transition corresponded to the second endothermic transition seen in the extracted lipids, which melted over a similar temperature range (Table 3 and Fig. 2B). Heating the LP to 37°C after cooling to 0°C melted half or less of the LP-TG that had crystallized at the lower temperature. Over all the LP samples, the melting enthalpies obtained on immediately reheating the LP after cooling to −3°C appeared to correlate with the proportion of saturated fatty acids in the LP-TG (Table 1). There was a positive association between the 16:0/18:1 ratio and ΔH over all the LP samples (r = 0.67), which just failed to reach significance at the 95% confidence level (where for N = 7, r = 0.70). A positive correlation between ΔH and FA saturation in LP-TG would be predicted since the more saturated the TG, the larger should be the fraction of TG that would be crystalline below T_c.

In separate experiments, the kinetics of crystallization of non-denatured chylomicrons were examined by varying the cooling rate and the time of storage at −3°C. When chylomicrons were cooled at 5°C/min, 12.4 cal/g

<table>
<thead>
<tr>
<th>TABLE 1. Triglyceride fatty acid composition and melting enthalpies of lymph chylomicrons and VLDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
</tr>
<tr>
<td>----</td>
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<tr>
<td>----</td>
</tr>
<tr>
<td>1</td>
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<tr>
<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>2</td>
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</table>

* Lipoproteins were isolated at 37°C as described in Methods.

1 TG-FA were analyzed by GLC of methyl esters after TLC separation of TG.

Mean (SD) of heating runs at 5°C/min from two to six continuous heating-cooling cycles between −3°C and 60°C, at a sensitivity of 0.1–0.2 mcal/sec. Samples were not held at either temperature limit.
TG was released during the exothermic liquid-to-crystalline transition. When the cooling rate was slowed to 0.3°C/min, the crystallization enthalpy was doubled (25.4 cal/g TG), implying that twice as much TG had crystallized during the slower cooling rate. Holding the LP at −3°C produced further crystallization. When chylomicrons were held at −3°C for 18 hr and then reheated at 5°C/min, the melting enthalpy reached 48.5 cal/g TG.

**TABLE 2.** Crystallization onset (T<sub>c</sub>) and peak (T<sub>p</sub>) transition temperatures during cooling of melted lymph lipoproteins and lipoprotein lipids

<table>
<thead>
<tr>
<th>Rat</th>
<th>Collection</th>
<th>T&lt;sub&gt;c&lt;/sub&gt;</th>
<th>T&lt;sub&gt;p&lt;/sub&gt;</th>
<th>T&lt;sub&gt;c&lt;/sub&gt;</th>
<th>T&lt;sub&gt;p&lt;/sub&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>°C</td>
<td>°C</td>
<td>°C</td>
<td>°C</td>
</tr>
<tr>
<td>Lipoprotein</td>
<td>1 0–8</td>
<td>18 (2)</td>
<td>7 (1)</td>
<td>17 (1)</td>
<td>10 (0)</td>
</tr>
<tr>
<td></td>
<td>1 8–24</td>
<td>22 (0)</td>
<td>14 (3)</td>
<td>19 (2)</td>
<td>6 (1)</td>
</tr>
<tr>
<td>Heat-denatured lipoprotein</td>
<td>1 0–8</td>
<td>20 (1)</td>
<td>ND</td>
<td>17 (1)</td>
<td>&lt;0</td>
</tr>
<tr>
<td></td>
<td>1 8–24</td>
<td>20 (4)</td>
<td>17 (0)</td>
<td>19</td>
<td>14 (0)</td>
</tr>
<tr>
<td>Extracted lipid</td>
<td>2 0–8</td>
<td>21 (2)</td>
<td>16 (0)</td>
<td>21 (2)</td>
<td>16 (0)</td>
</tr>
<tr>
<td></td>
<td>2 8–24</td>
<td>32 (1)</td>
<td>29 (0); 6 (0)</td>
<td>28 (0)</td>
<td>18;4</td>
</tr>
</tbody>
</table>

* No transitions were observed when lipoproteins, isolated at 37°C, were heated and cooled between 37–60°C.

* Lipoproteins containing 1.3–4.1 mg TG were cooled to −3°C and lipids were cooled to −23°C, at 5°C/min at a sensitivity of 0.1–0.2 mcal/sec.

* Lipoproteins were denatured by heating to 90°C.

* Two distinct transitions were observed, unlike lipoproteins, as shown in Fig. 2, Chylomicron TG 2.5 mg; VLDL TG 4.2 mg.

Data are means (SD) of one to four cooling runs; ND, not determined.
### Table 3. Temperature limits and peak temperatures for endothermic melting transitions of lymph lipoproteins and lipoprotein lipids

<table>
<thead>
<tr>
<th>Rat</th>
<th>Collection</th>
<th>Chylomicrons</th>
<th>VLDLa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hr</td>
<td>Tp</td>
<td>Tm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>°C</td>
<td>°C</td>
</tr>
<tr>
<td>Lipoprotein</td>
<td>1</td>
<td>0–8</td>
<td>21 (7)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>8–24</td>
<td>21 (5)</td>
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<tr>
<td></td>
<td>2</td>
<td>0–8</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8–24</td>
<td>31 (3)</td>
</tr>
<tr>
<td>Heat-denatured lipoprotein</td>
<td>1</td>
<td>0–8</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>8–24</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0–8</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8–24</td>
<td>28</td>
</tr>
<tr>
<td>Extracted lipid</td>
<td>2</td>
<td>0–8</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8–24</td>
<td>-9 (29)</td>
</tr>
</tbody>
</table>
* Tp and Ts define the start and end of the melting transition, Tm defines the temperature at which the lipids are half melted, and Tp defines the peak of the melting transition, as illustrated in Fig. 1.

Lipoproteins and lipids were heated at 5°/min from -3°C (LP and denatured LP) or from -23°C (lipid) at a sensitivity of 0.1–0.2 kcal/ sec.

Lipoproteins were denatured by heating to 90°C.

**Two transitions were observed, unlike lipoproteins, as shown in Fig. 2.

Data are means (SD) of one to four heating runs; ND, not determined.

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similar to the melting enthalpy (beta to liquid) for tripalmitin (53.1 cal/g) and tristearin (54.5 cal/g).

Overall, the data imply that the native LP was secreted with the lipids present as undercooled liquids at 37°C. When cooled to around 0°C, about half of the component TG crystallized rather rapidly; rewarmed the LP to 37°C melted about half of the crystallized TG, equivalent to only about 75% liquid after cooling and rewarmed. Heating to above the transition temperature (i.e., to 55–60°C) was necessary to melt all the TG once the LP had been cooled to 0°C. When LP was held at about 0°C for 18 hr, crystallization continued until possibly 90% of the TG had crystallized.

**X-ray diffraction**

To investigate the structural organization of the triglycerides in intact chylomicrons, x-ray diffraction studies were carried out at specific temperatures previously shown by calorimetry to produce a stable lipid state following a phase transition. Representative diffraction patterns obtained from chylomicrons of the 8–24 hr lymph collection from Rat 2 are illustrated in Fig. 3. At 37°C, immediately following isolation and concentration at the same temperature, the x-ray diffraction pattern exhibited a broad diffuse scattering, centered at 1/4.5 Å⁻¹ and superimposed on a steeply increasing background due to water scattering. This diffuse scattering at 1/4.5 Å⁻¹ is typical of triglycerides in the liquid state.

After rapid cooling to 0.8°C, a diffraction at 1/4.2 Å⁻¹, corresponding to the alpha polymorphic form of TG (22), became the dominant feature of the diffraction pattern. Three orders of long spacing corresponding to 43 Å were also observed at 0.8°C. On reheating to 20°C, the pattern changed to one exhibiting sharp diffractions at 1/4.6 Å⁻¹ and 1/3.8 Å⁻¹ in the wide angle region, together with three well-resolved orders of long spacing at 41 Å in the low angle region. These spacings, particularly those in the wide angle region, are typical of TG in the beta polymorphic crystalline form (22). Always discernable at 1/4.5 Å⁻¹, however, was a broad diffuse scattering suggesting a substantial residual liquid component. Heating to 32°C and 37°C produced no further changes in the diffraction pattern. Following heating to 60°C (the end point of the endothermic transition observed on heating in the calorimeter), the diffraction pattern once again transformed to that typical of liquid triglyceride. Subsequent rapid recouling to 11°C resulted in crystallization into the alpha polymorphic form, identifiable by a sharp diffraction maximum at 1/4.2 Å⁻¹. Again a substantial liquid component was observed at this temperature.

Kinetic aspects of the crystallization of chylomicron TG were followed in an experiment in which the chylomicron sample was cooled rapidly from the liquid state at 60°C to 1°C. Subsequent x-ray diffraction patterns were recorded at 30-min intervals with the sample held isothermally at 1°C (data not shown). Initial crystallization occurred into the alpha polymorphic form (diffraction maximum at 1/4.2 Å⁻¹), which slowly transformed into the beta polymorphic form (sharp diffractions at 1/4.6 and 1/3.8 Å⁻¹); by 7 hr no alpha form remained. At all times a liquid component was also present at 1/
4.5 Å⁻¹. These studies suggest that the stable beta TG polymorph of the native LP can be significantly under-cooled, and that the chylomicron TG first crystallizes into the alpha polymorph which transforms slowly to the stable beta form. A summary of the x-ray diffractions at long and short spacings appears in Table 4.

For comparison with whole LP, the x-ray diffraction pattern of the lipids isolated from lymph VLDL is shown in Fig. 4. The liquid pattern seen at 60°C persisted as the lipids were cooled rapidly to 37°C. On further cooling to 26°C, the beta polymorphic form appeared with wide angle spacings at 1/4.6 Å⁻¹. At −4°C, both alpha and beta forms were seen. On reheating to 23°C or to 37°C, the alpha form disappeared but the beta polymorph remained present, not melting until 55°C. The alpha to beta transition, which occurred close to 23°C, probably was responsible for the marked enthalpy change observed by DSC at the same temperature (see Fig. 2B). Holding the LP lipids at −10°C for 16 hr did not appreciably alter the proportion of alpha and beta forms seen when the lipids were first cooled to −4°C. This contrasts with the results obtained with whole LP, where the alpha form slowly disappeared at low temperatures. The x-ray diffraction data thus also suggest a constraining influence of the LP size, shape, or protein component on the physical properties of the LP lipids.

NMR

Proton NMR spectra of VLDL from the 8–24 hr lymph collection of Rat 2 were obtained sequentially at 38.5, 31.5, 26, 23, 18.5, 14.5, 13, and 8°C. The LP sample was then cooled in ice-water for 10 min and additional spectra were obtained at 23°C and 41°C. After heating to ~50°C for 15–20 min, the sample was stored at 37°C for 6 days and a final spectrum was obtained at 40°C under the previous conditions. This spectrum was identical in peak intensities and linewidths to the original spectrum obtained at 38.5°C.

A second series of temperature-dependent spectra was obtained one week later in the sequence 36, 31, 26.5, 23.5, 18.5, 16.5, 13, 10.5, 7.5, 0°C for 15 min, then 7.0, 15, 19, 22.5, 35.5, and 44°C. The aliphatic region of selected spectra obtained during cooling is shown in Fig. 5. For all peaks the linewidths increased and the intensities decreased markedly as the temperature was lowered through the thermal transition observed by DSC and x-ray diffraction when the LP was cooled (see Figs. 2A and 3). The intensities of the allylic peaks changed less with temperature than the intensities of α CH₂ peaks (see spectral inserts with higher vertical expansion, Fig. 5).

The integrated intensities for proton peaks at different locations along the fatty acyl chains (α CH₂ protons, allylic protons, and methyl protons) during the two heating and cooling runs are shown as a function of temperature in Fig. 6A, B, C. Agreement between the two trials was within the experimental measurement error of 10–20%. The intensities at any given temperature differed with the thermal history. During cooling all three intensities were constant between 38°C and 25°C and then decreased rapidly, while during heating they increased linearly between 8°C and ~45°C. At ~45°C the peak intensities were 80–90% of the original intensities observed at 37°C before the LP were cooled, suggesting that most TG had melted. This agrees with the DSC experiments which also demonstrated ~80% melting of cooled LP at 45°C (see Fig. 2A). The ratio (not shown) of the α CH₂ (Fig. 6A) to the CH₃ (Fig. 6C) peak areas, which represents the relative mobilities of protons at the carbonyl end compared with those at the terminal end of the acyl chains, remained constant during both heating and cooling. These data imply that both ends of the chain crystallized and melted simultaneously.

The temperature-dependent behavior of the allylic peak differed from those of the terminal methyl and the
TABLE 4. Summary of x-ray diffraction data

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Long Spacings*</th>
<th>Short Spacings</th>
<th>Polymorphic Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>Å</td>
<td>Å</td>
<td></td>
</tr>
<tr>
<td>Chylomicrons (Rat 2, 8–24 hr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37 (following isolation) 0.8</td>
<td>absent 43</td>
<td>4.5 diffuse liquid</td>
<td>4.2</td>
</tr>
<tr>
<td>20</td>
<td>41</td>
<td>4.5 diffuse liquid</td>
<td>4.6, 3.8 β</td>
</tr>
<tr>
<td>37 (following 0.8 and 20)</td>
<td>41</td>
<td>4.5 diffuse liquid</td>
<td>4.6, 3.8 β</td>
</tr>
<tr>
<td>VLDL lipid (Rat 2, 8–24 hr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37 (following 55–60) −4</td>
<td>absent 43</td>
<td>4.5 diffuse liquid</td>
<td>4.6, 3.8 β</td>
</tr>
<tr>
<td>23</td>
<td>44</td>
<td>4.5 diffuse liquid</td>
<td>4.6, 3.8 β</td>
</tr>
<tr>
<td>37 (following −4)</td>
<td>~48</td>
<td>4.6, 3.8 β</td>
<td>4.5 diffuse liquid</td>
</tr>
</tbody>
</table>

* The accuracy of the long spacing data was limited by the resolution of the x-ray detection system. Values are reported primarily to document further the observation of the sharp diffraction observed indicative of crystallization.

α methylene peaks. On cooling the LP from 37°C to 10°C, the CH₃ and α CH₂ peak areas decreased by 60–80% (Fig. 6C and 6A) while the allylic peak was only halved (Fig. 6B), implying that relatively more of the saturated acyl chains were immobilized during TG crystallization. The preferential crystallization of the satu-

![Fig. 4. Wide angle x-ray diffraction patterns of lymph VLDL lipids as a function of temperature. Lipids were isolated from heat-denatured VLDL obtained from the 8–24-hr lymph collection of Rat 2, as described in Methods. Diffraction patterns were obtained sequentially at the indicated temperatures. Unlike the intact LP, both alpha and beta polymorphs are present upon rapid cooling and there is no transformation during cold storage.](image)

![Fig. 5. The effect of cooling from 36°C to 10°C on the proton NMR spectrum of lymph VLDL. VLDL was isolated from the 8–24-hr lymph collection of Rat 2, as described in Methods. Starting with the lipoprotein in the native state at 36°C, spectra were recorded at decreasing temperatures down to 10.5°C. Each spectrum is the resultant of ten spectral accumulations obtained with a pulse interval of 4.0 sec, 8192 time domain points, and 2000 Hz spectral width. Chemical shift assignments were CH₃: 0.9 ppm; (CH₃)₂: 1.30 ppm; CH₂=CH₂CO: 1.57 ppm; CH₂C = C: 2.02 ppm; CH₂=CO: 2.23 ppm. Instrumental conditions were matched for all spectra; inserts show selected regions at 4-fold vertical expansion.](image)
obtained immediately on reheating to 37°C. The integrated intensities of all the peaks decreased rapidly after cooling to 6–7°C with little further change after 8 hr. The areas of the bulk CH$_2$ peak as a function of time, normalized to native uncooled LP at 37°C, are shown in the insert in Fig. 8. The final spectrum, obtained at 37°C after the LP was held at 6–7°C for 18 hr, shows that the intensities of all peaks were lower than in the initial 37°C spectrum; integration of the bulk CH$_2$ peak area indicated that about 25% of the TG had not melted. This is similar to the results for other resonances (Fig. 6A, B, C) and corroborates the data obtained by DSC and x-ray diffraction experiments. As shown in Fig. 8, the LP solidified at 6–7°C with a half-time of less than 0.15 hr. Ultimately, 70–75% of the TG solidified at that temperature, leaving 25–30% liquid.

**Electron microscopy**

Changes in chylomicron morphology as a function of temperature are shown in representative electron micrographs in Fig. 9 and are summarized in Table 5. Chylomicrons maintained at 37°C were round particles, extremely heterogeneous in size (Fig. 9A). After incubation at 4°C, 20–30% of the particles appeared flattened and polygonal (Fig. 9B). However, after incubating the chylomicrons at 58°C for 10 min the lipoprotein profile was again round (Fig. 9D) and the size distribution was similar to that of the native particles (Table 5).

VLDL exposed to low temperatures (not shown) displayed structural alterations similar to chylomicrons. At 4°C many particles became flattened and polygonal and this morphology could not be completely reversed by subsequent incubation at 37°C. Following a 10-min incubation at 58°C, however, the VLDL again appeared

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Fig. 6. Intensities of individual peaks in the proton NMR spectrum of lymph VLDL, illustrating supercooling. A: Intensity of methylene protons as a function of temperature. B: Intensity of methylene protons alpha to olefinic double bonds. C: Intensity of terminal methyl protons. D: Ratio of intensities of A/B expressed as a percentage of the ratio in the melted state. The rapid decrease in ratio below 25°C implies that, on cooling, the carbonyl region of the acyl chains, representative of both saturated and unsaturated fatty acids, is more immobilized than the central olefinic region of the unsaturated fatty acids. Symbols used: cooling, O, Δ; run I, O, ●; heating, ●, △; run II, Δ, ▲.

Fig. 7. Line widths (ν/ν) of bulk methylene protons as a function of temperature. Two separate NMR experiments are illustrated, with symbols as in Fig. 6.
spherical with a similar size distribution to the original VLDL. There was no evidence that heating LP to 58°C produced significant coalescence of the particles.

**Analytical ultracentrifugation**

Turbidimetric ultracentrifugation of lymph LP was performed first at 23°C, just above the TG crystallization temperature (Table 2), and then at 17.5°C, where about half the TG was crystalline (See Fig. 6). Previous studies by DSC (see Fig. 2A) revealed that cooling the LP from 23°C to 17°C was accompanied by a large exothermic (liquid to crystalline) phase change. Based on a TG content equal to 70% of the VLDL mass, a crystalline TG density 12% higher than that of liquid TG (23), and 50% of TG crystallized at the lower temperature, the density of the VLDL particles should have been approximately 4% higher at 17.5°C than at 23°C. The results of the first five scans at each temperature for rat lymph VLDL, taken 8 min apart, are shown in Fig. 10. There was a dramatic decrease in the rate of flotation of VLDL particles at 17.5°C compared with 23°C, reflecting the greater density of the VLDL at the lower temperature. Qualitatively similar results were obtained for chylomicrons (data not shown).

**DISCUSSION**

Recent discovery of lipid phase transitions in cholesterol ester-rich lipoproteins (1) indicated that lipids in the core of lipoproteins can behave similarly to bulk lipids. Cholesterol esters of biological origin, which have transitions around body temperature (1, 2, 6), display similar but not identical transitions when located in the core of small lipoprotein particles (2, 6). We wished to understand the physical state of the triglycerides located in the core of triglyceride-rich particles synthesized by the intestine, and how temperature affected the lipoprotein structure and properties. We have examined triglyceride-rich intestinal lipoproteins obtained from rats fed a diet rich in palmitic acid. The triglycerides of these lipoproteins contained about 74% saturated fatty acids, primarily palmitic acid. It is known that neutral triglycerides containing a high content of palmitic acid have melting points considerably above body temperature (8, 9, 22, 24). Recently it was shown that highly saturated VLDL secreted by isolated rat livers during perfusion with palmitate displays a major transition between 0-20°C (25).

Pupponi et al. (15) described triglyceride-rich particles, isolated from bovine plasma and lymph, that contained a high proportion of saturated fatty acid triglycerides, were too dense, were irregularly shaped, and that scattered light anomalously. It was suggested that these particles might contain crystalline triglycerides. Subsequently we demonstrated that crystalline triglycerides were present (26). Furthermore, triglyceride-rich particles from calf lymph, which contain highly saturated triglycerides, are spherical and float as VLDL when iso-
Fig. 9. Chylomicron morphology as a function of temperature. Chylomicrons were isolated at 37°C and were negatively stained as described in Methods. A, Freshly isolated at 37°C. Chylomicrons have round profiles and are extremely heterogeneous in size (see Table 5). B, After storage at 4°C for 24 hr, many particles became flattened and polygonal in shape as indicated by arrows. C, Heated to 37°C after 24 hr at 4°C. Persistence of polygonal particles is evident. D, Heated to 58°C for 10 min. Particles are again round in profile and are similar to the starting sample.

lated at 37°C, but become angular and more dense when cooled to 16° (26). Thus, it was suggested that cooling this VLDL to 16°C could initiate crystallization of the triglyceride and alter the shape and density of the particles.

The present report describes an in depth study of chylomicrons and VLDL isolated from intestinal lymph of rats fed a diet designed to enrich these lipoproteins with saturated fatty acids. The lipoproteins were isolated and maintained at 37°C so that they could be characterized first in their nascent state; the effects of changing the temperature were then evaluated. Chylomicrons and VLDL secreted by the intestine differed in their mean size but the fatty acid composition of the triglycerides was similar, with 74% of TG-FA saturated. The final melting point of the triglycerides isolated from these lipoproteins was 48–55°C, similar to that of the lipoproteins themselves (56 ± 6°C; ± ± SD). A variety of physical techniques was used to study the physical properties of the lipoproteins. Differential scanning calorimetry
TABLE 5. Chylomicron and VLDL size and shape as a function of temperature

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Polyangular</th>
<th>Spherical</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Long Axis</td>
<td>Short Axis</td>
</tr>
<tr>
<td>°C</td>
<td>#</td>
<td></td>
</tr>
<tr>
<td>Chylomicrons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A 37, freshly isolated</td>
<td>3514 (1342)</td>
<td>1416 (624)</td>
</tr>
<tr>
<td>B 4</td>
<td>3009 (709)</td>
<td>1103 (330)</td>
</tr>
<tr>
<td>C 37 after 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D 58 after 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLDL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A 37, freshly isolated</td>
<td>594 (193)</td>
<td>200</td>
</tr>
<tr>
<td>B 4</td>
<td>1827 (604)</td>
<td>897 (297)</td>
</tr>
<tr>
<td>C 37 after 4</td>
<td>2095 (664)</td>
<td>918 (249)</td>
</tr>
<tr>
<td>D 58 after 4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ Number of particles measured.
² Mean (SD).

Lipoproteins were negatively stained and examined at the temperatures indicated, and were photographed and measured at a magnification of ×60,000. Approximately 20–30% of the particles appeared polygonal under conditions B and C. More precise determination of the proportion of irregularly shaped LP was not possible due to clumping of the particles on the grids.

determined the temperature ranges and quantitated the enthalpies (ΔH) of the phase transitions. X-ray diffraction experiments defined the polymorphic forms of the crystalline material. NMR experiments determined the quantity of crystalline material present and the chemical type of the fatty acid molecules undergoing crystallization. Supporting evidence was obtained from electron microscopy, which was used to describe the sizes and shapes of particles, and from analytical ultracentrifugation, which defined their densities. The thermal histories of the particles were monitored meticulously so that in all experiments appropriate thermal histories were compared.

Our experiments have demonstrated that native VLDL and chylomicrons as isolated from lymph at ~37°C behave similarly and that their triglycerides are in the liquid state supercooled 0–15°C below their peak melting points. The particles are spherical and the particle density is appropriate for triglycerides in the liquid state. When the lipoproteins are cooled, crystallization is initiated at about 22°C, first to the alpha form of triglyceride which later undergoes a polymorphic transition to the stable beta form. When cooled very slowly to <7°C, or stored at laboratory coldroom temperatures (0–4°C), 75% or more of the triglyceride crystallizes in the beta polymorphic form, which distorts the normally spherical particle to angular, non-spheroidal shapes. Approximately 25% of the triglycerides remains in the liquid state at 6–7°C and these triglycerides are enriched in fatty acids with double bonds. When the partially crystalline particles are reheated, they undergo a gradual transition to the liquid state between about 20°C and about 60°C, with a peak temperature at 44 ± 6°C.

Cooled particles reheated only to 37°C still contain approximately 25% crystalline triglyceride. Thus, nascent triglyceride-rich lipoproteins containing highly saturated triglycerides are in a metastable, undercooled liquid state and isolation at temperatures below about 25°C causes partial crystallization of the lipoprotein triglycerides. Reheating to body temperature does not melt all of the crystallized triglyceride or restore the lipoprotein to its native state. Heating to 58°C causes complete melting.

![Graph](attachment:image.png)

Fig. 10. Analytical ultracentrifugation of lymph VLDL as a function of temperature. VLDL was isolated from the 8–24-hr lymph collection of Rat 2, as described in Methods. Analytical ultracentrifugation was performed in a d 1.0063 g/ml medium, first above the crystallization temperature (23°C) and then after crystallization of about half the TG (17.5°C). The figure, which shows the first five scans at each temperature (t1–t5) taken 8 min apart, illustrates the markedly reduced flotation rate at the lower temperature due to the increased density of the partially crystallized lipoprotein.

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and forms a particle morphologically similar to the nascent lipoprotein. Cooling from 58°C to 37°C re-establishes a metastable particle.

The implications of these studies are important. Virtually all standard techniques for isolating triglyceride-rich lipoproteins from plasma or lymph include some steps in which the preparation is cooled (e.g., the sample is collected over ice, isolations are carried out at or below room temperature, the samples are stored in a refrigerator). Thus, it is probable that all chylomicrons containing highly saturated triglycerides undergo partial crystallization during their isolation. Crystalization produces particles with abnormal shapes, and, in the case of the bovine lipoprotein (15), this results in the formation of a lipoprotein fraction that isolates in the intermediate density range (IDL). The metabolic fate of such partially crystallized particles when re-injected into animals is not known, but it may differ from that of the native lipoprotein isolated and maintained at 37°C. Floren and Nilsson (27), for example, noted that chylomicrons from butter-fed rats, isolated at low temperatures, behaved differently from chylomicrons isolated from corn oil-fed animals. Lipoprotein lipase appeared to hydrolyze the unsaturated triglycerides preferentially, leaving the saturated triglycerides in the remnant core. This selective lipolysis may have resulted from selective crystallization of the more saturated triglycerides which made them unavailable for the lipase reaction. Floren and Nilsson (27) noted further that more unhydrolyzed triglyceride remained in the liver when chilled chylomicrons from butter-fed rats were injected, compared with chylomicrons rich in polyunsaturated fatty acids. Possibly, chylomicron remnants that contained crystalline triglycerides were taken up by the liver but were not degraded normally. Future studies of chylomicron catabolism may require that the chylomicrons, particularly those obtained from animals fed saturated fat diets, are isolated at temperatures above their triglyceride crystallization temperature. We are at present comparing the metabolism of native chylomicrons isolated at 37°C with that of chylomicrons isolated at lower temperatures, which contain partially crystallized triglycerides and whose apoprotein profiles may differ from those of the nascent particles.2

The detailed effects of the degree of fatty acid unsaturation, the fatty acid chain length, or the triglyceride stereoisomerism on lipoprotein transition temperatures are not clearly known. However, we can predict that the transition temperature should be related to the degree of unsaturation. Triglycerides present in normal human VLDL are not highly saturated and do not show triglyceride or cholesterol ester transitions in the range of 10–80°C (28). The VLDL does, however, crystallize at about −10°C and melts between −20°C and 10°C (29). We have also shown that chylomicrons obtained from monkeys fed safflower oil have no transitions when isolated at 4°C.3 Just how much unsaturation is necessary to suppress the crystallization temperature below 4°C is presently under investigation.4

Until all possible effects of cooling LP below their crystallization temperatures on their physical, chemical, and metabolic properties are completely resolved, it seems advisable to perform all maneuvers above the LP crystallization temperature if this is known, or else at 37°C, which may require greater emphasis on sterility during handling. Because this approach is technically cumbersome, it is important to establish methods that will restore chilled particles to their original structure and composition. We have shown that heating to 55–60°C restores the physical properties of chilled particles. Possibly, similar treatment in the presence of cofactors (e.g., reheating chilled lymph to 60°C prior to chylomicron isolation) will also restore their chemical and metabolic properties. Such studies are under active investigation in our laboratory.

We thank Dr. Donald Puppione for the analytical ultracentrifugation, Ms. M. Prack for some of the lipid analyses, and Ms. I. Miller for typing the manuscript. This study was supported by National Institutes of Health Research Grants HL 18623, HL 07291, HL 18574, and AM 26658, and the National Livestock and Meat Board. Dr. Atkinson is an Established Investigator of the American Heart Association.

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3Lymph chylomicrons obtained from rats fed a diet with 63% of the fatty acids saturated also showed transitions around room temperature, although the LP was liquid when secreted, similarly to chylomicrons from rats fed the 75% saturated fatty acids. On cooling from 37°C in the DSC, crystallization began at 20–24°C. On reheating from 2°C, the crystallized lipids in the LP began to melt at 15–23°C, peak melting occurred at 46–50°C and melting was complete at about 60°C. Since diets containing fats which are about 60% saturated are commonly fed in dietary studies and LP separations usually have been performed below 20°C, reevaluation of earlier experiments seems advisable, particularly those reports which appeared to show that dietary TG composition influenced the differential formation and composition of LP classes separated by ultracentrifugation.
Solubilization and localization of triolein in phosphatidylcholine bilayers: A $^{13}$C NMR study

[triglyceride (triacylglycerol)/phospholipid bilayers/carboxyl groups/chemical shift]

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ABSTRACT Cosonicated mixtures of egg phosphatidylcholine and small amounts (<5% wt/wt) of triolein have been studied by $^{13}$C NMR spectroscopy. The 50.3-MHz $^{13}$C NMR spectrum of vesicle preparations containing 90% isotopically substituted [1-$^{13}$C]triolein showed two carboxyl resonances at chemical shift values that indicate hydrogen bonding of H$_2$O molecules with the carbonyl groups. The extent of hydration, estimated from the chemical shift values (173.07 ppm and 173.39 ppm) is ~50%. The data suggest that the triolein is located in the bilayer with the three carbonyl groups at the aqueous interface. The acyl chains are extended toward the bilayer interior, with a conformation of the glycerol region such that the primary ($\alpha$) carboxyls are closer to the aqueous medium than is the secondary ($\beta$) carboxyl. Thus, triolein is present in the bilayer in an orientation appropriate for enzymatic hydrolysis, with the second substrate (H$_2$O) in close proximity to the hydrolytic site, and with a conformation that could explain, in part, enzymatic specificity for hydrolysis at the $\alpha$ position. Spectra of vesicles containing 5-10% triolein showed two additional carboxyl peaks characteristic of pure (neat) triolein. This allowed a determination of the maximum solubility (~2.8%) of surface-oriented triolein in the bilayer phase. Beyond this limit all excess triolein partitions into a separate oil phase.

Long-chain triglycerides (triacylglycerols) are water-insoluble neutral lipids that serve as the main storage form of fatty acids in most animals and plants. During their metabolism and transport, triglycerides undergo enzymatic hydrolysis to release the constituent fatty acids. To achieve hydrolysis the lipolytic enzymes must gain simultaneous access to the water-insoluble triglycerides and to water. How this is possible is not currently understood. Carbonyl groups impart a slight polar character to triglycerides, permitting the molecules to spread at an air-water interface and to incorporate to a limited extent into phospholipid monolayers at an air-water interface (1, 2). Phase equilibrium techniques have shown that triglyceride emulsions contain a small amount (2-5%) of triglyceride in the surface phospholipid monolayer (3). Such observations suggest that triglyceride lipases may act on triglyceride molecules located at the surface of lipoproteins and certain cellular systems, such as the plasma membrane of the capillary endothelium.

This study utilizes $^{13}$C nuclear magnetic resonance (NMR) spectroscopy to determine the solubility and location of triolein in an aqueous phospholipid bilayer system. Hydrogen bonding with solvent molecules affects the $^{13}$C chemical shift(s) of carboxyl carbons in small organic molecules (4) and phospholipids (5, 6). Similarly, triglyceride molecules with carbonyl groups exposed to an aqueous environment should exhibit carbonyl $^{13}$C chemical shifts quite different from those in a hydrocarbon environment (7, 8). We have used the water-soluble triglyceride triacetin (triacylglycerol) to determine $^{13}$C chemical shifts in aqueous and nonaqueous environments. To detect and quantify the small amounts of triglyceride soluble in lecithin bilayers, we have employed 90% isotopically substituted [1-$^{13}$C]-((carboxyl-$^{13}$C)) triolein (trioleoylglycerol) in cosonicated triglyceride/phospholipid mixtures. The $^{13}$C nucleus provides a nondestructive, nonperturbing probe of carbonyl molecular environment and dynamics.

MATERIALS AND METHODS

Materials. Triacetin was obtained from Sigma; the $^{13}$C NMR spectrum showed no impurity peaks >1%. Egg yolk phosphatidylcholine (PtdCho) (lecithin) was obtained from Lipid Products (Nutley, England), and triolein from Nu Chek Prep (Elysian, MN). [1-$^{13}$C]Triolein was obtained from Koe Isotopes (Cambridge, MA). Purity (>99%) was verified by thin-layer chromatography and $^{13}$C NMR spectroscopy. A spectrum of [1-$^{13}$C]triolein in C$_2$H$_3$Cl$_3$ with signal-to-noise ratios of the carbonyl peaks of >100:1 showed only two carbonyl resonances, assigned to the $\beta$ (sn-2) chain and the $\alpha$ (sn-1 and sn-3) chains.

Vesicle Preparation. Egg PtdCho was dissolved in 2:1 (vol/vol) CHCl$_3$/CH$_3$OH, and triolein was dissolved in CHCl$_3$. All triolein used in the vesicle preparations was 90% isotopically substituted [1-$^{13}$C]triolein. The desired amounts of lipids were transferred into a 50-ml round-bottom flask, redispersed with 20 ml of CHCl$_3$, and dried as a thin film for 12 hr under reduced pressure. Then 1.6 ml of buffer (0.05 M KBr/0.01 M potassium phosphate/0.1 mM EDTA/0.1% sodium azide, pH 7.4), or 0.5-2.0% (wt/vol) aqueous KCl was added to the flask. In the latter case, distilled, deionized water was boiled to bring the pH to neutrality, and all subsequent steps were done under N$_2$ to keep the pH above 6.5. The sample was agitated for 1 hr at room temperature on a Vortex mixer and then transferred to a centrifuge tube, using 0.1 ml of buffer and 0.1 ml of H$_2$O to rinse the flask. The sample compositions are given as % triolein by weight of total lipid (i.e., 5% triolein, 95% PtdCho), with the PtdCho concentration ranging between 10 and 100 mg/ml. The sample was sonicated by using a Branson sonifier with a microtip, at power level 3 in a pulsing mode with a 30% duty cycle. The temperature, monitored by a thin thermocouple inserted into the sample, was <35°C. Samples were centrifuged for 30 min at low speed to remove titanium fragments. Selected samples were fractionated by ultracentrifugation for 10 hr at 140,000 g at 15°C in 0.53% KCl (r = 1.004 g/ml).

After NMR analysis, samples were analyzed for composition and purity. No (<1%) unesterified fatty acid or lysolecithin was detected by thin-layer chromatography. The PtdCho concentration of the samples in aqueous KCl was determined by a modified Bartlett method (9).

NMR Methods. Fourier-transform NMR spectra were obtained at 50.3 MHz with a Bruker WP200 spectrometer. A 90° $^{13}$C pulse (12 $\mu$sec), quadrature detection, and broad-band $^1$H

Abbreviations: PtdCho, phosphatidylcholine; NOE, nuclear Overhauser enhancement; Me$_3$Si, tetramethylsilane.
RESULTS

Fig. 1 shows the $^{13}$C NMR spectrum of neat and aqueous (3%, vol/vol) triacetin obtained in a single experiment. Dilution to concentrations below the critical micellar concentration (=2%, vol/vol) of triacetin (14) did not affect $\delta$ values. Aqueous solvation affects the chemical shift of every carbon resonance except the resonances for the methyl groups. However, the effect is small (=0.4 ppm) for the glycerol carbons and large (=3 ppm) for the carbonyl carbons.

Although triolein is insoluble in water, solvation effects on triolein carbonyl resonances were demonstrated by comparison of spectra to triolein in neat form and in organic solvents of differing polarity. Neat triolein exhibits resonances at 171.50 ppm (\(\beta\) carbonyl) and 171.77 ppm (\(\alpha\) carbonyls). These resonances are shifted to 172.83 ppm (\(\beta\)) and 173.24 ppm (\(\alpha\)) in CHCl$_3$ and to 173.47 ppm (\(\beta\)) and 173.90 ppm (\(\alpha\)) in 2:1 CHCl$_3$/CH$_3$OH. The latter values approximate those for aqueous triacetin carbonyl groups (Fig. 1). PtdCho carbonyl groups give resonances at 174.18 ppm (\(\alpha\)) and 173.74 ppm (\(\beta\)) in 2:1 CHCl$_3$/CH$_3$OH with or without added triolein.

The spectrum of a sonicated 5% [1-$^{13}$C]triolein/95% egg PtdCho mixture is shown in Fig. 2. This spectrum is identical (\(\delta\) values, \(\nu_{\text{rel}}\) values, relative intensities) to a spectrum obtained for sonicated PtdCho in the absence of triglyceride, except for four additional peaks in the carbonyl region. These peaks are readily attributable to triolein carbonyl carbons on the basis of peak intensities. The two narrow peaks at 171.58 ppm (O$_2$) and 171.85 ppm (O$_3$) have the same \(\delta\) and \(\nu_{\text{rel}}\) values as neat triolein and therefore originate from unhydrated carbonyl carbons in an oil phase. The two broader peaks at 172.39 ppm and 173.07 ppm are attributed to triglyceride \(\beta\) chain (S$_3$) and \(\alpha\) chain (S$_3$) carbonyl groups that are hydrogen bonded with water molecules at the aqueous interface of the bilayer surface (see Discussion). The \(\delta\) and \(\nu_{\text{rel}}\) values and intensities of the four triglyceride carbonyl resonances were identical at 18°C, 35°C, and 45°C. Because the \(\delta\) values of "oil" triolein peaks occur at the values for neat triolein and are temperature independent, the exchange between surface and oil triolein is slow (<75 exchanges per sec).

\(T_1\) and NOE values for carbonyl resonances in the spectrum of sonicated 5% triolein/95% PtdCho are presented in Table 1. \(T_1\) and NOE values of \(\alpha\) and \(\beta\) carbonyl peaks for neat triolein are presented for comparison. The two phospholipid carbonyl resonances reflect phospholipids on the outside (P$_3$) and inside (P$_1$) of the vesicle bilayer (5, 6). Because the carbonyl NOEs are identical, relative intensity measurements in the $^1$H decoupled spectrum reflect the number of carbon atoms contributing to each peak (10). Peak areas were measured under equilibrium pulsing conditions (=4 $\times$ \(T_1\)), although measurements could be made at shorter pulse intervals with only a small compromise in the accuracy, because of \(T_1\) similarities.

* Triolein exhibiting such carbonyl peaks will be designated "surface" triolein to distinguish it from triolein that is present in a hydrocarbon environment ("oil" triolein).
To determine the maximal solubility of triolein in the phospholipid bilayer, sonicated samples were prepared containing different initial amounts of triolein (1%, 2%, 3%, 4%, and 5%) under otherwise identical conditions. Samples prepared with 1% and 2% triolein had the same physical appearance as pure egg PtdCho vesicles at the same phospholipid concentration; they were transparent with a faint bluish-white Tyndall effect. The 3%, 4%, and 5% samples exhibited increasing amounts of turbidity, from a very faint (3%) to readily observable turbidity (5%). 13C NMR spectra obtained under identical conditions for all samples were indistinguishable except for the carbonyl region, as illustrated in Fig. 3. The δ and υ values of corresponding carbonyl resonances are independent of triolein content, but the integrated intensities differ markedly. The surface triolein carbonyl peaks (S1,3 and S2) increase in intensity as a function of triolein concentration up to 3% and then plateau, whereas the oil triolein carbonyl peaks (O1,3 and O3) are not observed below 3% triolein; at ≥3% triolein they increase in intensity with increasing triolein content. Integrated intensity (peak area) measurements for the carbonyl peaks at differing compositions are given in Table 2. Peak area ratios of total triolein to phospholipid calculated from the starting compositions are in good agreement with ratios obtained from the measured NMR peak areas. The ratio of the surface triolein carbonyl peaks to the PtdCho carbonyl peaks increases from 1.3 to ~3.2. The ratio of peak areas of surface/carboxyl to oil carbonyl groups decreases from infinity to almost 1.0. Repeat experiments with samples containing 2%, 4%, and 5% showed these results to be reproducible.

The maximal amount of surface triolein (2.8%) was the same (±0.1%) when determined by different methods. The average surface triolein/PtdCho intensity ratio of 3.2 yields a calculated composition of 2.9% triolein, 97.1% PtdCho. Plotting the in

Table 2. Ratios of carbonyl NMR peak areas in sonicated triolein/PtdCho mixtures with different relative compositions

<table>
<thead>
<tr>
<th>Composition</th>
<th>Calculated triolein/PtdCho</th>
<th>Triolein/oil PtdCho</th>
<th>Surface triolein/PtdCho</th>
<th>Surface triolein/oil triolein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1%</td>
<td>1.1</td>
<td>1.3</td>
<td>1.3</td>
<td>—</td>
</tr>
<tr>
<td>2%</td>
<td>2.3</td>
<td>2.5</td>
<td>2.5</td>
<td>—</td>
</tr>
<tr>
<td>3%</td>
<td>3.4</td>
<td>3.8</td>
<td>3.1</td>
<td>4.0</td>
</tr>
<tr>
<td>4%</td>
<td>4.6</td>
<td>5.0</td>
<td>3.3</td>
<td>2.0</td>
</tr>
<tr>
<td>4% fractionated</td>
<td>—</td>
<td>3.2</td>
<td>3.2</td>
<td>&gt;30.0</td>
</tr>
<tr>
<td>5%, trial 1</td>
<td>5.9</td>
<td>6.1</td>
<td>3.4</td>
<td>1.3</td>
</tr>
<tr>
<td>5%, trial 2</td>
<td>5.9</td>
<td>6.2</td>
<td>3.2</td>
<td>1.15</td>
</tr>
<tr>
<td>Average</td>
<td>2.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Measurements were made on spectra shown in Fig. 3 except 4% fractionated and 5% trial 2 entries. Composition is wt % of total lipid that is triolein.

Theoretical 13C NMR carbonyl peak area ratio of total triolein phospholipid calculated as shown for 1% triolein:

1 mg triolein × 1 mmol × 90 (90% abundance) × 3 carbonyl/mmol
884 mg

99 mg PtdCho × 1 mmol × 1.1 (1.1% abundance) × 2 carbonyl/mmol
807 mg

Area ratio of total triolein/PtdCho: (S1,3 + S2 + O1,3 + O3)/(P + P2); compare with calculated triolein/PtdCho in first column.

Area ratio of surface triolein/PtdCho: (S1,3 + S2)/(P + P2).

Area ratio of surface triolein/oil triolein: (O1,3 + O3)/(O1,3 + O3).

The 5%, 4%, and both 5% samples (those after saturation) were used to calculate the average area ratio for surface triolein/PtdCho.

Clear zone after fractionation by ultracentrifugation as in Materials and Methods; NMR spectrum as in Fig. 3, except 1000 accumulations.


The chemical shifts of triglyceride carbonyl carbons have a solvent dependence similar to that of carboxyls in small organic molecules (4) and in phospholipids (5, 6). As the hydrogen-bonding capacity of the solvent molecule(s) increases, carbonyl resonance frequencies shift downfield (4–6). Thus, the carbonyl chemical shift of triglycerides also depends on the extent of hydrogen bonding with solvent molecules.

The 13C NMR spectra of sonicated triolein/egg PtdCho systems exhibit unique carbonyl resonances intermediate between and well resolved from those for PtdCho in a vesicle and triolein in an oil phase. The chemical shifts of these peaks (173.07 ppm and 172.39 ppm) indicate that these carbonyls interact with water molecules and therefore are close to the aqueous surface of the bilayer. An estimate of the extent of hydration can be made, assuming a linear relationship between the extent of solvent hydrogen bonding with carbonyl and their chemical shifts (6). The fractional hydration \( f \) of these triolein carbonyls, 0.6 for \( \alpha \) carbonyls and 0.5 for \( \beta \) carbonyls, is similar to that for vesicle phospholipid carbonyls.

Because the hydrated triolein carbonyl groups are near the aqueous surface of the PtdCho bilayer, the triolein molecule must have an orientation similar to that of PtdCho, i.e., it must be present in the PtdCho monolayer with the fatty acyl methyls in the bilayer interior and the three hydrocarbon chains roughly parallel to the PtdCho chains. Two important consequences of such an orientation of the triolein molecule are (i) that the molecular motions would be more anisotropic than in an oil phase and (ii) that the conformation of the glyceryl backbone might be different from oil triolein. The anisotropic motions of an oriented triolein molecule could result in broader resonances without an effect on \( T_1 \) (7). Carbonyl \( T_1 \) and NOE values are the same for oriented PtdCho and liquid (near) triolein and also do not allow distinction between surface and oil triolein. For oil-phase triolein, carbonyl linewidths are very narrow (\( \approx 1.5 \) Hz), but they are significantly broader (5–7 Hz) for triolein.

\[ F_{\Delta\delta} = (8 - \delta)/(\Delta\omega - \delta), \]

where \( \delta \) is chemical shift, \( S \) is surface triolein, \( O \) is oil (near) triolein, and \( A \) is aqueous triacetin. The calculation is approximate because the measured \( \Delta\omega \) for triacetin was substituted for the \( \Delta\omega \) of triolein, which cannot be measured. An exact comparison cannot be made with the calculations for PtdCho (5) because different approximations were used in those calculations. Additionally, other mechanisms may contribute to chemical shift changes (although we assume they are quantitatively small) and diminish the accuracy of this calculation.
molecules in the PtdCho monolayer. The similar \( \nu_{\frac{1}{2}} \) values of surface triolein carbonyl and PtdCho carbonyl peaks may reflect similar anisotropic motions. Because PtdCho peaks contain unresolved \( \alpha \) and \( \beta \) carbonyls \( (6) \), and surface triolein carbonyl peaks may contain a contribution from exchange broadening (see below), the NMR linewidth data cannot conclusively demonstrate motional similarities. The orientation that we propose for surface triolein requires a different conformation of the glyceryl backbone from the "tuning fork" conformation proposed for liquid tristearin \( (17) \), in which one \( \alpha \) chain points in the opposite direction from the other chains. The significant change in the chemical shift difference between \( \alpha \) and \( \beta \) carbonyl resonances \( \Delta (\delta \alpha - \delta \beta) \) for triolein on going from an oil \( (0.27 \text{ ppm}) \) to the bilayer surface \( (0.70 \text{ ppm}) \) may indicate such a conformational change. Triacetin undergoes a similar but slightly smaller change in \( \Delta (\delta \alpha - \delta \beta) \) between neat \( (0.31 \text{ ppm}) \) and aqueous \( (0.54 \text{ ppm}) \) forms. This difference may also be related only to hydration differences between the \( \alpha \) and \( \beta \) carbonyl groups \( (6) \). Nevertheless, because the fractional hydration of the \( \beta \) carbonyl is less than that of \( \alpha \) carbonyls in surface triolein, the conformation is such that \( \alpha \) carbonyls are more accessible to water molecules.

The orientation of triolein in the PtdCho monolayer is appropriate for enzymatic hydrolysis, and the second substrate \( (H_2O) \) is in close proximity to the hydrolytic site. Rapid hydrolysis of triolein in sonicated, fractionated 1.5% triolein/egg PtdCho vesicles has been demonstrated with an acid lipase \( (18) \); therefore surface triolein is an effective substrate for enzymatic hydrolysis. In addition, the conformation of the molecule probably places the \( \alpha \) carbonyls closer to the surface in a position more favorable for enzymatic hydrolysis. In view of the known preference for hydrolysis of \( \alpha \) chains by several lipolytic enzymes \( (19, 20) \), this finding may be applicable to biological systems and explain (at least in part) such specificity.

Experiments using the shift reagent \( \text{Yb}^{3+} \) were designed to provide information of a more specific nature about the localization of triolein in the bilayer. Our results show that the triolein carbonyl groups are located further from the aqueous interface than are the PtdCho carbonyl and the PtdCho glyceryl backbone carbons or are less accessible to the shift reagent. Comparisons of the carbonyl chemical shifts in the absence of shift reagents suggest that triolein carbonyl groups are less accessible to \( H_2O \) molecules because surface triolein carbonyl peaks in the vesicle spectra occur relatively more upfield from PtdCho peaks than in organic solvent \( (C^2HCl_2/C^2H_2O_4H) \), in which the carbonyl environments are similar. The latter result could also indicate that triolein carbonyl groups are located lower in the bilayer surface than PtdCho carbonyls.

Another significant difference between PtdCho and surface triolein carbonyl resonances is that surface triolein carbonyl groups do not show individual carbonyl resonances representing triolein on the two sides of the bilayer,\(^4\) with or without added shift reagent. This finding could have three different explanations (not distinguishable by our studies): (i) unlike phospholipids, triolein is distributed on both halves of the bilayer in environments that are identical to the carbonyl carbons; (ii) all triolein is located on one side of the bilayer; and (iii) triolein molecules are distributed on the two halves in somewhat differing environments but the chemical shift differences are averaged by rapid exchange to give one observed shift. Although the triglyceride molecule is large, the latter explanation is physically plausible because of the lesser degree of polarity; the (minimum) exchange rate needed to produce a single peak with a width of \( ~5 \text{ Hz} \) is \( >5 \) exchanges per sec. Such flip-flop would, of course, provide a mechanism for transfer of triglyceride across a bimolecular membrane, and make additional triglyceride accessible to enzymatic hydrolysis at the outer membrane surface. Some triolein must be located on the outside layer because of the demonstrated enzymatic accessibility \( (18) \) and the observed line broadening with \( \text{Yb}^{3+} \).

Our NMR results for differing triolein-to-PtdCho starting ratios show that up to slightly less than 3% triolein can be incorporated into the bilayer surface. The environment of the triolein as reflected in NMR \( \delta \) and \( \nu_{\frac{1}{2}} \) values is the same at different relative amounts of triolein and at different temperatures \( (15-45^\circ C) \). The structure and dynamics of the phospholipids are unaltered by the presence of triglyceride, by the criteria of \( \delta \), \( \nu_{\frac{1}{2}} \), and relative intensities. After saturation of the bilayer, the excess triolein is present in a turbid suspension that can be separated by flotation from the vesicles. The exchange between these two pools is sufficiently slow to give unshifted resonances at any concentration or temperature studied.

Our value for the maximal amount of triolein in the PtdCho vesicle bilayer surface phase is similar to the amounts of triglyceride found in phospholipid monolayers isolated from triglyceride emulsion particles \( (3) \), showing that the latter values probably represent the surface composition of the intact particle. Thus, lipoprotein surfaces and certain biomembranes may contain small amounts of triglyceride available for transport and metabolism.

The authors thank Dr. David Atkinson for helpful discussion in both the experimental and writing stages and Dr. Trevor Redgrave and Dr. Helmut Hauser for a critical reading of the manuscript. This work was supported by U.S. Public Health Service Grant HL28335.

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4 We also considered assignment of the surface triolein peaks to outside and inside triolein, in analogy with PtdCho carbonyls. However, spectra of vesicles containing 1,2-dipalmitoyl-3-hexanoyl triacylglycerol synthesized with \( {1^2}C \) palmitic acid confirmed our assignments as above.
Physical Characterization of Lymph Chylomicra and Very Low Density Lipoproteins from Nonhuman Primates Fed Saturated Dietary Fat

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Differential scanning calorimetry (DSC) and x-ray diffraction studies were performed on chylomicra and very low density lipoproteins (VLDL), from nonhuman primates fed saturated fat, isolated from lymph at two different temperatures (15 and 39 °C). When heated from –10 to 60 °C, chylomicra and VLDL isolated at 15 °C had two endothermic transitions resulting from the melting of triglycerides (TG). Cooling resulted in the onset of crystallization of the TG core at 16–19 °C; 50% of the TG in the particles remained fluid at 4 °C. The initial DSC pattern was reproduced not on immediate reheating, but by storing samples at 4 °C overnight. Chylomicra isolated at 39 °C contained a metastable (undercooled) TG core until cooled to the onset of TG crystallization at 16–19 °C. Reheating the particles with crystalline TG to body temperature (39 °C) resulted in a partially crystalline TG core, not an undercooled liquid.

To ascertain the effect of particle structure on the TG physical properties, intact particles were compared with heat denatured particles and extracted lipids. The onset of crystallization was lower and the time necessary for isothermal crystallization was much greater for intact versus denatured particles or lipids. Only minor differences in physical properties as a function of particle size were found.

It was concluded that: 1) the physical state of chylomicra and VLDL TG core can be modified by isolation temperature; 2) particle structure affects crystallization but not melting of chylomicra and VLDL TG; and 3) chylomicra particle size does not markedly influence the physical properties of the TG core.

Approximately 40% of calories consumed by North Americans is predominantly triglyceride (1). Lymph chylomicrons and VLDL are the primary vehicles which transport dietary triglyceride from the intestine to peripheral cells (2). The triglyceride exists as a separate phase in the core of the chylomicron or VLDL particle, although a small fraction of the triglyceride is in equilibrium with the surface phase of the particle (3).

The phase behavior of purified triglyceride species has been extensively studied. A saturated triglyceride such as triolein may crystallize in at least three distinct polymorphic forms (α, β, β′) which depend on the mode of crystallization and previous thermal history. The α form, obtained by rapid cooling of a melted triglyceride, is characterized by a wide angle x-ray diffraction spacing of 4.1 Å indicative of hexagonal packing of the hydrocarbon chains (4). The α state is the lowest melting form and upon heating to its melting point rapidly recrystallizes to the most stable, highest melting β form (4). This form is characterized by wide angle x-ray diffraction spacings of 3.7, 3.9, 4.6 Å and triclinic packing of hydrocarbon chains (4). An intermediate melting crystalline form, β′, may be obtained by allowing the triglyceride melt to recrystallize isothermally a few degrees above the melting point of the α phase. Orthorhombic subcell packing of the fatty acyl chain and wide angle spacings of 3.8 and 4.2 Å are characteristic of the β′ phase (4). Triglyceride species containing fatty acyl chains with different degrees of unsaturation usually have more complicated phase behavior. For example, 2-oleyl-1,3-dipalmitin can exist in two different β′ phases, one melting at 26.5 °C and the other at 33.5 °C in addition to several α and β phases (5).

Although a vast literature exists for the phase behavior of pure triglycerides and triglyceride mixtures (6) and on the cholesteryl ester core of lipoproteins (7–11), there is a paucity of data on triglyceride phase behavior in lipoprotein particles. Using calorimetry and x-ray diffraction, Deckelbaum et al. (12) found that the core lipids in normal human VLDL were liquid between 10 and 60 °C. If the VLDL were cooled below 0 °C, crystallization of the core was initiated, and on reheating, the core lipids melted between –10 and 10 °C (13). Since chylomicra and VLDL are large lipoprotein complexes which contain TG as the major component, it was of interest to see if the physical properties of these particles approach that of bulk phase triglyceride systems. The purpose of the present study was to characterize the phase behavior of triglyceride-rich lipoproteins derived from thoracic duct lymph of nonhuman primates fed a saturated fat diet.

MATERIALS AND METHODS

Animals—Adult male African green monkeys (Cercopithecus aethiops) of the vervet subspecies or Macaca fascicularis monkeys were fed a semi-purified diet which consisted of 40% of calories as butter fat with 0.8 mg of cholesterol/kaol (14). An indwelling thoracic duct cannula was used for the diversion of lymph flow (15). The animals were allowed to recover from surgery at least 10 days prior to lymph collection. Lymph was collected at room temperature in a tube containing 0.1% EDTA, 0.02% azide, and 0.04% 5,5'-dithiobis(2-nitrobenzoic acid), pH 7.4 (final concentration). During lymph collection,
a liquid diet (40% of calories as butter fat) was infused via a duodenal cannula as described previously (15). In one experiment, an animal was infused with a similar diet in which safflower oil had been substituted isocarboxylally for butter fat. Lymph routinely was stored at 0°C until isolation of chylomicra and VLDL. In one study, lymph was collected at 39°C (body temperature of the monkey).

Isolation of Lymph VLDL and Chylomicra Subfractions—Isolation of lymph lipoproteins was performed using an L5-75 ultracentrifuge and SW 41 rotor (Beckman Instruments, Fullerton, CA). Ten ml of lymph were overlayed with d = 1.06 g/ml of NaCl solution (0.9% NaCl; 10% EDTA; 0.01% azide, pH 7.4) and fractionation was accomplished using the following conditions: S[50; w] > 5000, 22,000 rpm at 15,000 g (6 x 10^5 g/mL); S[50; w] = 2000–5000, 54 min at 15,000 rpm (1.5 x 10^5 g/mL); S[50; w] = 400–2000, 84 min at 27,000 rpm (7.5 x 10^5 g/mL); and S[50; w] = 20–400 (VLDL), 23 h at 30,000 rpm (1.5 x 10^6 g/mL). A single spin (7.5 x 10^6 g/mL) was used to isolate unfractonated chylomicrons (S[50; w] > 400). At each sequential spin, the compacted layer of lipoprotein at the top of the tube was carefully removed with a spatula. Isolation was done at 15 or 39°C. The centrifuge was stopped with the brake on.

The isolated lipoprotein layers were resuspended in a small volume of d = 1.006 g/ml of NaCl and stored at 4–6°C or 39°C.

Differential Scanning Calorimetry—Differential scanning calorimetry was done with a DSC-2 (Perkin-Elmer, Norwalk, CT) standardized with gallium and indium. Sample size was 50–75 μl (3–6 mg of triglyceride) with an equal volume of d = 1.006 g/ml NaCl as reference. Lipoprotein lipids (3–6 mg of triglyceride), obtained by extraction of aqueous samples with chloroform/methanol (2:1), were analyzed using an empty sample pan as reference. Scans were routinely done at a temperature of 0.1°C/min with 0.1–0.2 mcal/sec sensitivity. Scans were made at four different scanning speeds (1.25, 2.5, 5, and 10°C/min) and the peak temperature of transitions was extrapolated to 0°C/min scanning rate. All temperatures reported in the text have been corrected for scanning rate. Calorimetric enthalpies were determined by planimetric integration of the DSC curves. The coefficient of variation for calorimetric enthalpies varied from 2–9%.

DSC samples were analyzed according to the following thermal protocol unless stated otherwise. Samples were stored at 4°C for at least 24 h prior to all initial heating runs; these scans were from –10 to 60°C. The sample then was cooled from 60 to –10°C. A second heating (–10 to 60°C) was performed immediately after the cooling run. The sample was cooled again after the second heating and reheated immediately from –10 to 60°C. Heat denaturation of the sample was accomplished by holding samples at 100°C for 15 min.

Immediately following heat denaturation, the sample was cooled (100 to –10°C) and then reheated from –10 to 60°C. Extracted chylomicron and VLDL lipids were analyzed in the following manner. Initial heating runs were performed on samples stored at 4°C for at least 24 h. The initial heating was from –30 to 60°C followed by a cooling run (60 to –30°C). A second heating (–30 to 60°C) was done immediately after the cooling run.

Analytical and Preparative Chemical Procedures—Protein determinations were performed by the method of Lowry et al. (17) using bovine serum albumin, fraction V (Sigma) as the standard. Turbidity was measured by repeated extractions of the samples with hexane. Cholesterol determinations were done according to the procedure of Rudel and Morris (18). Phospholipids were quantitated by the method of Piskie and Subarrow (19). Triglyceride assays were done according to the method of Sardesai and Manning (20). Relative amounts of free and esterified cholesterol were determined by gas-liquid chromatography of chylomicron lipids (21). Triglycerides were isolated from lipid extracts by thin layer chromatography on Silica Gel H plates (Applied Science Laboratories, State College, PA) run in hexane/diethyl ether/acetic acid (78:22:1). Triglyceride bands were eluted from the plate with chloroform/methanol (2:1). Isolated triglyceride fatty acyl groups were methylated and quantitated by gas liquid chromatography as described by Morrison and Smith (23). For x-ray analysis, larger amounts of chylomicron triglyceride were isolated from lipid extracts by column chromatography on Florisil (24).

X-ray Diffraction—X-ray diffraction measurements were performed on chylomicrons (S[50; w] > 400) or isolated chylomicron triglyceride (S[50; w] > 400). The samples were sealed in a 1-mm I.D. Lindemann Glass tube (Lindemann Glass Corp., Indianapolis, IN). X-ray diffraction patterns were obtained over the range 1/60–1/2.5 Å⁻¹ using a Jarrell-Ash microfocus x-ray generator and a slit collimated Luzzati-Barro x-ray camera modified to include a single mirror focusing system. The diffraction patterns were recorded using a linear position sensitive counter (P.S.D. 1100, Tennelec, TN), coupled to a computer-based analysis system (TN 1710, Tracor Northern, WI).

RESULTS

Chemical Data—Fractionation of thoracic duct lymph in the ultracentrifuge resulted in four populations of triglyceride-rich particles which varied in chemical composition (Table I). As flotation rates varied from low (S[50; w] = 20–400; VLDL) to high (S[50; w] > 5000), the percentage of triglyceride increased while all other chemical components decreased. The percentage of protein composition was higher than values usually reported (25) because the chylomicra and VLDL were not washed repeatedly in the ultracentrifuge to decrease albumin content. This was confirmed by SDS-polyacrylamide gels (26) as the major protein band was albumin (data not shown).

The triglyceride fatty acid analysis of the chylomicra and VLDL is shown in Table II. The fatty acid composition of the butter fat diet fed to the monkeys also is listed for comparison. The most predominant triglyceride fatty acid was palmitic acid. Oleic acid accounted for one quarter of the fatty acids while stearic and myristic acid each approximated 10–15%. Little linoleic acid was found. By contrast, the chylomicra triglyceride (S[50; w] = 400–2000) isolated during infusion of the safflower oil diet had 67% linoleic, 13% stearic, 11% palmitic, and 4% myristic acid. Particle size had little effect on the fatty

<table>
<thead>
<tr>
<th>Component</th>
<th>VLDL</th>
<th>400–2000</th>
<th>&gt;5000</th>
</tr>
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<tbody>
<tr>
<td>Protein</td>
<td>11.9</td>
<td>7.5</td>
<td>2.2</td>
</tr>
<tr>
<td>Lipid</td>
<td>88.1</td>
<td>92.4</td>
<td>97.8</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>81.7</td>
<td>87.0</td>
<td>91.4</td>
</tr>
<tr>
<td>Ester cholesterol</td>
<td>2.7</td>
<td>2.0</td>
<td>1.6</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>1.1</td>
<td>1.0</td>
<td>0.7</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>14.5</td>
<td>10.1</td>
<td>6.2</td>
</tr>
<tr>
<td>Estimated particle diameter (Å)</td>
<td>600</td>
<td>850</td>
<td>1700</td>
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</table>

Table II

| Lymph VLDL and chylomicra triglyceride fatty acid composition |
|---------------------|-----|-----|-----|-----|
| VLDL | 400–2000 | >5000 |
| 120   | 1* | 0  | 3  | 1   |
| 140   | 12 | 9  | 13 | 12  |
| 160   | 38 | 35 | 35 | 37  |
| 181   | 2  | 4  | 3  | 3   |
| 185   | 15 | 15 | 14 | 12  |
| 200   | 26 | 27 | 26 | 26  |
| 202   | 2  | 4  | 3  | 2   |
| 3     | 3  | 5  | 5  | 10  |

* Percentage composition. Mean of triplicate determinations. Coefficient of variation for analyses was 8%.

S[50; w] range.

Fatty acid composition of butter diet. Fatty acid species shorter than 14 carbons were not quantitated.
acid composition and the diet and particle compositions were very similar.

**Differential Scanning Calorimetric Studies**—DSC traces of chylomicra and VLDL isolated from lymph at 15 °C and stored at 4 °C for various times (1 day to several weeks) are shown in Fig. 1. On heating from −10→60 °C in the DSC, no thermal transitions were observed for chylomicra derived from safflower infused animals (Fig. 1A). However, chylomicra derived from butter fed animals had two endotherms (Fig. 1B) with \( T_p \) of 20 and 38 °C. These endotherms resulted from the melting of core triglycerides as judged by DSC enthalpies and x-ray diffraction experiments (see below). The data for subfractions of decreasing particle size also are shown in Fig. 1, C-E. Decreasing particle size resulted in a lower \( T_p \) for the first endotherm (20→16 °C), an apparent decrease in the enthalpies (peak areas) of the endotherms, and the appearance of another endotherm at 48–50 °C. The highest temperature endotherm (\( T_p \) = 49 °C, 9.3 cal/g of protein) was due to protein denaturation as its area increased with particle protein composition (Table I). Also, the transition was not detected for extracted chylomicron and VLDL lipids. The protein transition was not seen for the safflower chylomicron sample (Fig. 1A) because the sample was washed previously in the ultracentrifuge.

Upon cooling samples from 60→−10 °C, one exotherm (\( T_p \)

---

**Table III**

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Calorimetric enthalpy and transition temperatures of lymph VLDL and chylomicra</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial heating −10→60 °C</td>
</tr>
<tr>
<td></td>
<td>( \Delta H ) ( ^oC )</td>
</tr>
<tr>
<td>VLDL</td>
<td>6.8 (16)</td>
</tr>
<tr>
<td>400–2000</td>
<td>8.2 (18)</td>
</tr>
<tr>
<td>2000–5000</td>
<td>10.3 (19)</td>
</tr>
<tr>
<td>&gt;5000</td>
<td>11.9 (20)</td>
</tr>
<tr>
<td>X ± S.E.</td>
<td>21.6 ± 1.9</td>
</tr>
</tbody>
</table>

\( ^oC \) = °C; \( ^oC \) = °C; \( \Delta H \) = \( \Delta H \); \( T_p \) = \( T_p \);

\( ^a \) Mean of 2–6 determinations on 2–3 samples. Coefficient of variation for calorimetric enthalpies ranged from 2–9%.

\( ^b \) Heat denatured at 100 °C for 15 min.

\( ^c \) Heat denatured at 100 °C for 15 min.

\( ^d \) Cal/g of triglyceride. Enthalpy of heating runs or exothermal enthalpy for cooling runs, \( \Delta H \) Total is total enthalpy of endothermal enthalpy for heating runs. For initial heating runs, \( \Delta H \) Total is simply the enthalpy sum of the two endothermal peaks for second heating runs, which contain both endothermal and exothermal transitions, it is equal to endothermal minus exothermal enthalpy.

\( ^e \) Numbers in parentheses are the peak transition temperatures (\( T_p \)) for heating runs or crystallization onset temperature (\( T_o \)) for cooling runs.

\( ^f \) Mean enthalpy and standard error of the mean of all 4 subfractions.
Physical Characterization of Monkey Lymph Lipoproteins

9–13 °C) was seen for all chylomicron subfractions and two exotherms (Tp = 11 and 2 °C) were found for the VLDL subfraction (Fig. 1, F–I). The degree of undercooling of the triglyceride from the peak of isotropic melting (37 °C) to the onset of recrystallization (16–19 °C) was large, which was suggestive of homogenous nucleation (27) of the triglyceride species within the particle.

A representative second heating profile showing an exotherm at Tp = 2 °C and a broad endotherm (15–45 °C; Tp = 37 °C) is illustrated in Fig. 2A. The second recrystallization profile was the same as the initial cooling run (Fig. 1F) and is not shown. The second heating pattern for intact particles was reproducible as long as the time between cooling and heating was short (several hours). However, if the samples were held overnight at 4 °C, the initial heating profile was reproduced (Fig. 1, B–E). The second heating profile was essentially the same for all chylomicron subfractions and VLDL.

Particles which were heat denatured at 0 °C for 15 min had a cooling profile markedly different from native particles (Fig. 2B). The onset of recrystallization (24 °C) was higher than native particles (16–19 °C; Table III) and two major exotherms were seen for the denatured particles (Tp = 4 and 23 °C). The heating profile for a heat-denatured sample was independent of particle size and similar to the second heating profile of the intact particles (Fig. 2, A versus C).

Initial and second heating and cooling profiles for extracted lipid are shown in Fig. 3 for a representative chylomicron subfraction. The initial heating profile (–30–+60 °C; Fig. 3A) was similar to that of the intact particles (Fig. 1B) except the Tp of the first endotherm was 8 °C lower for the extracted lipides (Table III). The cooling profile (Fig. 3B) for the extracted lipides was similar to that of heat-denatured particles (Fig. 2B) but different from that of intact particles (Fig. 1, F).

![Fig. 3. DSC tracings of initial and second heating (–30 to +60 °C) and cooling (60 to –30 °C) runs of extracted neat lipides (0 mg of triglyceride) from chylomiria (S, > 5000). Scans were made at 5 °C/min at a sensitivity of 0.5 mcal/s. Numbered arrows indicate temperatures at which x-ray scattering experiments were performed. Baselines have been indicated by dashed line. A, initial heating; B, initial cooling; C, second heating; and D, second cooling run.](image)

![Bragg Spacing, (Å)](image)

*Fig. 4. Wide angle x-ray diffraction patterns of butter chylomiria (S, > 400) upon initial heating (3–46 °C) and cooling (46–2 °C). Chylomiria (100 mg of chylomicron triglyceride/ml) were isolated from lymph at 15 °C and stored at 4 °C before experiments. A, 3 °C; B, 28 °C, sample heated from 3–28 °C; C, 46 °C, sample heated from 28–46 °C; D, 31 °C, sample cooled from 46–21 °C; E, 12 °C, sample cooled from 21–15 °C; and F, 2 °C, sample cooled from 12–2 °C.*

**X-ray Scattering Studies**—To investigate the structural arrangement of the triglyceride in the core of the chylomicron particle, x-ray diffraction experiments were carried out as a function of temperature. The wide angle diffraction patterns illustrated in Fig. 4 were obtained at the temperatures indicated in the DSC profiles by numbered arrows (Fig. 1). These points were selected as representative of temperatures where
TABLE IV

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Designationa</th>
<th>Temperature</th>
<th>Short Spacingb</th>
<th>Long Spacingc</th>
<th>Physical State</th>
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<tbody>
<tr>
<td>Heating</td>
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<td>3</td>
<td>3.8, 4.2, 4.6d</td>
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<td>β</td>
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<td></td>
<td>2</td>
<td>28</td>
<td>3.8, 4.3, 4.6</td>
<td>42</td>
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<tr>
<td></td>
<td>3</td>
<td>46</td>
<td>4.6</td>
<td>liquid</td>
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<td>Cooling</td>
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<td>21</td>
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<td>Undercooled liquid</td>
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<td>5</td>
<td>12</td>
<td>3.9, 4.2</td>
<td>42</td>
<td>β</td>
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<tr>
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<td>6</td>
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Isolated Triglycerides

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<th>Short Spacingb</th>
<th>Long Spacingc</th>
<th>Physical State</th>
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<tbody>
<tr>
<td>Melted and cooled slowly to −10 °C</td>
<td>7</td>
<td>−10</td>
<td>3.8, 4.2–4.3</td>
<td>42</td>
<td>β</td>
</tr>
<tr>
<td>Melted and cooled quickly to −10 °C</td>
<td>7</td>
<td>−10</td>
<td>4.1</td>
<td>46</td>
<td>α</td>
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<tr>
<td>Held at −10°C for 1 h after quick cooling of melt</td>
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<td>−10</td>
<td>3.9, 4.1</td>
<td>42, 46</td>
<td>β &amp; α</td>
</tr>
<tr>
<td>Heating to 5 or 10 °C from −10°C after quick cooling of melt</td>
<td>8</td>
<td>5</td>
<td>3.9, 4.2</td>
<td>42, 46</td>
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<td>9</td>
<td>10</td>
<td>3.9, 4.2</td>
<td>42</td>
<td>β</td>
</tr>
</tbody>
</table>

a Number designation corresponds to temperatures on DSC tracings (Figs. 1 and 3) at which x-ray diffraction experiments were performed.
b Short spacing measurements are accurate to ±0.1 Å based on the accuracy with which the peak could be located and the sample-detector distance.
c Long spacing measurements are accurate to ±2 Å.
d Diffuse diffraction pattern at 4.6 Å.

The triglyceride would be expected to be in a stable form following a phase transition. The short and long Bragg spacings are summarized in Table IV. Chylomicra isolated at 15 °C had diffraction patterns (Fig. 4A) typical of β' packing of triglycerides (Bragg spacings of 3.8 and 4.2 Å) at 3 °C. In addition there appeared to be a shoulder on the 1/4.2 Å⁻¹ peak at 1/4.6 Å⁻¹. This broad peak centered at 1/4.6 Å⁻¹ is indicative of liquid hydrocarbon fatty acyl chains.

When the sample was heated above the first endotherm to 28 °C (Fig. 4B), the diffuse scattering at 1/4.6 Å⁻¹ became stronger. At 46 °C, the diffraction pattern showed only a broad diffuse diffraction maximum at 1/4.6 Å⁻¹ typical of melted triglyceride (Fig. 4C). The x-ray pattern remained the same upon cooling from 46 → 21 °C (Fig. 4D) corroborating the DSC results, which suggested the triglycerides were in a metastable (undercooled) state. With the onset of crystallization, diffraction peaks reappeared at 1/3.8 and 1/4.2 Å⁻¹ (Fig. 4E) indicative of recrystallization into the β' phase. Cooling the sample from 12 → 2 °C did not result in an appreciable change in the x-ray diffraction patterns (Fig. 4F).

X-ray scattering experiments also were performed on the triglyceride isolated from chylomicron samples (S₁₀ > 400) which were isolated from lymph at 15 °C. The diffraction patterns as a function of temperature were essentially identical to those of the intact particle.

To determine whether the lower temperature endotherm seen in the extracted lipid samples (Fig. 3C; 0 °C) was the result of the melting of triglycerides in an α phase, x-ray studies were conducted on chylomicron (S₁₀ = 400) triglycerides at low temperature. Slow cooling of a sample with melted triglyceride to −10 °C resulted in a scattering profile typical of β' crystalline structure (Fig. 5A). However, following very rapid cooling to −10 °C, only one wide angle diffraction at 1/4.12 Å⁻¹ was observed (Fig. 5B) and the long spacing was 46 Å compared to 42 Å (Table IV). After holding the sample at −10 °C for 1 h, some of the triglyceride had undergone an α → β' conversion and the x-ray pattern had characteristics of both α and β' forms (Fig. 5C and Table IV). This clearly was discernible in the long spacing diffraction pattern (Table IV); long spacings typical of both α packing (46 Å) and β' packing (42 Å) were present. As the sample was warmed to 5 °C (Fig. 5D) and then 10 °C (Fig. 5E), all features of α crystalline packing were lost. Thus, the x-ray findings correspond well to the phase changes shown by DSC in Fig. 3C.
to crystallize when the temperature of the particles was lowered to \(-16^\circ\text{C}\) (Fig. 6E). Chylomicra and VLDL isolated from lymph which was cooled to 4 \(^\circ\text{C}\) had crystalline triglyceride (Fig. 1). If these particles were reheated to body temperature, the core still contained 20-30\% of the triglyceride in crystalline form (the percentage of crystalline triglyceride was determined by planimetric integration of DSC tracings as a function of temperature; Ref. 28). Thus, cooling the lymph during the isolation procedure resulted in changes which altered the physical state of the core relative to that of the "in vivo" particle.

Lymph chylomicra and VLDL were found to have complicated phase behavior which was dependent on the thermal history of the particle. Intact particles isolated at 15 \(^\circ\text{C}\) and stored overnight at 4 \(^\circ\text{C}\) had two endothermic transitions upon heating from -10 to 60 \(^\circ\text{C}\) (Fig. 1). The first transition \((T_p = 16-20 \, \text{\degree C})\) represented a \(\alpha \rightarrow \beta\) transition (Fig. 4). The first transition was reproducible as long as the particle temperature never exceeded 30 \(^\circ\text{C}\). The second endotherm \((T_p = 37 \, \text{\degree C})\) also represented a \(\beta \rightarrow \beta\) transition. However, because of the diffuse nature of the 1/46.6 Å \(^{-1}\) diffraction from the liquid triglyceride, spacings typical of \(\beta\) crystalline packing could not be detected. The predominant crystalline form of milk fat is \(\beta\) and a substantial liquid component is present in the diffraction pattern which obscures the \(\beta\) crystalline diffraction pattern (29). However, a high melting \(\beta\) crystalline fraction can be isolated from milk fat by solvent fractionation.

Once the chylomicron or VLDL particles were heated through the second transition \((45 \, \text{\degree C} \text{ or above})\), the initial heating profile was not reproduced by cooling and immediately reheating; to reproduce the initial heating profile the particles had to be maintained at 4 \(^\circ\text{C}\) overnight. The second heating profile was quite different from the initial heating profile in several features. Instead of two discrete endothermic transitions there was a low temperature exotherm representing an \(\alpha \rightarrow \beta\) transition (Fig. 5) and a broad endotherm \((15-45 \, \text{\degree C})\) resulting from a \(\beta \rightarrow \beta\) transition (Fig. 1). The total enthalpy obtained on the second heating (Table III; 15.2 cal/g) was less than that obtained on initial heating (21.6 cal/g). Thus, the core TG was in a more stable polymorphic form after being maintained at 4 \(^\circ\text{C}\) overnight relative to that found upon immediate recrystallization of the melt. Maintaining the sample at 4 \(^\circ\text{C}\) overnight allowed the less stable triglyceride phases to transform into more stable polymorphic forms which then absorbed more heat upon melting. This transformation was too slow to be seen for intact particles in the DSC but could be seen on the faster time scale of recrystallization of isolated chylomicron lipids. In this case, the transformation of the second heating profile to the initial heating profile was achieved by isothermal recrystallization of the undercooled liquid melt at the onset temperature \((23 \, \text{\degree C})\) of crystallization (Fig. 7).

The calorimetric enthalpies listed in Table III for chylomicra and VLDL are less than those reported in the literature for purified triglycerides in the \(\beta\) phase \((-40-50 \, \text{cal/g}; \text{Ref. 30})\). This was due to incomplete crystallization of the undercooled triglyceride, which contributed no enthalpy to the melting process. Lipid samples held at low temperature \((-40 \, \text{\degree C})\) for several weeks to allow complete triglyceride crystallization were found to have enthalpy values similar to purified triglycerides \((40 \, \text{cal/g})\). If 40 cal/g triglyceride is the total enthalpy of the completely crystallized sample, then the TG core of the lipoprotein samples was approximated 50\% liquid at low temperatures \((0-4 \, \text{\degree C})\).

The effect of particle structure on the physical properties of chylomicra and VLDL was investigated by comparing the melting and crystallization behavior of intact particles, heat denatured samples, and extracted lipids. The most pronounced effect of particle structure was on recrystallization patterns and kinetics. Intact particles had a recrystallization profile which was markedly different from that of denatured particles or neat lipids. This presumably was an effect on crystal nucleation as the degree of undercooling for the denatured particle and neat lipids was not as great as that of intact particles (Table III; To). This may be due, in part, to the ability of the particle structure (constraints) to limit the critical nucleus size (31). In addition, the constraints of particle structure increased the time necessary for isothermal crystallization at room temperature \((23 \, \text{\degree C})\) compared to isolated lipids (-2 weeks versus 30 min). Particle structure had little effect on the melting and crystallization enthalpies of chylomicra or VLDL triglycerides as there was only a 10-25\% increase in enthalpy upon thermal denaturation of intact particles. Because of the difference in temperature programming, comparison of calorimetric enthalpies for aqueous samples (intact or denatured particles) versus extracted lipids for effects of particle structure are misleading. Extracted lipids were routinely cooled 20 \(^\circ\text{C}\) lower \((-30 \, \text{\degree C})\) than either intact or denatured particles, thus crystallizing more of the lower melting liquid triglyceride. Heating the extracted lipids from -30 \(^\circ\text{C}\) resulted in the melting of the additional crystalline triglyceride \((\beta\) relative to aqueous samples), thus higher calorimetric enthalpies and lower \(T_p\) values were found. Lipid samples held at low temperatures for extended periods of time gave enthalpy values typical of purified triglyceride forms (see above).

Particle size also is an important variable in determining the physical characteristics of chylomicra and VLDL. Since the onset of crystallization temperature was similar for all subfractions (Table III), the most pronounced effect of particle size was on TG crystal growth. Intact VLDL had recrystallization profiles different from that of chylomicra subfractions (Fig. 1). The double recrystallization exotherm for intact VLDL versus the chylomicron single exotherm was presumably a size-related kinetic effect on crystallization. The \(T_p\) and enthalpy values for the intact particles were directly proportional to particle size (Table III; initial heating). Since the triglyceride fatty acid composition of all subfractions was similar, a satisfactory explanation for this observation is not apparent.

The changes in the core physical state may influence the metabolic fate of the chylomicra. Results of several studies have shown that lymph chylomicra derived from saturated fat-fed animals were metabolized differently from polyunsaturated fat-derived chylomicra (32-34). Nestel and Scow (33) have shown that the \(t_{1/2}\) for the mean rate of removal from plasma of cream chylomicra previously stored at 4 \(^\circ\text{C}\) was 76\% that of corn oil chylomicra. This preferential removal from plasma of saturated fat-derived chylomicra relative to unsaturated fat chylomicra was reflected in an increase in label from the saturated chylomicra found in the liver of the recipient animals. In a more recent study, Floren and Nilsson (34) have shown similar results with cream or corn oil chyle each labeled with \(^{1\text{H}}\)palmitate and \(^{1\text{C}}\)linoleate. They found that cream chyle isolated at 4 \(^\circ\text{C}\) was removed from the circulation by the liver more rapidly than corn oil chyle. Of the remnants remaining in the circulation at various time intervals, it was found that the cream chyle remnants had a relative enrichment of saturated triglyceride species \((\text{increased } ^{1\text{H}}/^{13\text{C}}\text{ ratio compared to injected chyle})\). A similar enrichment was not found for corn oil chyle. They performed additional studies using chyle which had not been cooled below room temperature. Contrary to the results found for chyle cooled to 4 \(^\circ\text{C}\), remnants derived from uncooled chyle
were found not to be significantly enriched in saturated triglyceride species. In addition, there was little preferential hydrolysis by lipoprotein lipase of the triglyceride species of uncooled chyle, i.e. the \(^{13}C/^{12}C\) was essentially unchanged. Based on our present results, the uncooled chyle of the Floren and Nilsson study presumably did not contain any crystalline triglyceride species while the chyle that was cooled to 4 °C and reheated to body temperature concomitant with re-injection did contain crystalline triglyceride. Therefore, the physical state of the triglyceride core of chylomicra and VLDL, which can be changed by the temperature of isolation, may affect both intravascular triglyceride hydrolysis and uptake of remnant particles. Presumably the metabolic differences between chylomicra with crystalline versus liquid undercooled cores are the result of lipid and/or apoprotein changes induced by the crystallization of the core or a decreased interaction of crystalline triglyceride with lipoprotein lipase.

Crystallization of triglyceride results in an ~10% increase in density (1, 35). Recently Small et al. (36) have described a bovine IDL particle which resulted from the crystallization of VLDL containing saturated triglyceride species. The highly fluid core of the chylomicra and VLDL of the present study appeared to keep the mean particle density from increasing above \(d = 1.006 \text{ g/ml}\); the calculated density for each subfraction is given in Table 1. The calculations were based on the density of 2-oleylglycerol in the \(d'\) polymorphic form at 15 °C (35) and on a 50% fluid, 50% solid triglyceride core (see above). Also included are the calculated density values for the same particles with a fluid triglyceride core at 15 °C. All subfractions studied had calculated particle densities of \(d < 1.006 \text{ g/ml}\). Ockner et al. (37) found no difference in the quantity of triglyceride or cholesterol transported in the \(d > 1.006 \text{ g/ml}\) fraction of rats infused with either palmitate or linoleic acid in micellar form. The \(d < 1.006 \text{ g/ml}\) triglyceride-rich fraction of rats infused with either palmitate or linoleic acid in micellar form. The \(d < 1.006 \text{ g/ml}\) lipoproteins isolated at 4 °C had 46–56% of the triglyceride fatty acids as palmitic acid. Thus, it appears unlikely that a significant amount of the \(d < 1.006 \text{ g/ml}\) lymph lipoproteins isolated at 15 °C from butter-infused animals was lost to the \(d > 1.006 \text{ g/ml}\) infrate because of triglyceride crystallization.

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National Cooperative Gallstone Study
The Effect of Chenodeoxycholic Acid on Lipoproteins and Apolipoproteins

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Subjects in the National Cooperative Gallstone Study undergoing 12 mo of therapy with chenodeoxycholic acid for the dissolution of gallstones (low-dose, 375 mg/day, n = 252; high-dose, 750 mg/day, n = 253) had a mean increase in serum cholesterol of 20 mg/dl as compared with a 5 mg/dl increase in the placebo group (n = 258). The effect of chenodeoxycholic acid on lipoproteins was determined in a random subset of the high-dose (n = 136) and placebo (n = 143) groups. For men, the mean baseline adjusted estimated low-density lipoprotein cholesterol level at 12 mo was significantly higher in the high-dose group than in the placebo group (159 vs. 146 mg/dl, p < 0.01), whereas among women this difference was not demonstrated. Change in low-density lipoprotein cholesterol level was inversely related to baseline cholesterol to an equivalent degree in each group among men and women. Women in the high-dose group had significantly lower very-low-density lipoprotein cholesterol levels than did the corresponding placebo group (27 vs. 32 mg/dl, p < 0.003). Very-low-density lipoprotein cholesterol levels did not differ significantly between the high-dose and placebo group in men. Treatment did not significantly affect the levels of high-density lipoprotein cholesterol or apoproteins A-I, A-II, or B. Chenodeoxycholic acid therapy produces an increase in total cholesterol and low-density lipoprotein cholesterol but does not alter high-density lipoprotein cholesterol levels.

Chenodeoxycholic acid (CDCA) decreases biliary cholesterol saturation (1,2) and has been shown in several studies (3) to be effective in the dissolution of cholesterol gallstones. This action is apparently brought about by reducing cholesterol secretion into bile (4) with resultant decrease in cholesterol saturation. A therapeutic trial, the National Cooperative Gallstone Study (NCGS), was designed and executed to determine in a clinical setting the effectiveness and safety of CDCA for dissolving cholesterol gallstones (5).

Because CDCA affects the metabolism of endogenous bile acids, there has been some concern that this agent might adversely affect cholesterol metabolism and increase the risk of developing coronary heart disease (6). For example, a normal pathway for removal of cholesterol from the body is by its conver-
sion to bile acids. This conversion pathway may be important for controlling low-density lipoprotein (LDL) levels, as shown by the lowering of the LDL levels that occurs when the conversion of cholesterol into bile acids is increased by bile acid sequestrants (2). Chenodeoxycholic acid feeding blocks bile acid synthesis and thus decreases the catabolism of cholesterol into bile acids and could lead to an increase in hepatic cellular cholesterol (6). Such an increase could lead to: (a) increased secretion of cholesterol into hepatic very-low-density lipoproteins (VLDLs); or (b) decreased hepatic uptake of cholesterol from plasma lipoprotein sources. It has been recently shown that a hepatic receptor for LDLs is present and that it is stimulated by cholestyramine treatment—a treatment that also enhances bile acid synthesis from cholesterol (8). Conversely, if CDCA blocks bile acid synthesis and increases cellular cholesterol, it may inhibit the expansion of the hepatic LDL receptor and subsequently augment plasma LDL. This latter response has been observed in two preliminary reports (9,10), and no studies have adequately tested the possibility.

The NCCS was designed to determine whether CDCA therapy for gallstones is associated with a significant alteration in the concentrations of serum lipids or of lipoproteins. At predefined intervals throughout the trial, serum total cholesterol and triglycerides were determined in patients with gallstones receiving placebo or one of two different doses of CDCA. The results showed that CDCA caused a significant increase in total cholesterol and a fall in triglycerides (5). Because of these findings, the present study was conducted to examine cholesterol concentrations in the different lipoprotein fractions: very-low-density lipoproteins, high-density lipoproteins, and low-density lipoproteins. Simultaneous measurements were made of apolipoproteins B, A-I, and A-II.

Methods

Study Design

The National Cooperative Gallstone Study (NCCS) was a multicenter, double-blind, clinical trial of the treatment of gallstones with chenodeoxycholic acid. The experimental design and implementation of the study have been described (11). The assignment of therapy was randomized among the three treatment groups: high-dose CDCA (750 mg/day); low-dose CDCA (375 mg/day); or placebo (identical starch-filled capsules containing 3 mg sodium cholate without CDCA). There were 305 patients in the groups receiving placebo and high-dose CDCA, and 306 patients in the group receiving low-dose CDCA for a total of 916 subjects. The criteria for eligibility and exclusion has been described in detail elsewhere (11). At prescribed intervals for up to 2 yr, periodic clinical, laboratory, and radiologic evaluations were conducted to monitor the patient's status (11).

The emerging results were reviewed on a regular basis by the NCCS Data Monitoring Committee and Advisory Board only. When an apparent rise in total cholesterol was observed by 12 mo in the high- and low-dose groups in excess of that seen in the placebo group, an ancillary study of the effects of CDCA (750 mg/day) on lipoprotein and apolipoproteins was recommended.

A sample of patients (150 receiving high-dose CDCA therapy and 150 receiving placebo) was drawn from among the study patients who had entered and 12-mo lipid measurements on the Biostatistical Center master file as of April 1, 1980. However, completely adequate entry and 12-mo specimens were available for 279 patients. The 279 subjects chosen for the lipoprotein study had demographic and clinical characteristics (Table 1) similar to those in the major study, which have been described in Reference 5. In the subsample, the characteristics of the high-dose group and the placebo group were nearly the same. No characteristics were significantly different either in comparison of the subset to the NCCS study or in the comparison within the subset of the high-dose group with the placebo group.

Lipids and Lipoproteins

Serum was obtained from subjects in the fasting state. After centrifugation and separation, all serum from a patient was pooled and divided into aliquots for use by the local clinic and the Central Serum Laboratory (CSL). Three 2-ml vials were shipped on dry ice to the CSL (Bio-Science Laboratories, Inc., Van Nuys, Calif.) for assays of serum glutamic oxaloacetic and serum glutamic pyruvic transaminases and serum lipids (total cholesterol and triglycerides). One of the three aliquots was used for these assays, the other two were stored at -70°C for future study. Internal procedures for quality control revealed that the coefficient of variation was 5% for cholesterol and 3% for triglyceride. For external quality control, masked duplicate aliquots of serum were submitted by each treatment center with every shipment. The coefficients of variation were 8% for cholesterol and 15% for triglyceride.

For the lipoprotein study, the designated subset of frozen samples was shipped on dry ice from the CSL to the Northwest Lipid Research Clinic (NWLRRC) in Seattle, Wash. These samples were kept frozen at -70°C until the day of analysis, when they were thawed at room temperature. To each 2 ml of serum was added 20 μl of preservation (5% sodium azide, 0.1% chloramphenicol, 0.05% gentamycin) with thorough mixing; then, 0.5 ml and 1.0 ml portions were removed for analysis of total serum cholesterol and triglyceride and of high-density lipoprotein (HDL) cholesterol, respectively. The remaining 0.5 ml was kept at 4°C in sealed vials for analysis of A-I, A-II, and B apolipoproteins. Cholesterol and triglyceride were determined by the methodology of the Lipid Research Clinics Laboratories on the AutoAnalyzer II (Technicon, Tarrytown, N.Y.) (4). Internal and external quality-control pools indicated that cholesterol and triglyceride were determined with a coefficient of variation of 0.4%-1.2% and 1.0%-1.4%, respectively.
Table 2. Cholesterol* and Triglyceride* after Twelve Months of Placebo, Low-Dose Chenodeoxycholic Acid and High-Dose Chenodeoxycholic Acid Treated Groups

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Sex</th>
<th>Group</th>
<th>n</th>
<th>Baseline</th>
<th>Baseline</th>
<th>Mean difference</th>
<th>Adjusted*</th>
<th>Probability*</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x ± SEM</td>
<td>x ± SEM</td>
<td>12 mo – baseline</td>
<td>12 mo</td>
<td>P vs. LD or HD</td>
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<tr>
<td>Cholesterol</td>
<td>M</td>
<td>P</td>
<td>114</td>
<td>222.2 ± 4.1</td>
<td>5.1 ± 3.0</td>
<td>226.3 ± 2.8</td>
<td>221.6 ± 2.8</td>
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<tr>
<td></td>
<td></td>
<td>LD</td>
<td>132</td>
<td>221.6 ± 3.2</td>
<td>19.9 ± 2.8</td>
<td>241.0 ± 2.6</td>
<td>238.7 ± 2.8</td>
<td>0.0019</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HD</td>
<td>115</td>
<td>218.5 ± 3.6</td>
<td>18.6 ± 3.0</td>
<td>237.1 ± 2.8</td>
<td>234.8 ± 2.6</td>
<td>0.0021</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>F</td>
<td>P</td>
<td>144</td>
<td>241.2 ± 3.8</td>
<td>9.2 ± 2.7</td>
<td>250.4 ± 3.1</td>
<td>247.3 ± 2.5</td>
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<tr>
<td></td>
<td></td>
<td>LD</td>
<td>120</td>
<td>232.1 ± 3.7</td>
<td>19.8 ± 2.7</td>
<td>252.5 ± 2.7</td>
<td>249.4 ± 2.5</td>
<td>0.0001</td>
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<tr>
<td></td>
<td></td>
<td>HD</td>
<td>138</td>
<td>238.3 ± 3.9</td>
<td>19.4 ± 2.7</td>
<td>257.0 ± 2.7</td>
<td>253.5 ± 2.5</td>
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<tr>
<td>Triglyceride</td>
<td>M</td>
<td>P</td>
<td>114</td>
<td>165.2 ± 8.6</td>
<td>-1.4 ± 9.5</td>
<td>157.0 ± 6.0</td>
<td>153.8 ± 6.0</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>LD</td>
<td>132</td>
<td>153.2 ± 6.8</td>
<td>-11.2 ± 5.3</td>
<td>142.5 ± 6.8</td>
<td>139.0 ± 6.0</td>
<td>0.0001</td>
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<tr>
<td></td>
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<td>HD</td>
<td>115</td>
<td>141.9 ± 6.4</td>
<td>-13.2 ± 4.3</td>
<td>135.3 ± 6.0</td>
<td>131.9 ± 6.0</td>
<td>0.0001</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>F</td>
<td>P</td>
<td>144</td>
<td>142.7 ± 6.8</td>
<td>1.4 ± 5.8</td>
<td>140.6 ± 6.4</td>
<td>137.3 ± 6.0</td>
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<tr>
<td></td>
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<td>LD</td>
<td>120</td>
<td>123.7 ± 5.1</td>
<td>-10.4 ± 4.1</td>
<td>122.0 ± 4.9</td>
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<tr>
<td></td>
<td></td>
<td>HD</td>
<td>138</td>
<td>143.1 ± 7.6</td>
<td>-18.4 ± 5.1</td>
<td>129.0 ± 4.5</td>
<td>125.7 ± 4.5</td>
<td>0.0001</td>
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</tbody>
</table>

* Measured at the Central Serum Laboratory (CSL) for all NCGS patients followed up to month 12. † Given as milligrams per deciliter. ‡ P = placebo; LD = low-dose CDCA treated group; HD = high-dose CDCA treated group. ‡ Adjusted 12 mo x – unadjusted 12 mo x – average within treatment slope x [baseline group x – overall baseline x]]. * Analysis of covariance with pairwise t-tests, p ≤ 0.0033 required for significance at the 0.01 level. † Analysis of variance of differences with pairwise t-tests, p ≤ 0.0033, which is required for significance at the 0.01 level.

Variables at entry, two-tailed Student's t-tests were used, taking into account the equality of sample variances. Differences between entry and month-12 values were calculated and used in t-tests for difference scores; however, because the regression slopes are not near unity the analysis of covariance was used to adjust lipid measurements at month 12 for any differences among individuals at entry. Linear least-squares regression models were used to quantify the relationship between changes in lipid levels as a function of baseline levels and also to relate changes in total cholesterol and in LDL-cholesterol levels.

Results

Summary of Results in the National Cooperative Gallstone Study

Serum lipid measurements of the NCGS patients were performed at randomization and thereafter at 1–3, 6, 9, 12, 16, 20, and 24 mo. Analyses of these measurements indicated significant differences in the baseline-adjusted mean values of serum cholesterol among the three treatment groups; those in the high- and low-dose group rose rapidly during the first 3 mo; and then in all groups rose slowly through month 16 (see Figure 10 of Reference 5). There were no significant differences between the high and low-dose groups. In contrast, the baseline-adjusted mean values of triglyceride in the high-dose group decreased over time and were significantly lower than those of the placebo group (see Figure 11 of Reference 5). Mean triglyceride levels of the low-dose group tended to be intermediate between those in the high-dose and placebo groups. These group differences persisted after adjusting for fluctuations in body weight during follow-up.

Serum Lipids of Men and Women Compared

To further explore the changes in lipid levels, additional analyses (not presented in Reference 5) were performed separately in men and women at month 12 (Table 2). After 12 mo of therapy, the mean cholesterol levels of both sexes significantly increased 19–20 mg/dl ± 3.00 (x ± SEM, p ≤ 0.01) in both the high- and low-dose groups. In the placebo group, the 1-yr mean change in cholesterol levels was not statistically significant among men or women, although among women it increased 9 mg/dl ± 3.0 (p ≤ 0.05, not significant by our criteria). The mean cholesterol levels for men, adjusted for baseline values, for both the high- and low-dose groups were significantly different from placebo values (p ≤ 0.0033), but these levels for women were not statistically significant by the NCGS significance criteria for paired comparisons.

After 12 mo, the mean triglyceride levels in the high-dose group significantly decreased (13–18 mg/dl ± 4.5, p ≤ 0.01) for both sexes. In the low-dose group, the decrease was only about 10–11 mg/dl ± 5.0. For the placebo group, the mean change in triglyceride over 12 mo was not significant for either men or women. The mean baseline-adjusted triglyceride levels of the high-dose group were significantly lower than those for the placebo group (p ≤
Table 1. Demographic Characteristics of the Subjects at Baseline on Day of Entry

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>NGC Study (n = 516)*</th>
<th>Lipoprotein study</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>x ± SEM</td>
<td>n</td>
</tr>
<tr>
<td>Sex (% men)</td>
<td>46.1 ± 1.6</td>
<td>422</td>
</tr>
<tr>
<td>Race (% white)</td>
<td>95.0 ± 0.7</td>
<td>870</td>
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<tr>
<td>Age (yr)</td>
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<tr>
<td>Men</td>
<td>53.4 ± 0.5</td>
<td>422</td>
</tr>
<tr>
<td>Women</td>
<td>56.0 ± 0.4</td>
<td>494</td>
</tr>
<tr>
<td>% Ideal body weight</td>
<td></td>
<td></td>
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<tr>
<td>Men</td>
<td>120.9 ± 0.8</td>
<td>422</td>
</tr>
<tr>
<td>Women</td>
<td>125.6 ± 1.1</td>
<td>494</td>
</tr>
<tr>
<td>Current smokers (%)</td>
<td>21.4 ± 1.4</td>
<td>196</td>
</tr>
<tr>
<td>Current cigarette smokers (%)b</td>
<td>17.5 ± 1.3</td>
<td>160</td>
</tr>
<tr>
<td>Current drinkers (g/wk)</td>
<td>41.1 ± 1.6</td>
<td>376</td>
</tr>
<tr>
<td>% Bile saturation</td>
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<td></td>
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<tr>
<td>Men</td>
<td>136.3 ± 2.8</td>
<td>272</td>
</tr>
<tr>
<td>Women</td>
<td>151.8 ± 3.6</td>
<td>262</td>
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</tbody>
</table>

*Total number of subjects in group. b Smoked at least six cigarettes, two cigars, or two pipefuls per day.

Values of cholesterol and triglyceride of the CSL sera were highly correlated with those obtained by the NWLRC (r = 0.940, n = 544 for cholesterol, and r = 0.988, n = 549, for triglyceride). Cholesterol values obtained by the CSL tended to be higher (236 ± 2 mg/dl) than those obtained by the NWLRC (230 ± 2 mg/dl, mean ± SEM), whereas triglyceride values obtained by the NWLRC (156 ± 4 mg/dl) were somewhat higher than those obtained by the CSL (144 ± 4 mg/dl).

High-density lipoprotein cholesterol was determined by measuring the cholesterol in supernatant obtained by precipitation of the lipoproteins containing apolipoprotein B (approximating the VLDLs and LDLs) with heparin and MnCl₂ (12,13). Analysis of samples stored frozen at -70°C for 1 yr were shown to be not significantly different from analysis of fresh plasma (14). An extension of this study indicated that sample storage at -70°C for up to 2 yr does not significantly affect the determination of HDL cholesterol or HDL triglyceride (correlation coefficient between initial value vs. 2-yr value = 0.98 for HDL cholesterol and 0.77 for HDL triglyceride, n = 16). Samples stored frozen for 2 yr had essentially the same value as sample size of 150 females afforded 66% power of detecting 48.6 mg/dl vs. 49.9 for HDL cholesterol and mean 11.8 mg/dl vs. 11.6 for HDL triglyceride. The NGCS samples were stored at -70°C from 2 to 41 mo. Turbid supernatants were filtered before analysis, as described (15). The LDL cholesterol was estimated as total cholesterol minus the sum (triglyceride/5 + HDL cholesterol) (16). Apolipoprotein B of total plasma was measured in triplicate by double-antibody radioimmunodiffusion assay with a between-assay coefficient of variation of 9% (17). Determinations of apolipoproteins A-I and A-II were performed in duplicate by radial immunodiffusion assay with a coefficient of variation of approximately 6% (18,19).

Sample Size

One of the objectives of this study was to find out if there was a significant shift in the levels of the LDLs and HDLs by month 12 in the high-dose group relative to the control group. In order to determine the sample size required for this study, a pilot study was conducted of 30 patients (15 high-dose and 15 placebo), the results of which indicated that the standard deviations of these differences were approximately 30 mg for LDLs and 12 mg for HDLs. On this basis, by using the methods outlined in Reference 20 it was determined that n = 300 would afford approximately 90% power (α = 0.05, one-sided) of detecting a difference between groups of 10 mg for LDLs and of 4 mg for HDLs. Later it was decided, however, that all analyses would be conducted separately for men and women, using the 279 of the original 300 selected patients for whom specimens were successfully assayed. The final sample size of 150 females afforded 66% power of detecting equivalent differences between groups in LDLs (10 mg) and HDLs (4 mg) and the sample size of 129 males afforded 60% power. These sample sizes afford over 80% power of detecting differences between groups on the order of 13 mg for LDLs and 5 mg for HDLs.

Significance Levels

A p ≤ 0.05 was required for significance in the analyses of a primary outcome variable (LDL and HDL levels) and p ≤ 0.01 for all other variables. For further analysis of the lipid data from the major NGCS study, the 0.01 significance level was employed and p ≤ 0.0033 was used for pair-wise comparisons.

Statistical Methods

All statistical analyses were conducted by standard methods (21) with the Statistical Analysis System (SAS) (22). To compare proportions, the two-tailed χ² test of association for contingency tables was used, continuity corrected. The Mantel–Haenszel procedure was employed to adjust the association between two variables for the effect of a third variable. To compare means of quantitative
Table 3. The Relationship Between the Change in Cholesterol or Triglyceride From Baseline to Month Twelve as a Function of Baseline Cholesterol or Triglyceride Level for Placebo, Low-Dose, and High-Dose Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Cholesterol</th>
<th>Triglyceride</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\beta_0$</td>
<td>$\beta_1$ R²</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>114</td>
<td>65.4 ± 14.9</td>
<td>-0.27 ± 0.07</td>
</tr>
<tr>
<td>LD</td>
<td>64</td>
<td>66.4 ± 22.5</td>
<td>-0.31 ± 0.10</td>
</tr>
<tr>
<td>HD</td>
<td>132</td>
<td>97.8 ± 15.4</td>
<td>-0.35 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>64</td>
<td>84.9 ± 16.3</td>
<td>-0.30 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>64</td>
<td>88.9 ± 17.2</td>
<td>-0.36 ± 0.08</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>144</td>
<td>66.1 ± 13.7</td>
<td>-0.24 ± 0.06</td>
</tr>
<tr>
<td>LD</td>
<td>79</td>
<td>58.3 ± 16.7</td>
<td>-0.23 ± 0.07</td>
</tr>
<tr>
<td>HD</td>
<td>120</td>
<td>84.3 ± 14.5</td>
<td>-0.28 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>138</td>
<td>105.8 ± 13.6</td>
<td>-0.37 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>84.2 ± 16.2</td>
<td>-0.31 ± 0.07</td>
</tr>
</tbody>
</table>

* First-order linear regression model: Δcholesterol = $\beta_0 + (\beta_1 \times$ baseline cholesterol), or Δtriglyceride = $\beta_0 + (\beta_1 \times$ baseline triglyceride); Δcholesterol = (cholesterol 12 mo - cholesterol baseline), and Δtriglyceride = (triglyceride 12 mo - cholesterol baseline). The estimated parameters, $\beta_0$ and $\beta_1$ of the model are regression coefficients. $\beta_0$ is the y intercept of the regression line; $\beta_1$ is the slope of this line. It indicates the change in the value of y per unit increase in x. R² is the proportion of the variance (the change in the cholesterol or triglyceride) that is accounted for by baseline value. Units of $\beta_0$ in mg/dl. Cholesterol and triglyceride analyzed at the Central Serum Laboratory (CSL). P = placebo group; LD = low-dose CDCA treated group; HD = high-dose CDCA treated group. Given as estimated parameter ± SEM. * F-test for regression significant at p < 0.0001. * Subset of major study that was analyzed for lipids, lipoproteins, and apoproteins at the Northwest Lipid Research Clinic, University of Washington, Seattle, Washington.

0.01) among females, but not among males. Furthermore, the mean triglyceride levels of the low-dose group were not significantly lower than those for the placebo group.

The change in cholesterol from baseline levels was studied by regression analyses at 3, 6, 9, 12, 16, and 24 mo in all groups. The change was significantly (p ≤ 0.05) inversely related to baseline cholesterol level, but at month 12 only 11% to 26% of the variation in Δcholesterol could be explained by this first-order linear regression model (Table 3). For patients of each sex there were no significant treatment-baseline interactions, indicating that the influence of baseline cholesterol on Δcholesterol is similar in all three treatment groups.

The change in triglyceride also was significantly (p ≤ 0.05) inversely related to baseline triglyceride levels. For women, but not men, there was a significant treatment-baseline interaction, indicating that among women, treatment influences the relationship between baseline triglyceride and Δtriglyceride levels.

Because CDCA therapy appeared to significantly increase serum cholesterol levels, the effect of 12 mo of CDCA therapy (750 mg/day) on lipoproteins and apolipoproteins was evaluated (Table 4). This included analyses of total cholesterol and triglyceride preformed by the NWLRC. The analyses of the 12-mo cholesterol and triglyceride values among the subsample are presented in Table 4. The mean baseline cholesterol of the women taking placebo tended to be higher than the baseline mean of the women on high-dose CDCA (p ≤ 0.06). The sampling plan did not include sex as a stratification factor, and therefore the higher mean value for the women can be attributed to sampling or random variations. There were no significant differences (p ≤ 0.01) between the high-dose and placebo groups' 12-mo mean cholesterol levels among men or women or for triglyceride among men. However, among women the high-dose group had a significantly lower mean triglyceride level (p ≤ 0.003) than the placebo group. The increase in cholesterol levels and decrease in triglyceride levels within the high-dose group were not as large as that observed in the complete sample, and due to the smaller sample size, these differences were not significant.

Lipoprotein and Apolipoproteins

For men, but not women, the mean baseline-adjusted LDL-cholesterol level at 12 mo was significantly higher in the high-dose group than in the placebo group (p ≤ 0.01, Table 4). Among women, the mean baseline-adjusted LDL-cholesterol level at 12 mo in the high-dose group was significantly lower (p ≤ 0.05) than that of the placebo group, but the mean increase in the high-dose group (15.1 mg/dl) was significantly greater than the mean increase observed in the placebo group (p ≤ 0.006).
### Table 4. Total Lipid, Lipoprotein, and Apolipoprotein Levels in Subjects Undergoing Chenodeoxycholic Acid Therapy for Twelve Months for Placebo and High-Dose Groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sex</th>
<th>Group</th>
<th>n</th>
<th>Baseline $\bar{x} \pm SEM$</th>
<th>Mean difference 12 mo – baseline $\bar{x} \pm SEM$</th>
<th>Adjusted$^b$ 12 mo $\bar{x} \pm SEM$</th>
<th>Probability$^c$ P vs. HD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>M</td>
<td>P</td>
<td>64</td>
<td>220.2 ± 4.8</td>
<td>−1.9 ± 4.1</td>
<td>217.8 ± 4.5</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>HD</td>
<td>64</td>
<td>218.8 ± 5.1</td>
<td>9.4 ± 3.6</td>
<td>228.6 ± 4.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>79</td>
<td>241.3 ± 5.5</td>
<td>3.4 ± 3.6</td>
<td>239.8 ± 4.3</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HD</td>
<td>70</td>
<td>227.2 ± 4.7</td>
<td>13.2 ± 3.1</td>
<td>240.6 ± 4.2</td>
<td></td>
</tr>
<tr>
<td>LDL-Cholesterol</td>
<td>M</td>
<td>P</td>
<td>64</td>
<td>145.4 ± 4.5</td>
<td>1.5 ± 3.9</td>
<td>147.5 ± 4.3</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HD</td>
<td>64</td>
<td>147.5 ± 5.0</td>
<td>12.3 ± 3.7</td>
<td>159.3 ± 5.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>79</td>
<td>161.6 ± 5.1</td>
<td>1.9 ± 3.6</td>
<td>167.7 ± 3.2</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HD</td>
<td>70</td>
<td>147.6 ± 4.0</td>
<td>15.1 ± 3.1</td>
<td>159.1 ± 4.0</td>
<td></td>
</tr>
<tr>
<td>HDL-Cholesterol</td>
<td>M</td>
<td>P</td>
<td>64</td>
<td>38.7 ± 1.3</td>
<td>0.5 ± 0.9</td>
<td>38.8 ± 1.0</td>
<td>0.46</td>
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<tr>
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<td>64</td>
<td>37.7 ± 1.1</td>
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<td>39.9 ± 1.0</td>
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<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>79</td>
<td>49.0 ± 1.7</td>
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<td>49.6 ± 1.1</td>
<td>0.94</td>
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<td>48.9 ± 1.7</td>
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<td>49.4 ± 1.2</td>
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<tr>
<td>VLDL-Cholesterol</td>
<td>M</td>
<td>P</td>
<td>64</td>
<td>36.4 ± 2.6</td>
<td>−2.3 ± 2.2</td>
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<tr>
<td></td>
<td></td>
<td>HD</td>
<td>64</td>
<td>33.1 ± 1.6</td>
<td>−4.3 ± 1.3</td>
<td>29.4 ± 1.5</td>
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<tr>
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<td>F</td>
<td>P</td>
<td>79</td>
<td>31.3 ± 1.9</td>
<td>0.3 ± 1.4</td>
<td>31.4 ± 1.1</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HD</td>
<td>70</td>
<td>30.7 ± 2.6</td>
<td>−4.4 ± 1.5</td>
<td>26.6 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Triglyceride</td>
<td>M</td>
<td>P</td>
<td>64</td>
<td>177.0 ± 13.1</td>
<td>−11.4 ± 11.1</td>
<td>162.5 ± 7.5</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HD</td>
<td>65</td>
<td>165.7 ± 8.8</td>
<td>−21.7 ± 6.4</td>
<td>147.1 ± 7.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>79</td>
<td>156.5 ± 9.4</td>
<td>−1.5 ± 6.9</td>
<td>157.2 ± 5.5</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HD</td>
<td>70</td>
<td>153.8 ± 13.1</td>
<td>−22.9 ± 7.6</td>
<td>132.9 ± 5.9</td>
<td></td>
</tr>
<tr>
<td>HDL-Triglyceride</td>
<td>M</td>
<td>P</td>
<td>64</td>
<td>12.7 ± 0.5</td>
<td>−0.5 ± 0.6</td>
<td>12.3 ± 0.6</td>
<td>0.44</td>
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<tr>
<td></td>
<td></td>
<td>HD</td>
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<td>12.7 ± 0.5</td>
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<td>12.9 ± 0.6</td>
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<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>78</td>
<td>15.0 ± 0.7</td>
<td>−0.1 ± 0.6</td>
<td>14.9 ± 0.5</td>
<td>0.01</td>
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<tr>
<td></td>
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<td>HD</td>
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<td>14.7 ± 0.6</td>
<td>−1.8 ± 0.6</td>
<td>12.9 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Apo-B</td>
<td>M</td>
<td>P</td>
<td>58</td>
<td>100.5 ± 2.9</td>
<td>−1.6 ± 4.6</td>
<td>99.5 ± 3.5</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
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<td>HD</td>
<td>56</td>
<td>104.8 ± 4.2</td>
<td>−8.1 ± 4.2</td>
<td>96.2 ± 3.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>76</td>
<td>100.4 ± 3.4</td>
<td>−2.2 ± 3.5</td>
<td>98.5 ± 2.9</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
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<td>HD</td>
<td>65</td>
<td>102.5 ± 3.4</td>
<td>−1.7 ± 4.3</td>
<td>100.5 ± 3.1</td>
<td></td>
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<tr>
<td>Apo-A-I</td>
<td>M</td>
<td>P</td>
<td>64</td>
<td>139.0 ± 3.3</td>
<td>0.2 ± 3.4</td>
<td>138.0 ± 2.5</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HD</td>
<td>65</td>
<td>133.9 ± 2.7</td>
<td>2.4 ± 2.7</td>
<td>137.0 ± 2.5</td>
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</tr>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>79</td>
<td>158.1 ± 3.2</td>
<td>−0.9 ± 3.2</td>
<td>156.9 ± 2.6</td>
<td>0.18</td>
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<td>151.7 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>Apo-A-II</td>
<td>M</td>
<td>P</td>
<td>64</td>
<td>34.5 ± 0.8</td>
<td>−0.4 ± 0.6</td>
<td>33.7 ± 0.7</td>
<td>0.08</td>
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<tr>
<td></td>
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<td>65</td>
<td>33.1 ± 0.7</td>
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<td>35.0 ± 0.7</td>
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<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>78</td>
<td>36.0 ± 0.7</td>
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<td>36.9 ± 0.6</td>
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<td>35.3 ± 0.7</td>
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<td>37.4 ± 0.6</td>
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</tr>
<tr>
<td>Apo-AI/A-II</td>
<td>M</td>
<td>P</td>
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<td>4.1 ± 0.05</td>
<td>0.007</td>
</tr>
<tr>
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<td>−0.1 ± 0.1</td>
<td>3.9 ± 0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>78</td>
<td>4.4 ± 0.1</td>
<td>−0.1 ± 0.1</td>
<td>4.3 ± 0.1</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HD</td>
<td>69</td>
<td>4.4 ± 0.1</td>
<td>−0.2 ± 0.1</td>
<td>4.1 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ All measurements are in mg/dl, and expressed as mean ± SEM. Lipids, lipoproteins, and apoproteins measured at the NWLRC, University of Washington. $^b$ Adjusted 12 mo $\bar{x}$ = unadjusted 12 mo $\bar{x}$ − [average within treatment slope × (baseline group $\bar{x}$ − overall baseline $\bar{x}$)]. $^c$ Analysis of covariance with pairwise t-tests, $p \leq 0.0033$ required for significance at the 0.01 level.

Comparison of the change in total-cholesterol and LDL-cholesterol levels after 12 mo on high-dose therapy suggests that the increase in cholesterol was attributable to an increase in LDL-cholesterol. Indeed, assuming a first-order linear regression model, Δcholesterol = [0.81 ± 0.07 (slope ± SEM) × ΔLDL-cholesterol] −0.6 mg/dl (r = 0.81) for men on high-dose therapy, and Δcholesterol = [0.72 ± 0.09 × ΔLDL-cholesterol] + 2.3 mg/dl (r = 0.71) for women on therapy. Table 5 shows that the change in LDL-cholesterol from baseline level to that of month 12 was significantly inversely related to baseline cholesterol and also baseline LDL-cholesterol levels. High-dose CDCA did not significantly affect the levels of total serum apolipoprotein B (Table 4). Because approximately 85% of the total apoprotein B
Table 5. The Relationship Between the Change in LDL-Cholesterol from Baseline to Month Twelve as a Function of Baseline Cholesterol or Baseline LDL-Cholesterol for Placebo and High-Dose Groups

<table>
<thead>
<tr>
<th>Sex</th>
<th>Group</th>
<th>n</th>
<th>( \beta_0 )</th>
<th>( \beta_1 )</th>
<th>( R^2 )</th>
<th>( \beta_0 )</th>
<th>( \beta_1 )</th>
<th>( R^2 )</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Baseline cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>M</td>
<td>Placebo</td>
<td>64</td>
<td>67.5 ± 21.1</td>
<td>-0.30 ± 0.09</td>
<td>0.14</td>
<td>62.2 ± 14.3</td>
<td>-0.42 ± 0.10</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>64</td>
<td>72.8 ± 18.6</td>
<td>-0.28 ± 0.08</td>
<td>0.15</td>
<td>71.0 ± 11.8</td>
<td>-0.40 ± 0.08</td>
<td>0.30</td>
</tr>
<tr>
<td>F</td>
<td>Placebo</td>
<td>79</td>
<td>58.2 ± 17.1</td>
<td>-0.23 ± 0.07</td>
<td>0.13</td>
<td>56.1 ± 11.9</td>
<td>-0.34 ± 0.07</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>70</td>
<td>51.5 ± 17.7</td>
<td>-0.16 ± 0.08</td>
<td>0.08</td>
<td>59.0 ± 12.7</td>
<td>-0.30 ± 0.08</td>
<td>0.16</td>
</tr>
</tbody>
</table>

*First-order linear regression model \( \Delta \text{LDL-cholesterol} = \beta_0 + (\beta_1 \times \text{baseline cholesterol or baseline LDL-cholesterol}) \). \( \beta_0 \) is the y intercept of the regression line, \( \beta_1 \) is the slope of the line. It indicates the change in the mean of the distribution of \( y \) per unit increase in \( x \). Units of \( \beta_0 \) in mg/dL and expressed as estimated parameter ± SEM. \( R^2 \) is the proportion of the variance (the change in the LDL-cholesterol) that is accounted for by the baseline cholesterol or the baseline LDL-cholesterol value. Cholesterol and LDL-cholesterol measured at the NWLRC, University of Washington. \( * \) F-test for regression significant at \( p \leq 0.0001 \).

is usually associated with LDLs and only about 15% with VLDLs (13), changes in apoprotein B levels may be expected to be correlated with changes in LDL-cholesterol levels in the absence of significant changes in VLDL levels. However, among men changes in LDL-cholesterol levels from baseline to month 12 were not significantly related to changes in apoprotein B. Among women, changes in LDL-cholesterol levels were significantly correlated with change in apoprotein B (\( r = 0.32, p = 0.004 \) for the high-dose group and \( r = 0.43, p = 0.0002 \) for the placebo group). The lower correlation for the high-dose group is presumably related to the decrease of VLDLs with therapy (see below).

Among women, but not men, the high-dose group had significantly (\( p \leq 0.003 \)) lower VLDL-cholesterol levels at month 12 than the placebo group (Table 4). Furthermore, like triglycerides, the change in VLDL-cholesterol level was inversely related to the baseline VLDL-cholesterol level (\( r = 0.57 \) for men on treatment, and 0.81 for women in the high-dose group, \( p \leq 0.0001 \)).

Treatment with CDCA did not significantly affect the levels of HDL-cholesterol or the serum concentration of apolipoproteins A-I, A-II (Table 4). For women, but not men, the high-dose group had significantly lower HDL-triglyceride levels than did the placebo group (\( p = 0.01 \)). Even though significant changes in HDL-cholesterol or apolipoproteins A-I or A-II did not occur with therapy, the mean A-I/A-II ratio was lower in the high-dose group than the placebo group in men (3.9 ± 0.05 vs. 4.1 ± 0.1, \( p = 0.007 \)); a similar difference was evident in women (\( p = 0.05 \)).

Changes in A-I were significantly related to changes in ΔHDL-cholesterol levels in the high-dose group, \( r = 0.31 \) for men and \( 0.33 \) for women, whereas for the placebo group \( r = 0.52 \) for men and \( 0.28 \) for women. Changes in A-II were not as strongly associated with HDL cholesterol (high-dose group, \( r = 0.19 \) for men and \( 0.22 \) for women; placebo group, \( r = 0.40 \) for men and \( 0.22 \) for women).

Discussion

Patients of the NCGS tended to be somewhat overweight; men averaged 121% of ideal weight, and women averaged 126% as compared with approximately 116% for normal adults (23). This tendency towards obesity may partly explain the higher baseline values for total cholesterol than those observed for the general U.S. population of similar age and the same sex (24). Whether there is a link between plasma cholesterol levels and the presence of gallstones has never been discovered. If there is, it is likely that obesity and increased intake of total calories contribute to both, but other factors also may be involved.

A finding of the NCGS was that both low-dose and high-dose CDCA therapy caused an increase in total plasma cholesterol for men and women. While patients were receiving CDCA medication, their serum cholesterol levels increased significantly above those of the placebo group; these also rose but not significantly above baseline levels. The magnitude of the rise was not great—approximately 5%–7% above that observed in the placebo group—but there is little doubt that the change was real. Because expansion of the bile acid pool with CDCA almost certainly decreases the conversion of cholesterol into bile acids in the liver, it was anticipated that hepatic and plasma concentrations of cholesterol might be increased (6). This possibility is in keeping with the observation that interruption of the enterohepatic circulation (EHC) with bile acid sequestrants leads to enhanced formation of bile acids from cholesterol and to a reduction in plasma cholesterol (8). With the exception of two preliminary studies (9,10), a rise in plasma cholesterol levels during CDCA treatment has not been reported in earlier studies, but in
all of these studies the number of patients studied has been relatively small and generally patients have not been studied for prolonged periods.

The finding that total cholesterol was increased by CDCA raised the question of which lipoprotein fraction was responsible for this change. To answer this question, frozen plasma from selected patients was analyzed for lipoproteins. Approximately one-third of the patient population in the trial was selected for lipoprotein studies. The general characteristics of the selected patients closely resembled those of the whole population.

The increase in total cholesterol on CDCA therapy appeared to be confined to LDL cholesterol among men and women, there being a significant increase in LDL cholesterol over placebo levels. The mechanism for the rise in LDLs during treatment with CDCA was not determined. It could be due to either increased synthesis of LDLs or a decreased clearance. On the basis of recent reports the latter seems the more likely. Current evidence suggests that some of the circulating LDLs are removed and degraded by the liver (25). The hepatic uptake of LDLs is probably mediated by specific receptors for LDLs; these receptors have been shown to exist in rats (26–28), dogs (29), and pigs (30). Furthermore, experiments in animals indicate that hepatic receptors for LDLs can be increased by interruption of the enterohepatic circulation of bile acids (8,31) and this may explain the reduction in LDL levels induced by this procedure. By the same token, expansion of the bile acid pool by CDCA reduces conversion of cholesterol into bile acids, which possibly suppresses hepatic receptors for LDLs, and thereby raises the LDL concentration.

The fact that CDCA reduces the rate of cholesterol catabolism into bile acids has caused some investigators to express concern that removal of cholesterol from the body would be impaired. This concern would be heightened if uptake of LDLs by the liver is inhibited. It has been reported that bile acid sequen- trants can cause partial resolution of tendon xan- thomas in patients with familial hypercholesterolemia, even when plasma cholesterol concentrations are minimally changed (32). This might be explained by an enhanced hepatic uptake of LDLs and conversion of LDL-cholesterol into bile acids. Conversely, reduced removal of LDLs by the liver during treatment with CDCA might interfere with normal mechanisms for removal of cholesterol from the body. Even if CDCA were to increase total body pools of cholesterol, this would not necessarily promote development of atherosclerosis beyond that caused by a rise in LDL levels. Still, this later possibility must be kept in mind.

Another finding of the NCCS was that CDCA induced a reduction in plasma triglycerides. This action has been observed previously in small groups of patients (33). Available evidence suggests that CDCA feeding interferes with the secretion of triglycerides in VLDLs (34). Whether the triglyceride-lowering action of CDCA offsets a potentially greater risk of developing coronary heart disease, resulting from a rise in cholesterol, is uncertain.

Chenodeoxycholic acid therapy did not significantly alter the levels of HDL cholesterol or serum levels of the HDL apolipoproteins A-I and A-II. However, the A-I/A-II ratio was significantly lower in men in the CDCA group than in the placebo group and a similar trend was observed in women. The A-I/A-II ratio varies considerably with the hydrated density of the HDL particle distribution, with the HDL2 subclass having significantly higher A-I/A-II ratio than the HDL4 subclass (35). Therefore, a decrease in the A-I/A-II ratio would be expected to be reflected in a decrease of the HDL2 subclass relative to the HDL3 subclass. Additional studies are needed to document the specific changes in the HDL subclass distribution induced by CDCA therapy.

In conclusion, this study has shown that CDCA therapy for cholelithiasis has an effect on plasma lipids. In many patients serum cholesterol and LDL-cholesterol levels are increased by the bile acid. Triglycerides are often decreased. Although HDL-cholesterol was unchanged, the distributions of apoproteins in HDLs were altered. There is no proof that these changes would increase the risk of developing CHD, but the rise in LDL levels in particular represents a cause for some concern. Therefore, in our opinion, CDCA should be used with caution. In any patient at high risk, treatment beyond 24 mo may not be justified.

References

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Ionization behavior of aqueous short-chain carboxylic acids: a carbon-13 NMR study

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Abstract  The $^{13}$C chemical shift of each carbon of aqueous acetic, propionic, and butyric acids has been measured as a function of pH or of added equivalents of base. A plot of chemical shifts for the carboxyl, $\alpha$, and $\beta$ carbons as a function of pH is sigmoidal and yields $pK_a$ values that agree closely with values obtained by potentiometric titration. In contrast, a plot of chemical shift as a function of added equivalents of base is linear and has a sharp break at the equivalence point. Based on this result, we propose that the local (microscopic) ionization state of the carboxyl group can be determined directly by NMR without need for pH or $pK_a$ determinations. In addition to titration curves, the effects of concentration, ionic strength, and temperature upon fatty acid chemical shifts are reported. For aqueous acids, changes in ionic strength and temperature have no effect on chemical shifts. However, changes in concentration do affect chemical shifts, probably as a result of changes in the relative degree of acid-acid and acid-water hydrogen bonding. Our results provide necessary background data for $^{13}$C NMR studies of higher fatty acids in lipid-lipid and lipid-protein systems. D. P. Cistola, D. M. Small, and J. A. Hamilton. Ionization behavior of aqueous short-chain carboxylic acids: a carbon-13 NMR study. J. Lipid Res. 1982. 23: 795–799.

Supplementary key words  $^{13}$C chemical shift • fatty acids • pH • ionic strength concentration • NMR titration curves

NMR studies have demonstrated that the ionization state of the carboxyl group has a significant influence upon the chemical shifts of $^{13}$C resonances of carboxylic acids (1, 2). The chemical shift difference between the fully ionized and un-ionized group allows an estimation of its $pK_a$ in a variety of carboxylic acids, amino acids, and peptides (3–6). Although NMR titration curves (chemical shift vs. pH) have been determined for many amino acids and have been applied extensively to ionization studies of peptides (1, 7, 8), there are no such curves for aqueous fatty acids other than acetic acid (1). In addition, the effect of other variables such as concentration, ionic strength, and temperature on $^{13}$C chemical shifts has not been systematically investigated for these acids.

We report $^{13}$C NMR titration curves for three water-miscible short-chain acids: acetic, propionic, and butyric acids. These acids are not complicated by the limited water solubility and complex phase behavior characteristic of the higher fatty acids (9). The titration curves, as well as the dependence of $^{13}$C chemical shifts upon concentration, temperature, and ionic strength reported herein, provide a detailed background necessary for interpretation of $^{13}$C chemical shifts of longer-chain fatty acids in water and in lipid-lipid and lipid-protein systems such as phospholipid bilayers (10, 11), membranes, lipoproteins, and serum albumin, and will aid in the description of the complex phase behavior of fatty acids in these systems.

EXPERIMENTAL

Materials

Glacial acetic acid (reagent A.C.S., Fisher), propionic acid (certified, Fisher), and butyric acid (reagent, Fisher) were diluted to appropriate concentrations with de-ionized, doubly-distilled water; concentrations are given as %v/v unless otherwise noted. The neat propionic acid used for temperature studies was obtained from Nu-Chek Prep, Elysian, MN. There were no impurity peaks in any of the $^{13}$C NMR spectra of the samples used. The pH of samples for concentration studies was adjusted with either 10 N NaOH, 10 N HCl, or solid NaOH.
a Beckman model 3560 pH meter equipped with an Altmex model 531167 5mm combination electrode; the pH meter was standardized to pH 4, 7, and 10 buffers depending on the pH range of measurement. The estimated uncertainty in pH values is ±0.05.

Titration

Ten-ml samples of dilute acid in a scintillation vial were titrated in a nitrogen atmosphere with 10 N NaOH or HCl using a 50 µl Hamilton syringe; sample dilution during titration was insignificant. At appropriate pH values, 2-ml aliquots were transferred to a 10-mm NMR tube. Sample pH values checked before and after each NMR run agreed within ±0.03 pH units.

NMR

Proton-decoupled Fourier transform $^{13}$C NMR spectra were obtained on a Bruker WP 200 spectrometer operating at 47 kGauss (50.3 MHz for $^{13}$C). The system was equipped with an Aspect 2000 data system with 32K data memory. Deuterochloroform and tetramethylsilane (TMS) in a coaxial insert were used as external lock and reference, respectively. The $^1$H irradiation for proton decoupling (1.0 watt) was centered at 3.2 ppm downfield from the $^1$H resonance of TMS. Chemical shifts were

![Diagram of chemical structures and pH vs. ppm graphs for A: Acetic Acid, B: Propionic Acid, C: Butyric Acid]
measured digitally, and their estimated uncertainty was ±0.05 ppm. Probe temperature was regulated with a Bruker B-VT-1000 variable temperature unit; sample temperature was measured using a thin, copper-constantan thermocouple and an Omega model 400A digital readout unit. Temperatures were recorded at 20-sec intervals following removal of sample from magnet and extrapolated back to 0 sec to obtain reported values with ±1°C uncertainty.

RESULTS

Representative spectra from the titration studies are shown in Fig. 1 for 4% butyric acid at pH values corresponding to protonated (spectrum A), deprotonated (spectrum D), and two intermediate states (spectra B and C). Linewidths of all resonances were narrow (<4 Hz) for butyric acid spectra, as well as for all acetic and propionic acid spectra.

Conventional titration curves are shown at the bottom of Fig. 2A (acetic), Fig. 2B (propionic), and Fig. 2C (butyric). The equivalence point corresponds to the steepest portion of the curves. Chemical shifts for each carbon resonance are plotted above the titration curve in each figure. The chemical shift for each carbon resonance increases linearly with added equivalents of base up to the equivalence point, after which the chemical shift remains constant, as shown in the insets of Figs. 2A–C. Thus, the equivalence point may be determined directly from these NMR plots, employing the carboxyl as well as the aliphatic (up to the β carbon) shift data.

Plots of chemical shift as a function of pH for each carbon resonance of acetic, propionic, and butyric acids are shown in Figs. 3A, B, and C, respectively. These curves are similar to those published for related compounds (1, 7, 8). The magnitude of the chemical shift difference between the ionized and un-ionized forms of each acid (titration shift) is largest for the carboxyl group and decreases for each successive carbon away from the carboxyl group. The pKₐ values can be determined from data for all carbon resonances except the γ carbon of butyric acid. Table 1 summarizes carboxyl titration shifts and corresponding pKₐ values; pKₐ values obtained by NMR agree with those obtained by potentiometric titration.

The effects of ionic strength and temperature on chemical shift were studied independently by a) changing ionic strength by adding NaCl at fixed pH, concentration, and sample temperature; and b) changing the NMR probe temperature at fixed pH, concentration, and ionic strength. For aqueous acids (10%, pH 2.5), large changes in ionic strength (up to μₑ = 2) and temperature (Tₑ = 30–55°C) had negligible effects upon chemical shifts (< 0.1 ppm). However, for neat propionic acid, variation of sample temperature from 8°C to 50°C produced a significant linear decrease in the carboxyl chemical shift (0.46 ppm) and smaller linear increases in aliphatic chemical shifts.

The concentration dependence of chemical shifts for each acid at pH 2.5 is shown in Fig. 4. The carboxyl chemical shift exhibited the strongest concentration dependence, while the α carbon exhibited the weakest concentration dependence for each acid. The sample temperature was controlled, but ionic strength increased (up to μₑ ≈ 2) with increasing concentration, since NaOH was added to maintain constant pH. For acetic acid (Fig. 4A), the carboxyl chemical shift decreased from 176.8 ppm at 0.032 mole fraction (10%) to a minimum at about 0.4 mole fraction (60%) and then increased to 177.9 ppm for undiluted glacial acetic acid; the methyl resonance was concentration independent, except at high concentrations (glacial acetic acid). Propionic acid (Fig. 4B) exhibited qualitatively similar results, with a minimum at approximately 0.3 mole fraction for the carboxyl shift. However, a larger chemical shift change occurred with the carboxyl carbon of propionic acid as compared with

| Table 1. Summary of carboxyl chemical shifts and pKₐ values |
|-----------------|------------------|---------------|---------------|---------------|
|                 | δₑₐₐt COOH       | δₑₐₐt COOH     | pKₐ (A)       | pKₐ (B)       | pKₐ (C)       |
| Acetic acid     | 181.45          | 176.80         | 4.7           | 4.6           | 4.75          |
| Propionic acid  | 184.95          | 180.02         | 4.9           | 4.8           | 4.87          |
| Butyric acid    | 184.10          | 179.22         | 4.8           | 4.8           | 4.82          |

Summary of carboxyl chemical shifts (ppm from external tetramethylsilane) for each acid in its fully deprotonated (δₑₐₐₜ) and fully protonated (δₑₐₐₜ) state and of pKₐ values derived from (A) NMR titration, (B) potentiometric titration, and (C) the literature (14). pKₐ (A) represents the pH values that correspond to ½ (δₑₐₐₜ – δₑₐₐₜ). pKₐ (B) values were determined from the potentiometric titration curves in Fig. 2. To ensure that δₑₐₐₜ was reached, samples were back-titrated with “10 N” HCl until chemical shifts reached a constant value. A plot of carboxyl chemical shift as a function of added μₑ of “10 N” HCl showed a sharp change in slope at δₑₐₐₜ (data not shown) similar to that observed at δₑₐₐₜ (Fig. 2).
Our results as plotted in Fig. 2 suggest a simple, direct method for obtaining microscopic ionization states without need for pH or $pK_a$ determinations. Since the carboxyl carbon gives rise to only one $^{13}$C resonance because of fast chemical exchange between protonated and unprotonated species, and since its $^{13}$C chemical shift increases linearly with added base up to the equivalence point, the ionization state of the carboxyl group can be described by the following simple equation:

$$\left(\frac{\delta - \delta_{\text{min}}}{\delta_{\text{max}} - \delta_{\text{min}}}\right) \times 100\% = \%\text{ ionization} \quad \text{Eq. 1}$$

where $\delta_{\text{max}}$ and $\delta_{\text{min}}$ are the carboxyl chemical shifts of the fully ionized and un-ionized acids, respectively, at a given concentration (e.g., 4%), and $\delta$ is the measured chemical shift for a given sample. Thus, a plot of chemical shift as a function of added titrant yields a direct measure of the ionization state of a specific chemical group. To our knowledge, this straightforward analysis has not been previously published; studies of similar compounds have always related $^{13}$C chemical shifts to pH (1, 3, 6, 7, 10, 11) and have assumed a linear relationship between ionization and chemical shift without actually demonstrating it (7, 11).

In our concentration studies, the carboxyl carbon showed the greatest chemical shift changes with concentration. A probable explanation is that concentration alters the relative degree of solute-solute and solute-solvent hydrogen bonding (17). At the lowest acid concentrations, hydrogen bonding between acid and water molecules is maximized, and at the highest acid concentrations, hydrogen bonding between acid molecules is maximized (17); our results show that the carboxyl resonance appears more downfield at concentrations where hydrogen bonding is maximized. Previous results for acetic acid in water are qualitatively similar to ours and also show a minimum chemical shift at approximately 0.3 mole fraction (Fig. 4); however, quantitative differences probably reflect the lack of pH control in the study of Maciel and Traficante (17). In another study, Hagen and Roberts (2) concluded that $^{13}$C chemical shifts of carboxylic acids are rather insensitive to concentration; however, they considered only a narrow concentration range (<0.1 mole fraction). It is clear from our results that carboxyl chemical shifts change significantly with concentration at values <0.3 mole fraction (Fig. 4). In addition, the concentration dependence of all resonances (except the $\alpha$ carbon) is greatest for the longest

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This result also replies to resonances $\alpha$ and $\beta$ to the carboxyl. This result assumes that concentration changes are minimal during titration and that each molecule has only one ionizable group. If more than one group is present, computer curve fitting can be used (7, 16).
acid (butyric), in agreement with previous findings over a much smaller concentration range (2).

In contrast to the marked effects of ionization and concentration upon chemical shifts, large changes in ionic strength and temperature have no significant effect upon chemical shifts for aqueous acids when other variables are controlled. This insensitivity of chemical shift to ionic strength and temperature is important for two reasons. First of all, addition of NaOH during our titrations resulted in unavoidable changes in ionic strength and consequential changes in sample temperature (13). The above findings for ionic strength and temperature demonstrate that these changes do not affect the chemical shifts obtained from our titration studies. Secondly, in contrast to our results, others have found chemical shift changes with ionic strength for aqueous sodium acetate (18), aqueous sodium octanoate (18), and (aqueous) phospholipid/fatty acid vesicles (11). Our results suggest that these changes reflect either a lack of pH control (sodium acetate) (18), a difference in the ionization behavior of longer-chain fatty acids (18), or a difference in the molecular environment near the carboxyl group (11).

Free fatty acids are a major product of triglyceride lipolysis in the intestine, bloodstream, and tissues. In the bloodstream, released free fatty acid can remain in association with serum albumin and lipoproteins, or can cross the capillary endothelium to enter the underlying tissue. One way to probe the molecular environment of fatty acids in these different locations is by NMR chemical shift measurements. However, in order to properly interpret chemical shifts in these complex systems, it is necessary to understand the simplest model systems. The data presented in this paper will help to distinguish the effects of hydrogen bonding and ionization on chemical shifts in complex fatty acid-lipid and fatty acid-protein systems.

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REFERENCES

THE BEHAVIOR OF BIOLOGICAL LIPIDS

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Abstract - Lipids active in biological systems demonstrate a broad range of behavior in water, from hydrocarbons which are insoluble, to molecules such as bile salts that possess potent detergent properties and interact with water rather dynamically. The purpose of this paper is to describe some of the physical properties of lipids with respect to their interaction with aqueous systems, and to classify lipids based on these interactions. The hydrocarbon part of a lipid molecule may be aliphatic or cyclic/aromatic. In predominantly aliphatic lipids, the hydrocarbon part consists of a chain(s), containing eight or more carbons. All such lipids have specific properties related to the hydrocarbon chain packing, while cyclic/aromatic lipids possess unique properties related to the specific hydrocarbon structure. Three basic types of aliphatic chain packing can be found in most predominantly aliphatic chain molecules: 1) a tightly packed aliphatic chain lattice with specific chain-chain interactions and minimum specific volume, 2) an intermediate form of crystalline packing in which specific chain-chain interactions are lost, but the chains are packed in a centered hexagonal lattice, 3) a liquid state in which the \(-\text{CH}_2\)- groups can move more or less freely. Transitions between these states are characterized by abrupt changes in volume and excess specific heat (enthalpy). The partial specific volume of the \(-\text{CH}_2\)- group in the aliphatic chain increases from ~23Å\(^3\)/\(-\text{CH}_2\)- in tightly packed chains to ~26Å\(^3\)/\(-\text{CH}_2\)- in hexagonal packing to ~29Å\(^3\)/\(-\text{CH}_2\)- in the liquid state. The enthalpies of the transition are: ~1 kcal/\(-\text{CH}_2\)- from the crystalline lattice (specific chain-chain interaction) to liquid and ~0.5 kcal/\(-\text{CH}_2\)- from the hexagonal chain packing lattice to liquid. Thus, a change in volume of 4Å\(^3\)/\(-\text{CH}_2\)- requires about 0.17 kcal.

The transition from crystalline to liquid can occur from either tightly packed or more loosely packed crystalline structures. These transitions are known by a variety of names depending upon the lipid system. For instance, it is called the melting point in alkanes, fatty acida, fatty alcoholis, di- and triglycerides, waxes, etc.; the order-disorder transition or gel-liquid crystal transition in soaps, monoglycerides, phospholipids, etc.; the critical micellar temperature or Krafft point in soaps and detergents. This transition temperature is governed by the length of the hydrocarbon chain and the presence of double bonds, cyclic structures, or branches within the chain. The hydrophilic part of the lipid also plays a major part in the chain transition temperatures, as well as in the interaction with aqueous systems. The crystal-liquid chain transition for a given chain length increases in the following order: alkanes < chlorides < alkanes < bromides < aldehydes < alcohols < fatty acids < triglycerides < monoglycerides < Na soaps < phosphatidylcholines < phosphatidylethanolamines < Ca soaps.

Lipids in which the aliphatic chain is in the fluid state, may be classified empirically as nonpolar and polar based on their interaction at the air-water interface and in bulk systems. Nonpolar molecules are insoluble in aqueous systems and do not spread at the air-water interface. Nonpolar molecules include hydrocarbons, waxes, and sterol esters. Polar molecules may be divided into three distinct classes: I. Insoluble Non-swelling Amphiphiles. These molecules spread at the air-water interface to form a stable monolayer but are insoluble in the bulk. This class includes long-chain fatty acids, primary amines, alcohols, cholesterol, di- and triglycerides. II. Insoluble Swelling Amphiphiles. These molecules form stable monolayers, but swell in water to form liquid crystalline...
phases. Membrane lipids, such as phospholipids and cerebroside as well as monoglycerides and acid-soaps, fall in this class. III. Soluble
Amphiphilic Molecules. These molecules form unstable monolayers, have some bulk solubility as monomers and form micelles at a critical micellar
centrification. Aliphatic molecules of this type swell at low water
centrifugation to form liquid crystals, whereas cyclic/aromatic molecules
usually form crystals which dissolve directly to form micelles without
first swelling.

INTRODUCTION

Hydrocarbons and bile salts are examples of biological lipids that possess extremely
different physical properties. Hydrocarbons are water insoluble, do not spread at air-water
interface, and are soluble in organic solvents. Bile salts are water soluble, spread
actively at air-water interface, and are insoluble in organic solvents. The purpose of this
paper is to describe some of the physical properties of biological lipids interacting with
aqueous systems and to classify lipids based on these interactions.

THE PHYSICAL STATES OF LIPIDS

Lipids are medium sized molecules of molecular weight between 150 and 3000 that contain a
substantial hydrocarbon moiety. Many lipids also contain a water soluble moiety giving the
molecules an amphiphilic character; that is, one part of the molecule is hydrocarbon soluble,
the other, water soluble. The hydrocarbon portion of the lipid molecule may be aliphatic or
cyclic/aromatic. In predominantly aliphatic lipids the hydrocarbon part usually consists of a
single chain. However, two chains are present in some phospholipids and sphingolipids and
three chains are present in triacylglycerols. By definition the aliphatic chains should
contain at least eight or more carbons. Such lipids have specific properties related to the
hydrocarbon chain packing, while cyclic/aromatic lipids derive their unique properties from
specific hydrocarbon-hydrocarbon interactions. Aliphatic chain packing assumes three
basic configurations: 1) a tightly packed aliphatic chain lattice with specific chain-chain
interactions and a minimum specific volume, 2) an intermediate crystalline packing of the
hydrocarbon chains in which specific chain-chain interactions are lost, but the chains are
packed approximately in a centered hexagonal lattice and, 3) a liquid state in which there
is no lattice and the -CH₂- groups move about more or less freely. The characteristics of
these configurations are given in Table 1 along with some of their commonly used names.

TABLE 1 Characteristics of Chain Packing

<table>
<thead>
<tr>
<th>Aliphatic Chain Interaction</th>
<th>Subcell Lattice (a)</th>
<th>Vol/ -CH₂- Surface Area/-CH₂-</th>
<th>Motion</th>
<th>Common Names (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific</td>
<td>Orthorhombic</td>
<td>23-24Å² 18-19Å²</td>
<td>Very Restricted</td>
<td>Alkanes = Orthorhombic perpendicular, triclinic, monoclinic, α, γ</td>
</tr>
<tr>
<td></td>
<td>perpendicular</td>
<td></td>
<td></td>
<td>Acids and Amides = A, B, and C forms</td>
</tr>
<tr>
<td></td>
<td>Orthorhombic</td>
<td></td>
<td></td>
<td>Mono, dl, and tri-</td>
</tr>
<tr>
<td></td>
<td>parallel</td>
<td></td>
<td></td>
<td>acylglycerols = β and β'</td>
</tr>
<tr>
<td></td>
<td>Monoclinic</td>
<td></td>
<td></td>
<td>forms</td>
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<tr>
<td></td>
<td>parallel</td>
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<tr>
<td></td>
<td>Triclinic</td>
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<td></td>
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<tr>
<td></td>
<td>parallel</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Hybrid cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonspecific</td>
<td>Hexagonal or near hexagonal</td>
<td>~ 26Å² ~20Å²</td>
<td>Restricted</td>
<td>Alkanes = Rotator phase α</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Acids, Alcohol, Glycerides = α phase</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Phospholipids = gel phase, ordered phase, hexagonal phase, l₆ (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Soaps = gel phase</td>
</tr>
</tbody>
</table>


The behavior of biological lipids

TABLE 1 Characteristics of Chain Packing (Continued)

<table>
<thead>
<tr>
<th>Liquid</th>
<th>No lattice, but domains of roughly 29 - 30 Å³</th>
<th>Fluid</th>
<th>Alkanes, Acids, Alcohols, di- and triacylglycerols = melt, neat liquid, isotropic liquid phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Monoacylglycerols and Phospholipids = Liquid crystal phase (lamellar or Lα, cubic, hexagonal I and Hexagonal II, etc.)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Soap = neat, viscous isotropic, or middle phase; liquid phases</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cholesterol esters = liquid crystal, fluid crystal or mesophase (smectic A, nematic, cholesteric), ordered (as opposed to isotropic liquid)</td>
</tr>
</tbody>
</table>

Note a. For a good review of simple and hybrid subcells, see Ref. 1. Hybrid subcells often occur in complex lipids.

Note b. Nomenclature for different states of lipids is complicated and not consistent between lipid classes. This table should be helpful in orientation since many aliphatic molecules undergo transitions from various crystalline states to more liquid-like states. For further discussion of specific states see Refs. 1-4.

![Graph](image)

**Fig. 1.** The volume of the \(-\text{CH}_2-\) group vs. the temperature for a homologous series of odd carbon numbered hydrocarbons. Orthorhombic perpendicular \(= \odot\); alpha \(=\) a hexagonal "rotator" phase. Each hydrocarbon undergoes two transitions; one from orthorhombic perpendicular to rotator phase (alpha), and one from alpha to a liquid. The coefficient of expansion of the orthorhombic perpendicular phase is very small, however, that of the alpha phase is appreciable (not shown). The volume per \(-\text{CH}_2-\) group in the orthorhombic perpendicular phase before the transition to alpha is approximately 23.6 Å³ for each of the hydrocarbons and the minimum area in the alpha phase is approximately 25.8 Å³. In all cases the volume per \(-\text{CH}_2-\) group in the liquid state at the melting point is approximately 29.4 Å³. Corresponding surface areas per \(-\text{CH}_2-\) group are given on the right of the diagram.
The simplest molecules to undergo polymorphic transitions between crystals with specific chain-chain interactions and the more loosely packed hexagonal state are normal alkanes as illustrated in Fig. 1 (4).

The odd-numbered hydrocarbons in this homologous series undergo orthorhombic perpendicular to hexagonal transitions and hexagonal to liquid transitions. There are marked and sudden changes in volume for each specific hydrocarbon at a specific transition temperature. Many complex lipids undergo similar transitions and similar abrupt volume changes occur (4). Even hydrated phosphatidyl cholines undergo similar changes at their gel-liquid crystal transition (5).

The changes in excess specific heat (enthalpy) occurring at such transitions are given for a variety of different lipids in Fig. 2. A plot of $H$ versus the number of carbons in the hydrocarbon chain gives a slope which is equivalent to the $\text{H}/\text{-CH}_2$ group. The enthalpy of transitions between the three general states of lipid chains outlined in Table 1 are very similar in a wide variety of saturated aliphatic chain lipids (4). When double bonds are present the enthalpy appears to decrease, but the type of chain packing has not been documented in most cases. As a general rule, a change in volume of $1\text{Å}^3$ per $\text{-CH}_2$ group requires approximately 0.17 kcal.

Fig. 2. The enthalpy ($\Delta H$) of chain melting transitions of a variety of lipids plotted against the number of carbons in the aliphatic chain. Triglyceride = triglyceride $\beta$ to liquid; odd hydrocarbons = orthorhombic perpendicular to liquid; odd hydrocarbons $\alpha$ (rotator phase) to liquid; PC = hydrated phosphatidylcholines, $\alpha$ (lg) to liquid crystal; and Na soap = sodium soaps, hexagonal to liquid crystal. The enthalpies of the transitions from the tightly packed crystalline forms ($\beta$, orthorhombic perpendicular) - liquid are about 1 kcal/$\text{-CH}_2$, whereas the transitions from the more loosely packed hexagonal (alpha phase) to the liquid are roughly 0.5 Kcal/$\text{-CH}_2$. The numbers given in parentheses are the enthalpies per $\text{-CH}_2$ group (4).
The hydrophilic portion of the molecule also plays a major part in chain transition temperatures. The transition temperatures of a homologous series of several different aliphatic chain molecules are given in Fig. 3. Those with the strongest polar lattices appear to have the highest melting transitions. Thus, both the polar and hydrocarbon parts of the molecules affect the transition temperatures.

![Graph showing Chain Melting Point from Most Stable Crystal](image)

Fig. 3. The major chain melting transition temperatures of a variety of lipids are plotted against the number of carbons in the aliphatic chain. The stronger the interaction of the polar group the higher the melting point. Note that the melting transitions increase with the increasing hydrocarbon chain length, and even in water (see soaps and phospholipids) the transition temperature increases with chain length. However, the presence of water in general lowers the chain transition (4).

A CLASSIFICATION OF LIPIDS BASED ON INTERACTION WITH WATER

The interactions of lipids in aqueous systems can be discussed with respect to their bulk properties (behavior in water) and surface properties (behavior at the air-water interface). In water, some lipids are completely insoluble, while others swell, undergo lyotropic mesomorphism, (that is, form liquid crystals) or dissolve to form micellar solutions. At the interface, lipids may not spread, may spread to form stable or unstable monolayers, or may dissolve. The surface and bulk properties of a given lipid depend upon the relative strength of the hydrophilic and the lipophilic or hydrocarbon portions of the molecule; that is, the hydrophilic-lipophilic balance (HLB). If the hydrophilic part is stronger than the hydrocarbon part, then the molecule tends to be water-soluble. Strong hydrophilic groups include ionized carboxyls (H₂COO⁻), sulfates, phosphates, quaternary amines, sugars, etc.

Conversly, if the hydrocarbon part is stronger than the hydrophilic part, the HLB is tipped towards the lipophilic side and the molecule tends to be water-insoluble. Small (6,7) has proposed a simple classification which groups molecules according to their behavior at an air-water interface and in bulk aqueous systems (Table 2).
<table>
<thead>
<tr>
<th>Class</th>
<th>Surface properties at air-water interface</th>
<th>Bulk properties in water</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonpolar</td>
<td>Will not spread to form monolayer</td>
<td>Insoluble</td>
<td>Long-chain, saturated or unsaturated, branched or unbranched, aliphatic hydrocarbons with or without aromatic groups, e.g., dodecane, octadecane, hexadecane, paraffin oil, phytane, pristane, carotene, lycopen, gadusene, squalene. Large aromatic hydrocarbons, e.g., cholestane, benzpyrenes, coprostan, benzophenanthroenes. Esters and ethers in which both components are large hydrophobic lipids, e.g., sterol esters of long-chain fatty acids, oxides of long-chain fatty acids and long chained normal monoaclcohols, ethers of long chained alcohols, sterol ethers, long chained triethers of glycerol.</td>
</tr>
<tr>
<td>Polar</td>
<td>Spread to form stable monolayer</td>
<td>Insoluble or solubility very low</td>
<td>Triglycerides, diglycerides, long chained protonated fatty acids, long chained normal alcohols, long chained normal amines, long chained aldehydes, phytols, retinols, vitamin A, vitamin K, vitamin E, cholesterol, deoxycholesterol, sitosterol, vitamin D, un-ionized phosphatidic acid, sterol esters of very short chain acids, oxides in which either acid or alcohol moiety is less than 4 carbon atoms long.</td>
</tr>
<tr>
<td>Class I</td>
<td>Insoluble nonswelling amphiphilic lipides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polar</td>
<td>Spread to form stable monolayer</td>
<td>Insoluble but swells in water to form lyotropic liquid crystals such as lamellar cubic, hexagonal</td>
<td>Phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, sphingomyelin, cardiolipin, plasmalogenes, ionized phosphatidic acid, cerdebroside, phosphatidyl serine, monoglycerides, &quot;acid-soaps&quot;, alpha hydroxy fatty acids, monoethers of glycerol, mixtures of phospholipids and glycolipids extracted from cell membranes or cellular organelles (glyco- lipids and plant sulfolipids).</td>
</tr>
<tr>
<td>Class II</td>
<td>Insoluble swelling amphiphilic lipides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class IIIA</td>
<td>Soluble amphiphiles with lyotropic mesomorphism</td>
<td>Soluble; form micelles above a CMC. At low water concentrations forms liquid crystals.</td>
<td>Sodium and potassium salts of long chained fatty acids, many of the ordinary anionic, cationic, and nonionic detergents, lyssolecithin, palmytol and ole-1 coenzyme A and other long chained thioesters of coenzyme A, gangliosides, sulfo cerebroside.</td>
</tr>
<tr>
<td>Class IIIB</td>
<td>Soluble amphiphiles, no lyotropic mesomorphism</td>
<td>Soluble but form micelles but not liquid crystals</td>
<td>Conjugated and free bile salts, sulfated bile alcohols, sodium salt of fusidic acid, rocin soaps, saponin, sodium salt of phenanthrene sulfonyl acid, penicillin, phenothiazines</td>
</tr>
</tbody>
</table>
In applying this classification to any specific lipid, one must consider the water concentration and the temperature. Certain molecules may be quite insoluble at low temperature, but on passing to a higher temperature undergo chain melting which allows water to penetrate to the hydrophilic region, thus causing the formation of liquid crystals or micelles. The transition temperature from the tightly packed crystal or even the relatively loosely packed hexagonal chain to the more liquid state determines the physical characteristics of the lipid. Although little data are available, it is possible that acyl chain lipids packed in a crystal with specific chain-chain packing can hydrate appreciably without undergoing chain melting to a less tightly packed lattice. Certainly, lipids with predominantly hexagonal chain packing can hydrate appreciably without undergoing chain melting. A change in tilt or perturbations in the lattice that cause rippling of the basic crystalline bilayer may accompany hydration (2,8,9). The following classification applies only to lipids with fluid chains.

Nonpolar lipids (Fig. 4)
Lipids belonging to this class are insoluble in the bulk and do not spread to form a monolayer on the surface; that is, they have a negative spreading pressure. These molecules either have no polar constituents (pure hydrocarbons) or possess a hydrophilic part so small or so buried in the center of the molecule that it cannot interact with water, thereby preventing the molecule from spreading. This is the case with esters of long chain fatty acids and bulky monohydryalcohols (waxes) and sterol esters.

![Non-polar lipids diagram](image)

Polar lipids
These lipids are surface soluble and form stable or unstable monolayers.

Class I: Insoluble, nonswelling amphiphiles (Fig. 5): Lipids of this class are virtually insoluble in the bulk but will spread at the interface to form a stable monolayer. Thus, they have a positive spreading pressure. Triglycerides, long-chained un-ionized fatty acids, cholesterol, and many fat soluble vitamins (vitamins A, D, E, and K) are members of this class.

![Polar lipids class I diagram](image)
Class II: Insoluble, swelling amphiphiles (Fig. 6): These lipids are virtually insoluble in water. However, they undergo lyotropic mesomorphism to form liquid crystalline phases. In other words, while the lipid is not soluble in water, water is soluble in the hydrophilic part of the lipid, and therefore it swells. Some lipids have a finite capacity to swell, particularly those with smaller polar groups which form an inverted hexagonal phase (e.g. phosphatidylethanolamines). Phosphatidylcholines (8-10), sphingomyelin (11,12), and cerebrosides (13) which form a lamellar phase incorporate up to 45% water by weight into the polar region of their liquid crystal structures. Other lipids, however, appear to swell to a much greater extent. Ionized phosphatidylserine swells to form lamellar liquid crystals which are at least 90% water by weight (14). These lipids form stable, insoluble monolayers and have a positive spreading pressure. The principal lipids in this class are the lipids that constitute biological membranes, such as phospholipids and cerebrosides.

Fig. 6

POLAR LIPIDS-Class II
Insoluble swelling amphiphiles

Forms a stable monolayer
Bulk phase: swells to form liquid crystals in water

Class III: Soluble amphiphiles: Because of their bulk solubility, soluble amphiphiles form unstable monolayers at the interface and micelles in aqueous solutions. There are two general types of these compounds. Type IIIA lipids (Fig. 7) exhibit lyotropic mesomorphism at low water concentration, and form liquid crystals, as do Class II lipids. However, at higher water concentrations, these liquid crystals dissolve to form rod shaped or spherical micelles. Aliphatic molecules, such as Na+ or K+ soaps, lysolactic acid, and many detergents, are representative of Type IIIA lipids. Type IIIB lipids (Fig. 8), bulky aromatic ring systems often comprise the hydrophobic component of the molecule. These compounds form micelles, but as a general rule do not form liquid crystals. Molecules typical of this system are bile salts, rosin soaps, and a variety of pharmacological agents.

Fig. 7

POLAR LIPIDS-Class III A
Soluble amphiphiles with lyotropic mesomorphism

Forms an unstable monolayer
Bulk phase: forms liquid crystals at low water concentration; forms micelles at higher water concentrations
Interactions between different classes of lipids in aqueous systems may in part be predicted from the classification presented above. Phase equilibria studies of interactions between different classes have been presented (6). For instance, a Class I lipid such as cholesterol may be solubilized appreciably in a lamellar liquid crystalline phase by phospholipids such as lecithin and sphingomyelin. Cholesterol and Class IIIA lipids combine in roughly equal molar ratios to form a lamellar liquid crystalline phase and the two behave together as a Class II-like lipid. Only when large amounts of Class IIIA detergents are added is cholesterol dissolved into micelles. The usefulness in biology of such phase equilibria systems has been pointed out (6,7) and presumably processes such as membrane budding and fusion may ultimately be explained by the composition and states of lipids taking part in these processes.

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REFERENCES
The Phase Behavior of Triolein, Cholesterol, and Lecithin Emulsions

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The lipid phase behavior of triolein (TO)–cholesterol (C)–egg yolk phosphatidylcholine (L)–water was studied at 22–24°C and pH 7. Coarse emulsions were centrifuged at high speeds (14–28,000 rpm) to separate the oil and surface monolayer phases. The equilibration procedure and purity of the phases were monitored by analyzing the data with triangular coordinate phase diagrams. The L-rich surface monolayer annealed to form multilamellar bilayers following coalescence of the droplets. L was confined to the surface, but TO and C partitioned between the phases. The emulsion region consisted of a quadrilateral zone on the triangular coordinate phase diagram whose four corners are 100% TO, 98% TO–2% C, 3.5% TO–96.5% L, and 2% TO–33% C–65% L. The tie lines were located within this two-phase region. In the majority of this region the surface-oil distribution ratio of C was 28 and that of TO was 0.025–0.04. Apparently, when C was at its highest concentration in the TO oil phase (1–2%, by weight), C molecules associated to form dimers. Above the limit of solubility of C in the emulsions, C monohydrate crystals were formed.

INTRODUCTION

Triglyceride (TG), cholesterol (C), and phospholipid (PL) are major components of biological emulsion droplets: TG-rich lipoproteins, dietary fat droplets, and intracellular lipid storage droplets (1). Long-chain TG emulsions stabilized by PL, have been used as model substrates in tests of the activity of lipolytic enzymes. From these studies it has been established that the activity of lipolytic enzymes is sensitive to the total chemical composition of the emulsion. For example, fatty acids are required to maximally activate intestinal lipase (2), whereas, C inhibits the hydrolysis of TG by lipoprotein lipase (3). However, the data cannot be related to the surface concentration of the activator or inhibitor, because the phase behavior of the emulsions was not defined.

At present, only the gross features of the structural organization of biological emulsions are known (4). PL and C orient at the surface of TG-rich lipoproteins (4–7) because they stabilize the dispersed lipids by lowering the interfacial energy of the particles (8). TG and cholesterol esters occupy the oil phases or “cores” of the droplets (4–7) because they have greater oil–water interfacial tensions than the more polar lipids (8). However, lipids which are soluble in both regions would be expected to partition between the phases to establish equilibrium (9–11). For example, C, which is soluble in TG oils (12–14), also partitions into the oil phase of chylomicrons (9, 10). Likewise, TG has limited solubility in PL air–water monolayer (15) and vesicles (16), and is soluble in the surface monolayer of lipoproteins (17).

In this paper, we describe the phase behavior of triolein (TO), C, and egg yolk phosphatidylcholine (L) in water emulsions at 24°C. The phase diagram of these lipids was constructed from the compositions of emulsion phases isolated by high-speed centrifugation. The positions of the tie lines which join the compositions of equilibrated phases were located within the two-phase emulsion region. The knowledge of the tie line positions permits study of interparticle
lipid equilibria and calculation of the percentages of each of the lipids carried in the surface and oil phases as a function of the size of the emulsion particle.

MATERIALS AND METHODS

TO and C were purchased from Nu Chek Prep, Inc. (Elysian, MN). L was obtained from Lipid Products (Nutfield Ridge, England). The purity of the lipids was checked by thin-layer chromatography (TLC), and they were verified to be >99% pure, as stated by the manufacturer. The acyl chain composition of the L was determined by preparing fatty acid methyl esters using boron trifluoride–methanol reagent, Supelco Inc. (Bellefonte, PA) (18). Methyl esters were separated on a 6-ft, 5% DEGS column, Supelco, Inc., mounted in a Hewlett-Packard #5710A gas chromatograph with a HP3385A automation system, Hewlett-Packard Company (Avondale, PA). The L contained 2% 12:0, 2% 14:0, 36% 16:0, 2% 16:1, 11% 18:0, 27% 18:1, 17% 18:2, and 3% other fatty acids. [9,10-3H] trioleyl glycerol (TO), [4-14C] C, [3H] and [14C] toluene, and Aquasol liquid scintillation fluid were purchased from New England Nuclear, Inc. (Boston, MA). The purity of the radio-labeled lipids was maintained at >98%. Solvents were redistilled, and water was twice-distilled and then deionized before use.

Preparation of Emulsions

Mixtures of 75 or 100 mg of the emulsion lipids were prepared in chloroform–methanol (2:1) (CM). Specific activities of the [3H] TO and [14C] C were set in the range of 1,000–2,000 dpm/µg for all experiments. The errors in the specific activity values were ±2–3%. Mixtures were added to 15 × 45-mm glass vials, V.W.R. Scientific, Inc. (Medford, MA). The samples were dried under N2 on a warm water bath (45°C) and were vacuum desiccated at 4°C for at least 16 hr. Either 0.67 or 0.9 ml of water was added to the dried lipids to make mixtures of 10% lipid, by weight. The water was boiled, and then gassed with N2 as it cooled, to bring it to pH 7.

Samples were sealed under N2 and were agitated at a moderate rate of speed (∼200 rpm) using a vortex mixer set to vortex continuously, Fisher Scientific Company (Pittsburgh, PA). An insulating styrofoam rack was attached to the vortex mixer to hold the vials. Agitation was performed for 24 hr at ambient temperature (22–24°C). During this time, aliquots were taken from some of the samples to determine the length of time required to attain equilibrium. Other emulsions were checked by TLC to determine if the lipids decomposed during agitation. No degradation products were detected during 2 days of equilibration, and the pH of the emulsions remained between 6.5 and 7. The chemical compositions of the emulsions were similar (within ±5% for each lipid) to the compositions of the mixtures in the solvents. Thus, no lipid component selectively adhered to the glass vials. The composition measured after 24 hr of equilibration was taken as the initial emulsion composition.

Centrifugation of Emulsions

Aliquots of 65 µl were transferred to glass capillary tubes (i.d. = 1.1–1.2 mm × L = 75 mm) purchased from Sherwood Medical Industries, Inc. (St. Louis, MO). The tubes were flame-sealed at one end and were centrifuged at 24°C inside plastic adaptors which were machined to fit into the buckets of a Beckman SW 41 swinging bucket rotor, Beckman Instruments, Inc. (Palo Alto, CA). Low-speed centrifugations were performed for 30 sec at 500 rpm in an International Refrigerated Centrifuge (Model #PR-2), International Equipment Company (Boston, MA). Emulsions were broken by centrifuging samples between 14,000–28,000 rpm in a Beckman SW 41 rotor using a Beckman Ultracentrifuge (Model #L2-50). Following centrifugation, the tubes could be examined by microscopy (see below) and/or scored.
with a diamond knife and broken into sections to recover emulsion creams, or separated emulsion phases. In most experiments, the oil phase was recovered after one 12-hr centrifugation. The surface phase was usually resuspended in water by gentle vortexing and was recentrifuged 12 hr at the speed of the first centrifugation. Unless otherwise indicated, 65–100 μl of water per surface phase was added to resuspend the lipids.

Morphology of Emulsions and Emulsion Phases

Emulsions and isolated phases were examined by direct and polarized-light microscopy using a Zeiss NL polarized-light microscope manufactured by Carl Zeiss, Inc. (New York, NY). Samples were examined either in situ in the capillary tubes or after they were removed from the tubes and placed on microscope slides. The anisotropic liquid crystalline L (19, 20) and crystalline C monohydrate phases (21) were identified by their birefringence and morphology. Isotropic regions were identified as either oil or aqueous phases by adding water to one side of the sample on a microscope slide and watching to see if the approaching liquids formed a boundary or mixed together.

For three emulsions, the surface material obtained after one centrifugation step was resuspended in water and studied by X-ray diffraction experiments. Samples were loaded into glass X-ray capillary tubes (o.d. = 1 mm), Lindeman Corporation (Indianapolis, IN), and were exposed to nickel-filtered CuKα X-rays, λ = 1.54 Å, produced by a Jarrel–Ash Microfocus X-ray generator and slit-collimated by a Luzzati–Baro camera. Diffraction maxima were detected by a position sensitive counter, (Model P.D.S. 1100), Tennelec Corporation (Oak Ridge, TN), and recorded by an analysis system (Model TN 1710), Tracer Northern Company (Middletown, WI). The small- and wide-angle spacings of the surface lipids were calculated using the Bragg equation, \( nλ/2 \sin θ = d \).

Lipid Analysis

The percent composition of lipid samples was determined by liquid scintillation counting (LSC) and chemical assay. Aliquots of lipid mixtures in CM were dried and weighed using a Cahn Automatic Electrobalance (Model #25), Cahn Instruments, Inc. (Cerritos, CA). Other aliquots were prepared for counting in a Beckman LS 250 liquid scintillation counter, Beckman Instruments, Inc. (Fullerton, CA), which was calibrated for double-label LSC. The detected cpm in the samples was converted to μg of TO and C using quench curves. The mass of L in the samples was determined either by the difference between the total lipid mass transferred to the vial and the combined masses of detected TO and C, or by chemical assay (22). The two methods gave the same lipid percent compositions.

RESULTS

Physical Characteristics of the Emulsions

Oil-in-water emulsions were produced by 24 hr of agitation. The samples contained microscopically visible droplets which were extensively aggregated. When emulsions were examined by polarized-light microscopy following 24 hr of agitation, no birefringent multilamellar liposomes were observed. Samples which contained excess C possessed birefringent, parallelogram-shaped C monohydrate crystals, as well as emulsion droplets.

Study of the Mechanism of Phase Separation

Emulsion A was composed of 78% TO, 2% C, and 20% L. Twelve aliquots were centrifuged at 20,000 rpm in the SW 41 rotor. The centrifuge was stopped and three tubes were removed after 45 min, 2, 5, and 12 hr
of centrifugation. The percent volumes of the five regions which formed were calculated (I–V, Fig. 1). The compositions of Region I, the oil phase, and Region V, the surface phase, were determined. The phase compositions were constant and independent of the number of hours of centrifugation (data not shown).

The centrifuged tubes adopted the morphology shown in Fig. 1 within 45 min of centrifugation. Region I contained an isotropic oil composed of 99.5% TO, 0.5% C, and no L. When the oil was spread against water, no myelin figures (L) or C monohydrate crystals precipitated. The volume of Region I increased rapidly at first (dashed line) and then grew linearly over the next 11 hr of centrifugation.

Region II contained a birefringent L-rich surface phase suspended in the isotropic TO oil. The volume of Region II decreased during centrifugation (Fig. 1). However, no net decrease in the combined volumes of Region I + II occurred after the first hour of centrifugation. The combined volumes of Region I + II were similar to the total volume of TO in the starting emulsion (8.7%). Therefore, Region II was converted to Region I by drainage of the surface phase out of Region II.

Below Region II is a turbid, nonbirefringent zone, Region III, which contained granular particles. When centrifuged tubes were placed on their sides, the particles diffused into Region IV, the aqueous infranatant. Therefore, intact oil-in-water emulsion drop-
lets accumulated in Region III. The volume of Region III was constant (±1%) during the 12-hr experiment.

Region IV had a flux of lipids through it during centrifugation. After brief (<30 min) centrifugation periods, birefringent droplets were found in Region IV. After longer centrifugations, these were not detected. The remaining Tyndall scattering is attributed to microemulsion droplets which are present in Region IV (23). These accounted for ~10% of the lipid mass of the centrifuged emulsion.

The emulsion surface phase, Region V, sedimented to the bottom of the centrifuge tube. In the sample shown in Fig. 1, the upper 2 mm of Region V is turbid, whereas the lower 0.7 mm is optically transparent. However, both parts exhibited strongly birefringent neat phase texture when viewed between crossed polars. The data indicate that the upper section contains the surface phase and excess water, and the lower section contains a single maximally-hydrated (lamellar liquid crystalline) L phase (19).

The rate of increase in the surface phase volume of emulsion A is shown in Fig. 1. Because the surface phases contained trapped water, the volumes of Region V did not correspond closely to the expected volumes of the L phase (±2%). Like Region I, the surface phase accumulated rapidly at first (dashed line). After this initial stage of growth, the volumes of Region I and Region V increased at comparable rates.

If the growth of Region I continued at the same, nearly linear rate, then all of the oil phase lipids would appear in Region I by approximately 40 hr of centrifugation. However, for other emulsions, the rate of oil accumulation was observed to decrease between 12 and 24 hr of centrifugation. Even after prolonging the centrifugation to 48 hr, less than half of the total TO oil was present in Region I (data not shown). Because the mass of oil in Region I at 12 hr of centrifugation was sufficient to perform chemical analyses, this length of centrifugation was selected for most experiments.

To determine the structure of the surface phase, three emulsions with different percentages of C were centrifuged to obtain their surface lipids. The surface lipids (collected with one centrifugation) contained L; 0, 10, and 20% C; and <7% TO. The sedimented lipids were resuspended in water and studied by X-ray diffraction. Wide-angle diffraction maxima were present at a distance of 4.6 Å⁻¹. The position of these diffuse bands corresponded to the separation distance between adjacent acyl chains and steroid nuclei. The small-angle diffraction maxima of these samples corresponded to lamellar d spacings of 65–66 Å. Therefore, the surface monolayers annealed following droplet coalescence and formed liquid-crystalline bilayers (19, 20, 24).

**Equilibration of Emulsions**

A mixture of 80% TO, 2% C, and 18% L was agitated to form emulsion B. Its chemical composition was measured after 3, 10, and 24 hr of agitation. At each of these time points, 65 µl of emulsion B was centrifuged for 30 sec at 500 rpm. These centrifugation conditions did not break the emulsion, but produced creams which were divided into three portions, originating from the top, middle, and lowermost regions of the cream. A fourth fraction, the remaining aqueous infranatant, was also removed for analysis. At each time point, another 65-µl sample was spun in the ultracentrifuge to isolate oil and surface phases.

The compositions of the emulsion subfractions are plotted in Fig. 2a. The data points plot on a single tie line given by the compositions of the isolated oil (O) and surface (S,) phases. The cream subfractions plot from left to right along the tie line in the order of the cream top (T), middle (M), and lowermost (L) regions. The infranatant (I) fractions plot further toward the L apex of the diagram. The compositions of all data points fall on the tie line because only the relative masses of the surface and oil phases in the subfractions vary and not the com-
FIG. 2. (a) Graph of the compositions of subfractions taken from the cream and infranatant (I) regions of emulsion B. Samples were centrifuged following 3 (×), 10 (●), and 24 (▲) hr of equilibration. The emulsion creams were separated by low-speed centrifugation into top (T), middle (M), and lowermost (L) regions representing approximately 20, 40, and 40% of the total cream volumes, respectively. For the sample centrifuged following 24 hr of equilibration, only one point is shown for the top and middle fractions, since these data points are superimposed on this diagram. Points O and Sₚ represent the oil and recenterfuged surface phases, respectively, obtained from a 65-μl sample centrifuged at 20,000 rpm following 24 hr of equilibration. (b) Graph of the compositions of the emulsion (E), oil (O), and surface (S) phases isolated from emulsion B. The data points for the emulsion compositions represent the mean of three determinations. The oil phases contain 0.5% C (n = 1 for the 3- and 10-hr samples, and n = 3 for the 24-hr data point). The surface phase compositions were determined from n = 1 (Sₚ) and n = 2 (Sₛ) samples. Sₚ represents the composition of the purified surface phase isolated from the recenterfuged Sₛ samples.

positions of the phases (25). In Fig. 2b, the compositions of the emulsions and the phases isolated at each time point are plotted. The data points fall on the same tie line given by the subfraction compositions in Fig. 2a. The scatter of the compositions of the starting emulsions can be attributed to sampling variations. The results shown in Figs. 2a and b indicate that the distribution of C between droplets and the surface and oil phases of individual droplets had reached mass equilibrium within 3 hr.

The Effect of Resuspension and Recentrifugation on the Composition of the Surface Phase

Emulsions C, D, E, and F were studied to determine if a pure surface phase sample was obtained with one centrifugation step. Six aliquots of each emulsion were centrifuged for 12-hr periods at 20,000 rpm. Two samples served as control tubes which were removed after 24 hr (two centrifugations), and 36 hr (three centrifugations) to measure their oil and surface compositions. The oil and surface phases of four tubes were pooled and sampled after the first 12 hr of centrifugation. The surface lipids were resuspended in water and recenterfuged 12 hr. These steps were repeated a second time. In summary, the compositions of the surface phases were determined after 0, 1, and 2 recenterfugation steps. The control tubes were spun for the same lengths of time, 12, 24, and 36 hr, but without resuspension and recenterfugation of their surface lipids.

The compositions of the surface phases obtained with each centrifugation step are presented in Table I. For each emulsion, the surface lipids isolated from the control tubes had similar compositions regardless of the number of hours of centrifugation. The oil phase compositions did not change over this time period either (data not shown). However, the compositions of the material which

sedimented from the resuspended surface lipids did change following the second centrifugation. The TO percentages decreased by ~50%, but the C:L ratios remained constant. The second resuspension and centrifugation steps produced little or no further changes in the chemical compositions.

After each recentrifugation of the lipids, a small mass of birefringent lipid floated to the top of the tubes. The mass of the floated material present after the second centrifugation had decreased by five-fold from the amount present after the first purification step. For some emulsions (emulsions G and H) the composition of the initial floating "impurity" was determined. In Fig. 3, the compositions of the emulsion (E), oil (O), initial surface (S₁), final surface (S₂), and floating impurity (Iₚₙ) (emulsions G and H, only) regions are plotted on a triangular coordinate diagram. The compositions of all data points for a given emulsion fall on the same tie line. Therefore, we conclude that resuspension and recentrifugation removes regions of trapped oil from the initial surface lipid fraction without changing the composition of the true surface phase.

Phase Behavior of TO–C–L–Water, pH 7, 22–24°C

The phase diagram of TO–C–L emulsions containing 90% water is shown in Fig. 4. The data were taken from emulsions centrifuged at 20,000 rpm. The oil phase lipids were obtained by one 12-hr centrifugation. The surface phase lipids were resuspended in water and recentrifuged for a second 12-hr period. Water is not plotted on the diagram but is present along with all regions shown.

The surface phase, Region I (labd), contains 3.5 ± 0.2% TO and 96.5 ± 0.2% L (n = 3) in the absence of C (point a). As the C content of the surface increases, the TO content varies between 2.3–4.0%. The surface TO:L ratio ranges between 0.025–0.045, and the surface boundary has been drawn at TO:L = 0.036. The boundary extends to point b, which indicates the maximum solubility of C in the surface phase. Because a C-saturated surface phase could not be prepared free of C monohydrate crystals, it was shown to contain in the range of 30–35% C, the composition of point b was tentatively calculated. It was assumed that 2% TO is incorporated into a surface having a L:C ratio of 2:1. Therefore, point b consists of 2% TO, 33% C, and 65% L.

The oil phase, Region II (TOe), consists

<table>
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<tr>
<th>Emulsion</th>
<th>h</th>
<th>No.</th>
<th>Percent TO</th>
<th>Percent C</th>
<th>Percent L</th>
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</table>

* Samples were centrifuged at 20,000 rpm and 24°C.
* Total number of hours of centrifugation.
* The number of 12-hr recentrifugation steps, each of which required that the surface phase be resuspended in water prior to performing the recentrifugation.
* The compositions of the samples taken after 12 hr of centrifugation represent the mean values of four pooled samples. All other samples represent single samples. We suggest that appropriate limits of error for the values of each lipid are ±1 S.D. of TO, 0.5–1% for C, and 1–2% for L.
of TO and 0–1.96 ± 0.07% C (n = 4) (point e). No L was detected in this phase. The maximally C-saturated oil (point e) was obtained in homogeneous form.

Region III is the zone in which emulsions consisting of both oil and surface phases are formed. The compositions of the equilibrated phases found in the emulsions (such as point f) are given by the intersections of the tie lines with the phase boundaries. The tie lines are not parallel to the TO–L border of the figure, because C is much more soluble in the surface phase. Throughout most of Region III (see below), the distribution ratio, $K_C$ (26),

$$K_C = \frac{x_C}{S_C},$$  

where $x_C$ and $S_C$ designate the weight fraction solubilities of C in the surface and oil phases, is equal to 28 ± 5 (n = 16).

Region IV is delineated from Regions I, II, and III by the bold dashed line joining points e and b. Above the dashed line, emulsion droplets are saturated with C and exist in equilibrium with C monohydrate crystals. Region IV has zero degrees of freedom, and mixtures (such as point g) always consist of phases with compositions at points e, b, and c.

Finally, Region V (which was not extensively studied), is a zone composed of two phases, the maximally C-saturated L phase (bd), and C monohydrate crystals (point c). The TO content of this surface phase can vary between 0–2% (points d and b, respectively).

**DISCUSSION**

The technique of high-speed centrifugation has been applied to separate and isolate the phases that are present in emulsions composed of TO, C, L, and water. The emulsion droplets which are formed by vortexing mixtures of the lipids in water are large, mostly micron-sized droplets which are easily forced to coalesce by the compressive forces in the centrifuged samples (for a review of the theory of demulsification, see Refs. (27, 28)). Based upon the data for the chemical compositions of emulsion B and its subfractions (Figs. 2a and b),

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emulsions attain chemical equilibrium within 3 hr of vortexing (25). The equilibration time of 24 hr was chosen for all other experiments because samples were better dispersed by longer periods of agitation.

As is shown in Fig. 1, centrifugation of emulsion A at 20,000 rpm caused most of the emulsion to be compressed to about 9–10% of its initial volume within 1–2 hr (see Regions I + II). During this time period, the volumes of the oil (Region I) and surface (Region V) phases increased rapidly, and then between 2–12 hr of centrifugation, increased at slower, approximately linear rates. The observed rate of increase in Region I during the linear phase underestimates the rate at which the emulsion actually coalesced. By 45 min of centrifugation, a large volume of oil was already present in the continuous phase of Region II. As for emulsions of mineral oil (29, 30) or benzene (31) stabilized by sodium dodecyl sulfate, the rate of increase in Region I is determined by the drainage of surface phase out of Region II as well as by the addition of oil from newly coalesced droplets.

The surface lipids prepared by one centrifugation period were freed of contaminating oil phase by resuspension in water and recentrifugation (Table I and Fig. 3). Apparently, the oil sediments during the first centrifugation period because it is combined with surface phase lipids in aggregates of net buoyant density > 1 g/ml. The oil does not escape Region V, even during prolonged centrifugation periods (Table I), because of the high viscosity of the surface lipids. When the initial surface phase is resuspended in water, it is broken into smaller fragments. Within this suspension, there is an increased probability that the oil regions are associated with smaller quantities of the surface lipids. The net buoyant densities of these aggregates are then <1 g/ml, and they float during recentrifugation.

The data points for the initial and recentrifuged surface phases fall close to one another on the tie lines (Fig. 3). Therefore, only a small amount of floating lipids are removed by recentrifugation. The relative masses of the floating impurity (Iₙ) and the true surface phase (S₂) in the initial surface lipids (S₁) is determined by the ratio, S₁S₂/IₙS₂, where S₁S₂ and IₙS₂ are lengths of the line segments measured along the tie line. For emulsions G and H, the impurity contributes 10 and 5%, respectively, to the masses of the initial surface lipids. The percentages of oil (O) in the initial surface lipids are calculated using S₁S₂/O S₂. For emulsions G and H, 5 and 2% of the initial surface lipid mass is the oil phase. The percentage of oil in the floating impurity is calculated using IₙS₂/O S₂. For emulsions G and H, 52 and 34% of the masses of the floating lipids are the oil phases. The remaining masses are the pure surface phases (S₂).

Elevated hydrostatic pressure is known to increase the phase transition temperature of pure PL (32), and to induce long-chain alcohols to migrate from the oil phase to the interface of oil-in-water droplets (33). Experiments were performed to determine if the composition of the emulsion phases was influenced by the speed of centrifugation in the range of 14,000–28,000 rpm. This range of speeds corresponds to pressures of 1–25 atm in the oil phase (Region I) and 170–680 atm in the surface phase (Region V). In summary, no effects could be attributed to the number of hours of exposure to high pressure (Table I), the number of repeated centrifugations (of the surface phase) conducted at high pressure (Table I), or the speed of centrifugation itself (data not shown).

TO was the major component of the oil cores, and a minor component of the surface monolayers of the emulsion droplets. The percentage of TO in the surface varied between 2.3–4.0% and was independent of the surface C:L ratio. The distribution ratios K_TO (Eq. [1]) were in the range of 0.025–0.04 for emulsions in Region III (Fig. 4). L-TO vesicles (16) and the emulsion surface phase incorporate the same percentage of
TO per unit weight of L. Thus, the molecular organization of lipids at the lipid–water interface may be similar. TO molecules may orient with their glycerol backbone and carbonyl regions hydrogen bonded with water and their acyl chains parallel to the L acyl chains (16).

Because \( K_C > K_{TO} \), the surface affinity of C is greater than that of TO. C may hydrogen bond more strongly at the interface than TO because its 3-hydroxyl group is at the end of the molecule where it is accessible to water. Because C is less polar than L, C is soluble in the TO oil. The maximum solubility of C in the emulsion oil phase at 22–24°C (1.96%) is identical to the maximum solubility of C in TO–C–water systems (14). In that study, the authors reported that the amount of C dissolved in the anhydrous TO–C oils was decreased by adding water. Apparently, the presence of the water phase also influences the solubility of C in the oil phase of TO emulsions. Perhaps water molecules enter the oil while hydrogen bonded to C molecules, and these hydrated complexes are less soluble.

To determine if the incorporation of C into the emulsions altered the relative affinities of the phases for this molecule, values for the molar distribution ratios, \( x_{m_C} \), were calculated using the mole fractions of C in each phase (26). The equation for determining
Fig. 5. Illustration of the method of determining phase compositions from the plots of emulsion compositions. The tie lines $W$, $X$, $Y$, and $Z$ were plotted by using $K_c = 28$ (Eq. [1]) to calculate the C compositions of points such as $X_0$ and $Y_0$ on the oil phase boundary (TOE) and points such as $X_i$ and $Y_i$ on the surface phase boundary ($ab$). The surface phase composition ($S$) of emulsion ($E$) is determined as is explained in the text using line $M$, which is parallel to $ab$, and intersects the tie lines $X$ and $Y$ at points $j$ and $k$, respectively. Tie line $L$ can then be drawn for emulsion $E$ through points $E$ and $S$. The intersection of tie line $L$ with the oil phase boundary occurs at point $O$ (see the figure inset). The calculation applies only to the region (TOZ$_a$Z$_a$) of the diagram.

$kmc$ is analogous to Eq. [1] and is

$$kmc = \frac{x_C}{x_{C_o}}, \quad [2]$$

where $x_C$ and $x_{C_o}$ now represent the mole fractions of C in the surface and oil phases of the emulsions, respectively. The data for the sixteen emulsions were compared, and it was noted that the quantity of C in the oil phases was limited to 0–1%, by weight, when the quantity of C in the surface phases varied between 0–28%, by weight. Across the range of values, the distribution ratios calculated using Eq. [2] were approximately constant and equal to $22 \pm 3 \ (n = 16)$. However, for emulsions saturated with C (Fig. 4, Region IV), the average value of $kmc$ was only $11 \pm 1 \ (n = 4)$ which differs significantly from the value of 22.

It was assumed that the part of Region III corresponding to emulsions having between 1–2% C, by weight, in the oil phase (Region $Z_aebZ_a$ of Fig. 5) may be a region in which C molecules associate in the oil phase. If this is true, then the value of $kmc$ should be calculated using

$$kmc = \frac{x_C}{[x_{C_o}]^{1/n}}, \quad [3]$$

where $n$ represents the number of C molecules per association complex (26). When $n = 2$, the average value of $kmc$ calculated with Eq. [3] was 23 for the systems saturated with C. This value does not differ significantly from the values for $kmc$ in (Region TOZ$_a$Z$_a$) of Fig. 5, and suggests that C molecules dimerize at higher concentrations (1–2%) in the oil phase. Because Eq. [3] does not apply to all of Region III, it appears that molecular associations begin when there is approximately 1% C in the oil phase and lead to a doubling of the capacity of the TO to solubilize C. It has been shown that C forms association complexes in the nonpolar
solvents, carbon tetrachloride and chloroform (34–36). In carbon tetrachloride, complexes may contain up to six molecules of C (36). In the more polar solvent, chloroform, the interactions between C molecules are weaker, and at most, C dimers form. If the association of C in nonpolar media is a general phenomenon, then within lipoproteins, for example, similar association complexes may form between C molecules which are dissolved in the particle core (17).

The phase compositions of emulsions plotting within Region (TOZCZαβ) of Fig. 5 can be determined graphically. If the weight fraction of C in one of the phases of an emulsion plotting in this region is known, the corresponding weight fraction of C in the other phase can be calculated using Eq. [1]. Then, the tie line for this emulsion can be drawn between the two points on the phase diagram. The method for determining the tie line of an emulsion such as indicated by point E (Fig. 5), will now be demonstrated. It depends upon the use of a set of tie lines calculated with $K_C = 28$: lines $W$, $X$, $Y$, and $Z$. One first draws line $M$, parallel to the line $ab$ (for which the ratio of TO:L was set equal to 0.036) through point $E$. The intersection points of line $M$ with lines $X$ and $Y$ are points $j$ and $k$, respectively. One then determines the ratio $\frac{Ej}{yk}$ and establishes point $S$ by setting $\frac{Ej}{yk} = \frac{SY}{Xk}$. The value for the oil phase C composition, point O (Fig. 5, inset), is determined by substituting the value of the percent C found at point S (17.5%) into Eq. [1], with $K_C = 28$. The value for the C content of point O is thus 0.6%, by weight. The total compositions of points O and S are then read from the graph. The phase compositions could alternatively be determined experimentally by subfractionating emulsion E, plotting the compositions of the subfractions on the phase diagram, and drawing a tie line through the subfraction compositions intersecting the phase boundaries (see Fig. 2a).

The results of the study of TO–C–L–water emulsions indicate that TO and C partition between the surface and oil phases of the particles. The percent of the total particle C or TO found in each phase is a function of the size of the droplets. In large droplets $D > 0.1 \mu m$, >20% of the total particle C is carried in the oil phase. Therefore, in biological emulsion droplets of similar size, substantial amounts of C may be carried in the cores of the particles. Because TG is soluble in the surface of TO and lipoprotein lipid emulsions (17), lipases may hydrolyze TG within the surface monolayer without penetrating to the particle core. To further model the structure of biological particles, other lipids such as cholesterol esters and fatty acids will be included in emulsions to study their effects upon the phase compositions of the particles.

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Interactions of the Carboxyl Group of Oleic Acid with Bovine Serum Albumin: A $^{13}$C NMR Study* 

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The interactions of the carboxyl group of oleic acid with bovine serum albumin (BSA) were studied by $^{13}$C NMR spectroscopy at 50.3 MHz using 90% isotopically substituted [1-$^{13}$C]oleic acid. $^{13}$C NMR spectra were obtained as a function of the mole ratio of oleic acid to BSA (from 0.5–10.0) and, for selected mole ratios, as a function of pH (between pH 3.0 and 10.6) and temperature (between 15 and 55 ºC and thermally denatured at 95 ºC). Except for spectra of highly acidic (pH ≤ 3.9) and denatured samples, spectra of oleic acid/BSA complexes showed multiple narrow resonances from the oleic acid carboxyl carbon in a region (179–184 ppm) downfield from protein carbonyl and carboxyl carbon resonances. At low oleic acid/BSA ratios (0.5 and 1.0), at least two oleic acid carbonyl carbon peaks were observed; at high ratios (≥3.0), at least four peaks were present. The intensities of individual peaks, but not their chemical shifts, varied with the oleic acid/BSA ratio. The chemical shift of individual oleic acid peaks was invariant between pH 6.0 and 10.6; below pH 6.0, one of the oleic acid resonances exhibited an NMR titration curve with an apparent pKₐ of ~4. Thus, BSA binding sites for oleic acid are heterogeneous as monitored by the magnetic microenvironment of the oleic acid carboxyl carbon. The number of different oleic acid environments and the relative population of oleic acid molecules in these environments is dependent on the mole ratio of oleic acid/BSA.  

Our results suggested that the anionic form of oleic acid is bound to BSA at physiological pH and that the multiplicity of NMR peaks for [1-$^{13}$C]oleic acid resulted from, at least in part, different electrostatic and hydrogen bonding interactions between the oleic acid carboxyl group and specific amino acid residues of BSA.

Albumin is one of few proteins which can bind unesterified fatty acids and is the major vehicle for transport of fatty acid in plasma (1, 2). In human plasma, the mole ratio of fatty acid to albumin normally varies between 0.5 and 1.5 (1, 3) but is much higher under certain conditions, such as heparin administration (4) or extreme exercise (5).

In spite of the obvious importance of fatty acid/albumin interactions, studies by physical techniques have often been hampered by technical problems. The low water solubility of long chain fatty acids has made it difficult to obtain accurate binding constants for fatty acid/albumin complexes (1). Spectroscopic studies at physiological mole ratios of fatty acid to albumin using the fatty acid as a probe are either impractical or impossible because of the simplicity of the fatty acid molecule (e.g. lack of intrinsic chromophoric or fluorescent properties) or the relatively low abundance, compared to the background abundance from albumin, of spectroscopic signals (e.g. proton or $^{13}$C NMR signals). Consequently, fatty acids containing nitrooxide groups or conjugated double bonds have been used in electron spin resonance (6–10) and fluorescence (11, 12) spectroscopic studies, respectively. Natural abundance $^{13}$C NMR spectroscopy has been used to study interactions of long chain alkyl sulfates at very high molar ratios of surfactant to albumin (13, 14).

Bovine serum albumin and human serum albumin have similar structures, based on the amino acid sequences and on the proposed location of disulfide linkages (15–17). Each protein molecule has three homologous cylindrical domains and a total of nine loops (2, 15–17). Recently this model of structural organization has been utilized for interpretation of fluorescence and ESR results. Thus, it has been suggested that the first two fatty acids bind to a single specific domain (domain III) (11) and that each of the remaining two domains can bind two fatty acid molecules (11, 18).

This study demonstrates the feasibility of using $^{13}$C NMR spectroscopy to probe interactions between albumin and a biological fatty acid, oleic acid. Of the numerous fatty acids found in association with albumin, oleic acid is the most abundant in both human serum albumin (19) and BSA (20) and also appears to be the most tightly bound (1, 21). To achieve adequate spectral sensitivity and to focus specifically on the oleic acid carboxyl carbon, 90% isotopically substituted [1-$^{13}$C]oleic acid was used. The use of the $^{13}$C nucleus as a nonperturbing probe should avoid some ambiguities encountered with perturbing probes, as discussed by Perkins et al. (10) for the case of nitrooxide spin-labeled fatty acids. The NMR chemical shift (δ) of the carboxyl carbon is highly sensitive to ionization and hydrogen bonding of the carboxyl group (22–25). We have studied the effect on the oleic acid carboxyl $^{13}$C NMR signal of (a) varying the molar ratio of oleic acid to BSA, (b) varying the temperature, and (c) varying the pH.

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¹ The abbreviations used are: BSA, bovine serum albumin; T₁, spin lattice relaxation time; NOE, nuclear Overhauser enhancement.
Interactions of Oleic Acid with Albumin

Fig. 3. Carboxyl and carbonyl region of the proton-decoupled Fourier transform $^{13}$C NMR spectra of 0.5–7 mol of fatty acid (FA)/BSA at pH 7.4. BSA was added to neat 99% [1-$^{13}$C]oleic acid and the pH was adjusted to 7.4. Spectra accumulation conditions were the same as in Fig. 2 except 4000 spectral accumulations were obtained for each spectrum in the bottom and top rows. Spectra shown in the middle row are from samples with mole ratios indicated in the bottom line of the figure. The spectra (middle row) for 0.5, 2, and 3 mol of oleic acid/mol of BSA were obtained following 20,000, 24,000, and 31,327 accumulations, respectively, and are printed with higher vertical gain. The spectrum (middle row) for 1 mol of oleic acid/mol of BSA is a 2-fold vertical expansion of the bottom spectrum (4,000 accumulations). Major oleic acid carboxyl peaks are labeled as a–d. The broad signal is primarily from protein backbone carbonyl groups.

measured by removal of the sample from the probe and insertion of a thin thermocouple (25). Unless otherwise stated, all spectra were obtained at 36 °C.

RESULTS

Fig. 1 shows the elution profile from a Sephadex G-150 column of a solution containing 5 mol of [1-$^{13}$C]oleic acid/mol of BSA. The majority (74%) of the protein eluted in the monomeric size range ($M_w = 66,000$) just before the total volume of the column. There was no distinguishable dimeric peak, but small amounts of polymeric species of BSA were present. Using analytical columns with [9,10-$^{13}$H]oleic acid/BSA complexes (1–6 mol of oleic acid/mol of BSA), it was found that 65–74% of the radiolabeled oleic acid was bound to monomeric BSA and the remainder was bound to polymeric forms. Free oleic acid was not recovered in the total volume, indicating that oleic acid remained bound to BSA and was not liberated as free un-ionized oleic acid or as potassium oleate. Recovery of radiolabeled fatty acid from the column was 105 ± 12% of that added.

The $^{13}$C NMR spectra of fatty acid-free BSA and a preparation of 5 mol of oleic acid/mol of BSA before and after fractionation are shown in Fig. 2. Both 5:1 complexes (unfractionated material, Fig. 2B, and pooled, concentrated material from the monomeric peak of the preparative G-150 column, Fig 2C) gave essentially identical spectra. Compared to the spectrum of fatty acid-free BSA (Fig. 2A), the spectra of [1-$^{13}$C]oleic acid/BSA complexes had at least four additional resonances between 180.6 and 185.8 ppm. The relatively weak resonance at 180.9 ppm in the spectrum of BSA (Fig. 2A) is probably from glutamic acid carboxyl groups in the protein (34). Protein resonances were generally much broader than the oleic acid resonances and did not differ significantly at different oleic acid/BSA ratios or at different pH values, except as noted. The 170–180 ppm region encompasses the protein peptide backbone carbon groups and some side chain carboxyl groups (23, 34). The peak at ~130 ppm is from certain protein aromatic groups, the broad signal at ~40 ppm from u-carbons in the protein backbone, and the 14–35 ppm region from methylene and methyl groups of amino acid side chains (23). The relatively narrow resonance at 39.54 ppm is from the c-carbon and the b-carbon of lysine and leucine (23), respectively, both of which are abundant amino acid species in BSA (15).

Studies at Differing Oleic Acid/BSA Mole Ratios—The region of the $^{13}$C NMR spectrum containing protein carbonyl and carboxyl and oleic acid carboxyl resonances (170–190 ppm) is shown in Fig. 3 for oleic acid/BSA mole ratios varying from 0.5–7.0 at constant pH (7.4) and constant BSA concentration. At low oleic acid/BSA ratios, narrow peaks ($\nu_{\text{2H}} \sim 10$ Hz) were present at 180.9 ppm and 182.4 ppm (0.5 mol of oleic acid) and at 180.8 ppm and 182.4 ppm (1.0 mol of oleic acid). In these spectra, the peak at 180.8 may include an appreciable contribution from protein carboxyl groups (Fig. 2A). At higher oleic acid/BSA ratios, the intensity of the oleic acid carboxyl peaks increased, and at least four resonances between 180.5 ppm and 184.0 ppm were detected in spectra of samples containing >2 mol of oleic acid/mol of BSA. There were no detectable differences in the 170–180 ppm region between the fatty acid-free BSA spectrum and spectra at any oleic acid/BSA ratio. For convenience, four oleic acid peaks which showed little or no chemical shift change as a function of the oleic acid/BSA ratio were designated a–d, as indicated in Figs. 3 and 4. Peak a was not present in spectra of <3 mol of oleic acid/BSA (Fig. 3) and peak d could not be detected in a spectrum of 10 mol of oleic acid/mol of BSA (spectrum not shown).

The peak heights of the oleic acid carboxyl resonances increased relative to protein resonances (e.g. the protein carboxyl envelope; Fig. 3) with increasing oleic acid/BSA mole ratios. The increase was disproportionate, with a large increase for peak c and a small increase for peak a, as shown in plots of peak heights versus the oleic acid/BSA mole ratio (Fig. 5). The peak height was used as a relative measure of peak area because individual peaks were not sufficiently resolved for area measurements. The sum of the peak heights

The comparison of peak intensities using peak heights is not intended to be rigorously quantitative because there appear to be differences in peak widths (e.g. between peaks b and c) and there were some detectable changes in line widths at different mole ratios, as in the spectrum of 4 mol of oleic acid/mol of BSA, in which peak d showed an anomalous line broadening and consequently a smaller peak height.
region in particular, were unaffected by pH changes between pH 6.5 and 10, except for small changes in resolution which made it difficult to distinguish between resonances b and c at some pH values. At high pH (pH 10.4 and 10.6), peaks b and c were not distinguishable and appeared as a single broadened resonance at the chemical shift corresponding to peak b; in addition, the lysine c-carbon peak shifted downfield slightly (see "Experimental Procedures"). At low pH (<6), significant changes in the oleic acid carboxyl region were observed, as illustrated by the selected spectra shown in Fig. 7. Qualitatively, the carboxyl region first showed a loss of resolution, changes in relative intensities, and an apparent line broadening of the major resonances with decreasing pH (e.g. pH 5.0 and 4.4; in Fig. 7); at pH 4.4, a peak at 179.5 ppm (a chemical shift value different from those for peaks a-d) first appeared and the intensity of the new peak increased while all other peak intensities decreased to yield a single narrow peak at pH < 4.0 (e.g. pH 3.9 in Fig. 7).

The carboxyl chemical shift values are plotted as a function of pH in Fig. 8A. At pH > 6.0, four resonances were detectable (corresponding to a, b, c, and d of Figs. 3 and 4) which showed little or no chemical shift change between pH 6.0 and 10. Since peaks b and c were in general poorly resolved above pH 7.4, the chemical shift values are connected by a dotted line; even if crossover of the two peaks occurred, the chemical shift change would be small (<0.5 ppm). Note that similar chemical shift values were obtained whether oleic acid was added to BSA as un-ionized oleic acid (circles) or ionized potassium oleate (squares). In addition, the NMR results were identical when the pH was decreased from 10.6 to 3.9 (open circles) and when pH was increased from 3.9 to 10.6 (closed circles). Significant decreases in chemical shift values occurred in the pH range (pH < 5.5) in which qualitative spectral changes and gross sample changes (turbidity) were observed (see below). In Fig. 8A, the chemical shift values for the most intense peak (corresponding to peak c at pH > 5.5) were connected. It thus appeared that the major oleic acid peak had a sigmoidal behavior of chemical shift versus pH. The smaller resonances a, b, and d either coalesced into the single intense resonance or broadened beyond detection at low pH.

To study the low pH region in greater detail, additional titrations of oleic acid/BSA complexes were carried out by adding BSA to oleic acid and titrating from pH 6.0 to pH 3.4. Fig. 8B shows the plot of carboxyl chemical shift values as a function of added equivalents (or moles) of HCl for a representative experiment (5 mol of oleic acid/mol of BSA). The chemical shift values of the most intense peak showed a linear dependence on the number of added HCl equivalents, with two break points. The general form of the curve resembled that for the 13C NMR titration of aqueous carboxylic acids (25); the linearly decreasing chemical shift values with added HCl were consistent with protonation of the carboxyl group and the onset of protonation occurred at ~20 μl of HCl (Fig. 8B). The total chemical shift change (δmax - δmin = 2.8 ppm)
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Fig. 2, B and C). It is interesting to note that our results contrast with results of fluorne NMR spectroscopy, which could not detect any magnetic inequivalence in the first 14-15 binding sites of long chain trifluoroalkylsulfate ions on BSA (38).

One remarkable feature of the oleic acid/BSA interactions was the insensitivity of the oleic acid carbonyl function to changes in bulk pH (Fig. 6, a and b) between pH 6.0 and 10.6, as indicated by the constancy of the chemical shifts of the individual oleic acid carbonyl peaks. This result shows that the oleic acid carbonyl groups (bound to BSA) did not titrate in this pH range, in marked contrast to aqueous oleic acid in the absence of albumin (apparent pK = 7.2, Ref. 39) and to oleic acid in egg phosphatidylcholine vesicles (apparent pK = 7.6, Footnote 3). This result is physiologically significant because small changes in bulk pH near pH 7.4 result in changes in the ionization state and physical state of protein-free aqueous oleic acid (39).

An NMR titration curve was obtained for the major oleic acid carbonyl peak (peak c) at low pH (Fig. 8, a and B). This titration curve may be incomplete, based on the smaller total chemical shift change (δ_{max} - δ_{min}) compared with aqueous short chain carboxylic acids (28). The apparent pKc estimated from the NMR titration curve (pKc ~ 4) was somewhat lower than pKc values for carboxylic acids (25). Since BSA undergoes a conformational change which expands the structure in the same pH range (40), it is not clear whether the protonation of oleic acid molecules occurred as a result of the disruption of specific lipid-protein interactions and exposure of oleic acid carbonyl groups or whether the protonation occurred independent of protein changes. The chemical shifts of oleic acid carbonyl peaks a, b, and d were more resistant to pH changes than peak c, suggesting that oleic acid molecules in binding sites corresponding to peaks a, b, and d were more protected by specific lipid-protein interactions and/or steric hindrances. Highly acidic (pH ≤ 4.0) conditions removed specific oleic acid carbonyl interactions with BSA and resulted in a more homogeneous population of oleic acid molecules.

The relatively minor changes in tertiary structure induced by temperature changes below the denaturation temperature (35) produced minor changes in the oleic acid carbonyl spectrum. The loss of resolution and apparent broadening of peaks b and c at higher temperatures may have been caused by protein conformational changes or by an increased rate of exchange of oleic acid between these magnetically similar sites. Following gross structural changes induced by thermal denaturation, the multiple oleic acid peaks collapsed to a broad peak centered at 181.0 ppm. Thus, the magnetic environment of all peaks except d changed markedly. The upfield shift of peaks a, b, and c could indicate a change in the net ionization state of these populations of oleic acid carbonyl groups as a result of increased exposure of these groups to solvent molecules.

Our results provide evidence that the ionized form of oleic acid is bound to BSA at pH 7.4. This conclusion can be inferred from the pH dependence of the oleic acid carbonyl chemical shift and is consistent with the chemical shift changes following protein denaturation. The observation of several distinct resonances for the oleic acid carbonyl carbon may have been a result of differences in electrostatic and hydrogen bonding interactions between the oleic acid carbonyl group and specific amino acid groups. However, in addition to the reported large effects of ionization and hydrogen bonding on fatty acid carbonyl carbon chemical shifts in lipid systems (22-25), other extrinsic factors may affect the chemical shift of oleic acid in the presence of protein, so that a precise interpretation of chemical shift values cannot be made at present. Several previous studies have suggested that basic amino acid residues, including arginine (41), lysine and arginine (42), and tyrosine (43), are located at or near fatty acid binding sites on albumin. The ionization of tyrosine residues was hindered by the addition of fatty acid (43), suggesting close and specific interactions between the fatty acid carbonyl group and tyrosines. In addition, hydrogen bonding interactions between ligands and binding sites have been proposed from studies of steroid (44) and alcohol (45) binding to BSA.

3C NMR spectra of oleic acid/BSA complexes at pH 7.4 contained oleic acid carbonyl resonances with line width values as low as 10 Hz, in contrast to spectra of aqueous oleic acid (at the same oleic acid concentration but with no BSA) at pH values near physiological pH. In the latter case, the resonances were severely broadened, probably as a result of slow reorientation of lamellar liquid crystalline structures and/or highly restricted internal molecular motions of the oleic acid carbonyl group. The narrower oleic acid carbonyl resonances in the oleic acid/BSA complex may be a result of rapid internal motions or overall reorientation of the complex or both. Although the relaxation mechanism(s) of the fatty acid carbonyl carbon is not known and a detailed analysis of molecular motions cannot be made, resonances of nonprotonated carbons relaxing either by a dipolar mechanism or chemical shift anisotropy mechanism (46, 47) will be much narrower than resonances for protonated carbons with the same molecular motions. In fact, using the longer correlation times for anisotropic overall reorientation of BSA, it is clear that narrow (≤10 Hz) carbonyl resonances could be observed even with rigid attachment of the carbonyl group to BSA binding sites. Studies of the relaxation behavior of fatty acid α-CH₂ or β-CH₃ carbons should provide an understanding of whether the head group region is rigidly attached or whether motions which are significantly more rapid than the overall reorientation of the fatty acid/BSA complexes are present.

The present study showed that carbonyl carbons of 90% [1-3C]oleic acid yielded observable NMR resonances at physiological mole ratios of oleic acid/BSA. The oleic acid carbonyl resonances were in a region that was nearly devoid of protein resonances, a result which allowed quantitation of NMR features of the oleic acid resonances. The observation of multiple oleic acid carbonyl peaks suggested that specific ionic and/or hydrogen-bonding interactions may be an essential aspect of fatty acid binding to albumin.

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REFERENCES

4 It is not possible to rule out other forms of heterogeneity in the population of albumin monomers, such as sulphhydryl group heterogeneity (20, 28), as a source of minor resonances in the 13C NMR fatty acid spectrum. However, the vast majority of albumin binding studies, including those from which specific binding models have been derived (12, 18), have not considered such forms of albumin heterogeneity.
Interactions of the Carboxyl Group of Oleic Acid with Bovine Serum Albumin: A \(^{13}\)C NMR Study*

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The interactions of the carboxyl group of oleic acid with bovine serum albumin (BSA) were studied by \(^{13}\)C NMR spectroscopy at 50.3 MHz using 90% isotopically substituted [1-\(^{13}\)C]oleic acid. \(^{13}\)C NMR spectra were obtained as a function of the mole ratio of oleic acid to BSA (from 0.5–10.0) and, for selected mole ratios, as a function of pH (between 3.0 and 10.6) and temperature (between 15 and 55 °C and thermally denatured at 95 °C). Except for spectra of highly acidic (pH ≤ 3.9) and denatured samples, spectra of oleic acid/BSA complexes showed multiple narrow resonances from the oleic acid carboxyl group in a region (179–184 ppm) downfield from protein carbonyl and carboxyl carbon resonances. At low oleic acid/BSA ratios (0.5 and 1.0), at least two oleic acid carboxyl peaks were observed; at high ratios (≥3.0), at least four peaks were present. The intensities of individual peaks, but not their chemical shifts, varied with the oleic acid/BSA ratio. The chemical shift of individual oleic acid peaks was invariant between pH 6.0 and 10.6; below pH 6.0, one of the oleic acid resonances exhibited an NMR titration curve with an apparent pK of ~4. Thus, BSA binding sites for oleic acid are heterogeneous as monitored by the magnetic microenvironment of the oleic acid carboxyl carbon. The number of different oleic acid environments and the relative population of oleic acid molecules in these environments is dependent on the mole ratio of oleic acid/BSA. Our results suggested that the anionic form of oleic acid is bound to BSA at physiological pH and that the multiplicity of NMR peaks for [1-\(^{13}\)C]oleic acid resulted from, at least in part, different electrostatic and hydrogen bonding interactions between the oleic acid carboxyl group and specific amino acid residues of BSA.

Albumin is one of few proteins which can bind unesterified fatty acids and is the major vehicle for transport of fatty acid in plasma (1, 2). In human plasma, the mole ratio of fatty acid to albumin normally varies between 0.5 and 1.5 (1,3) but is much higher under certain conditions, such as heparin administration (4) or extreme exercise (5).

In spite of the obvious importance of fatty acid/albumin interactions, studies by physical techniques have often been hampered by technical problems. The low water solubility of long chain fatty acids has made it difficult to obtain accurate binding constants for fatty acid/albumin complexes (1). Spectroscopic studies at physiological mole ratios of fatty acid to albumin using the fatty acid as a probe are either impractical or impossible because of the simplicity of the fatty acid molecule (e.g. lack of intrinsic chromophoric or fluorescent properties) or the relatively low abundance, compared to the background abundance from albumin, of spectroscopic signals (e.g. proton or \(^{13}\)C NMR signals). Consequently, fatty acids containing nitroxide groups or conjugated double bonds have been used in electron spin resonance (6–10) and fluorescence (11, 12) spectroscopic studies, respectively. Natural abundance \(^{13}\)C NMR spectroscopy has been used to study interactions of long chain alkyl sulfates at very high molar ratios of surfactant to albumin (13, 14).

Bovine serum albumin and human serum albumin have similar structures, based on the amino acid sequences and on the proposed location of disulfide linkages (15–17). Each protein molecule has three homologous cylindrical domains and a total of nine loops (2, 15–17). Recently this model of structural organization has been utilized for interpretation of fluorescence and ESR results. Thus, it has been suggested that the first two fatty acids bind to a single specific domain (domain III) (11) and that each of the remaining two domains can bind two fatty acid molecules (11, 18).

This study demonstrates the feasibility of using \(^{13}\)C NMR spectroscopy to probe interactions between albumin and a biological fatty acid, oleic acid. Of the numerous fatty acids found in association with albumin, oleic acid is the most abundant in both human serum albumin (19) and BSA (20) and also appears to be the most tightly bound (1, 21). To achieve adequate spectral sensitivity and to focus specifically on the oleic acid carboxyl carbon, 90% isotopically substituted [1-\(^{13}\)C]oleic acid was used. The use of the \(^{13}\)C nucleus as a nonperturbing probe should avoid some ambiguities encountered with perturbing probes, as discussed by Perkins et al. (10) for the case of nitroxide spin-labeled fatty acids. The NMR chemical shift (δ) of the carboxyl carbon is highly sensitive to ionization and hydrogen bonding of the carboxyl group (22–25). We have studied the effect on the oleic acid carboxyl \(^{13}\)C NMR signal of (a) varying the molar ratio of oleic acid to BSA, (b) varying the temperature, and (c) varying the pH.

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1 The abbreviations used are: BSA, bovine serum albumin; T1, spin lattice relaxation time; NOE, nuclear Overhauser enhancement.