

1-1-1990

## Removal of cholesterol from animal fat

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Removal of cholesterol from animal fat

By

Ying Chen

A Thesis Submitted to the  
Graduate Faculty in Partial Fulfillment of the  
Requirements for the Degree of

MASTER OF SCIENCE

Department: Food Science and Human Nutrition

Major: Food Technology

Signatures have been redacted for privacy

Iowa State University  
Ames, Iowa

1990

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## INTRODUCTION

Coronary heart disease is the leading cause of death in the United States. More than 1.25 million heart attacks occur each year, and of those having heart attacks more than 500,000 die (National Heart, Lung and Blood Institute, 1984).

Numerous studies have demonstrated that risk factors associated with atherosclerosis are age, sex, genetic factors, hypertension, cigarette smoking, diabetes, and hypercholesterolemia. Diet generally is accepted as the most important controllable factor in the development of hypercholesterolemia. It has been proven that dietary cholesterol, fat, proteins, carbohydrates, fibers, minerals, and vitamins can affect the concentration of plasma cholesterol. Reduction in dietary lipids containing saturated fatty acids and cholesterol is a basic precaution that is recommended for the prevention, arrest, and reversal of atherosclerosis.

Several processes to reduce the cholesterol level in various food products have been proposed: molecular distillation of milk fat, solvent extraction of dried egg powder, vacuum steam stripping and supercritical carbon dioxide extraction of various animal fats, and bacterial reduction of cholesterol. But these chemical and physical

technologies all are expensive, complicated, and difficult to use on a commercial scale.

The cholesterol removal method examined in this thesis is based upon the reaction between the hydroxy group of cholesterol and cyclic anhydrides, succinic anhydride or glutaric anhydride. When this reaction occurs, the anhydride ring opens and succinic anhydride (or glutaric anhydride) is esterified with the hydroxy group of cholesterol by one of its carboxylic groups. The second carboxylic group is left free as an acid. Thus, each free cholesterol molecule in a fat is converted into an acid ester that can be easily removed from fats by the traditional alkali refining steps that are commonly used to remove free fatty acids from fats and oils. This method provides an effective and economic way to reduce the free cholesterol in animal fats.

## LITERATURE REVIEW

### Atherosclerosis

Atherosclerosis is characterized by a thickening (or irregular fatty deposits) in the internal layer of the walls of major blood vessels, especially arteries. This process results in a constriction of the vessel's lumen, a restriction of blood flow, and loss of vessel elasticity (Linder, 1985). These changes promote the formation of occlusive blood clots and can result in fatal injury to vital organs. Fatty deposits in the interior of arteries have been found to contain 70% of cholesterol (Linder, 1985).

Many studies have shown that in groups of humans whose blood levels of cholesterol are high, the incidence of atherosclerosis also is high (Hulley, 1988). Cholesterol inside bodies is obtained mainly from two sources: diet and biosynthesis. The majority of cholesterol is synthesized within the body, mainly in the liver and intestine (Turley and Dietschy, 1982). Diet supplies less than half of the total amount of cholesterol present in a typical individual. However, numerous dietary studies in animals have indicated that addition of substantial amounts of cholesterol and saturated fat to the diet will induce atherosclerosis (Page,

1954). In humans, it has been reported that a drastic lowering of cholesterol intake (from 600 mg/day to less than 50 mg/day) will reduce plasma cholesterol 15%-20% (Connor, 1980). This kind of information has had a major effect on the consumption and price of animal products that are the primary dietary sources of cholesterol.

With the increasing concern about the risk of atherosclerosis, more and more new food products that are low in cholesterol and saturated fat and high in polyunsaturated fat are purchased by consumers. At the same time, clinical treatments based on clofibrate, niacin, neomycin, cholestyramine and other drugs and resins also have been used to lower plasma cholesterol concentrations. By a combination of diet and pharmacologic agents, a drastic (25%-30%) reduction in plasma cholesterol has been obtained (Linder, 1985).

## **Cholesterol**

### **Biology**

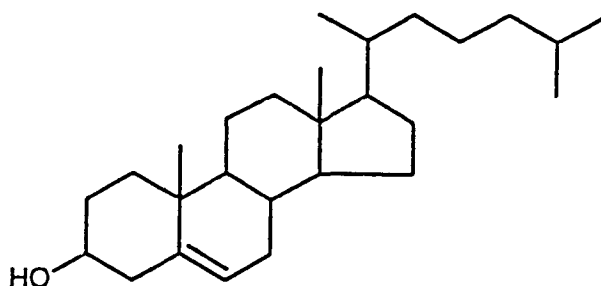
Cholesterol, in spite of its negative effects on atherosclerosis, is a necessary body constituent that is found in all animal tissues (Stryer, 1988). Cholesterol is a sterol that modulates the fluidity of eukaryotic membranes.

Nutritionists believe that cholesterol is needed for the synthesis of myelin, which surrounds nerve fibers and is essential for the normal functioning of the central nervous system. This is especially important early in human life. Cholesterol is a precursor of bile acids and steroid hormones such as progesterone, testosterone, estradiol and cortisol (Stryer, 1988). Cholesterol presents in all human tissues as free cholesterol and cholesteryl esters (Linder, 1985). The greatest concentrations of cholesterol are found in the brain, adrenal glands, and soft tissues of the body (Sabine, 1977). The lowest concentrations are in the heart and skeletal muscles (Kahn et al., 1963). Adipose tissue is a major cholesterol storage depot (Farkas et al., 1973; Schotz et al., 1953), whereas other tissues store cholesterol in their membranes (Takasugi and Imai, 1966). Cholesterol enters the body pool from two sources. It may be absorbed from the diet at the rate of about 300 to 500 mg/day (Turley and Dietschy, 1982) or be synthesized within the various organs at about 700 to 900 mg/day. There are a number of ways for the removal of cholesterol from the body. Cholesterol is secreted into the gastrointestinal tract from the liver at about 600 mg/day. Cholesterol is lost through the sloughing of skin at about 85 mg/day. After being converted to bile acids, about 400 mg/day is lost in feces, and biosynthesis of steroid hormones uses



about 50 mg/day. During active growth, some cholesterol (about 1.5 g cholesterol/kg body weight gained) is used to build new body tissue (Dietschy, 1984). The rate at which the body acquires cholesterol from all sources must be balanced by the rate at which the body can excrete the sterol molecule. Elaborate regulatory and transport mechanisms maintain this balance.

### Chemistry



Cholesterol was first described at the end of the eighteenth century by the French chemist de Fourcroy (1789). Early in this century, with the development of X-ray crystallography and synthetic chemistry, its final structural formula was established (Wieland and Dane, 1932).

As a steroid, cholesterol has a special ring structure, which is relatively stable thermodynamically, and it is considered to be inert in most chemical reactions.

However, cholesterol also is an alcohol by virtue of the hydroxy group in the third position, and it exhibits the reaction typical of this group (Sabine, 1977).

Organic chemists and pharmacologists have devised several syntheses of cholesteryl hemisuccinate, because it is important for pharmacological, enzymatic and biological studies (Brasar et al., 1983). Cholesteryl hemisuccinate is especially used in the salt form as a water-soluble standard for serum cholesterol determinations. The most recent method of cholesteryl hemisuccinate synthesis requires equimolar amounts of cholesterol and succinic anhydride to be refluxed in dry pyridine for 8.5 hours after which the solvent is evaporated under reduced pressure (Brasar et al., 1983).

#### **Previous Attempts to Reduce Cholesterol in Food Products**

A great deal of attention has been focused on the reduction of the cholesterol level in various food products. The methods used can be classified as microbiological, physical, chemical, and enzymatic.

Chosson et al. (1988) have reported reduction of cholesterol in edible fats with bacteria. This was done by incubating the fats with cholesterol-degrading bacteria, e.g., Norcardia actinomadura. If milk fat was added to a culture of

Nocardia CNCM I-636 and incubated for 12 hrs at 28°C, the cholesterol content was decreased 89% from 234 to 25 g/100g. So far, the few reports using this approach have been carried out on a small scale, and the method has not been reduced to a practical process.

Molecular distillation has been used to obtain a butter-like low cholesterol product (Bracco, 1978). This was prepared by molecular distillation of anhydrous milk fat in a two-stage falling film evaporator with an active heat-exchange surface of 0.1 m<sup>2</sup> and a rotation rate of 25-2500 rpm. The first stage was maintained at 70-90°C and 0.5-1 mm, and the oil was introduced at 50-60°C. The second stage was maintained at 172-220°C and 0.5-3 μm. The condenser temperature was 48-55°C. The cholesterol content was said to be reduced by 70%-90%. Vegetable oils, flavors, colors, vitamins, and milk had to be added to give a final product of satisfactory quality. The apparatus and procedure were relatively expensive.

An alternative physical method, steam stripping, was said to remove substantially all of the non-esterified cholesterol from fish oils as well as achieving deodorization (Marschner and Fine, 1988). Menhaden oil having 0.47% cholesterol and 0.08% free fatty acid was deaerated at 25 mm Hg and subjected to nitrogen sprage. Dry steam was introduced

to form a steam-oil mixture, which was heated to 460-480°C. The mixture was allowed to flow into a chamber where volatile matters (comprising free fatty acids, organic compounds, cholesterol, etc.) were flash-vaporized and separated from the liquid phase. The liquid phase was then subjected to thin film countercurrent steam stripping to remove residual fatty acids and cholesterol. The final product had 0.14% cholesterol and 0.03% free fatty acid. This method was thought to be less expensive than molecular distillation. Even so, it is still a very expensive and complicated procedure.

A chemical method, such as solvent extraction, has been used to reduce the cholesterol level and remove other lipids from dried egg yolk or egg powder (Melnick et al., 1971; Levin, 1975). Dried egg yolk solid (containing 2980 mg/100 g cholesterol) was extracted at a temperature <71°C with a nonpolar solvent, such as hexane or ethanol-ethyl ether. Egg yolks containing 410 mg cholesterol/100 g product was obtained.

Low cholesterol dried egg powder was said to be obtained by the following process. One volume of raw, whole liquid egg was mixed with four volumes of ethylene dichloride in a homogenizer, and the emulsified mixture was then introduced into a pressure vessel as a fine spray below the surface of a

boiling body of ethylene dichlorine maintained at 35°C and about 215 mm. Water was removed from the egg-solvent mixture through an azeotropic distillation processing. After substantial dehydration, the ethylene dichloride was drained from the solids, and fresh solvent was added to the dried product. The mixture was stirred, and solvent was again removed. Butylated hydroxytoluene, 0.02% based on the weight of fat present, was mixed with the egg product and five volumes of water was added to give a flowable mixture. This mixture was then homogenized and spray dried. The free fat content of the product was 1% by weight and there was a corresponding reduction of cholesterol. The ethylene dichloride content of the dried product was <100 ppm.

Larson and Froning (1981) used a hexane-isopropanol solvent mixture to extract an egg oil with a high level of cholesterol (40 mg/g). After the crude oil fraction was degummed, refined and bleached, the cholesterol content was reduced by 40%. The resulting fractions were used to make mayonnaise, but none of the fractions (egg oil, protein, and phospholipids) alone, or in combination, produced emulsions as stable as those from native yolk. This is because organic solvents remove all the lipids, including the phospholipids responsible for many of the yolk's functional properties. Also in these processes proteins were denatured, which greatly

impaired functional properties. Such chemical extractions can result in product contamination with residual solvents.

There has been some research using supercritical carbon dioxide extraction to remove cholesterol from butter, eggs, milk, and even whole steaks (Hardardottir and Kinsella, 1988; Froning et al., 1990). So far much of this work has not been reported in the scientific literature in detail. Generally, the carbon dioxide was pumped into a pressurized extraction vessel, and passed through the food particles at temperatures and pressures such that the carbon dioxide was in a supercritical state. Then the discharged carbon dioxide containing extracted lipids was pumped into a separator vessel where the pressure was reduced and the dissolved lipids come out of solution. The carbon dioxide was recycled for the next extraction. The extraction removes lipids other than cholesterol from the food. The chief advantage is that the carbon dioxide is regarded as a safe, nontoxic solvent. Even though it is feasible to produce cholesterol-reduced butter in this way, consumers may be unwilling to buy it, since this process adds 20¢/lb of the price. Some researchers, such as those from SKW Chemical Inc. in Georgia, have claimed that they did not find supercritical carbon dioxide cholesterol removal economically attractive after exploring it for several years (Anon., 1989).

Enzymes offer another alternative for remove cholesterol from food. Dr. Donald Beitz at Iowa State University is experimenting with cholesterol reductase enzymes, which are found naturally in cucumber leaves (Anon., 1989). This enzyme converts cholesterol into coprostanol, which is an innocuous sterol that is supposedly not absorbed as it passes through the digestive system. Although it is suggested that the enzyme could be added to milk, further studies are needed to assess the economical feasibility, nutritional and food safety aspects of this approach.

#### **Possible Additional Effects and Advantages of Treating Fats with Cyclic Anhydrides**

The reaction between cyclic anhydrides and hydroxy groups that is the focus of this thesis (see Introduction p. 2) may have additional effects and possibly advantages aside from its use in removing cholesterol from fats.

One of the unusual characteristics of milk fats is that it contains gamma- and delta-hydroxy fatty acids in its triglycerides. When these hydroxy fatty acids are released from milk fats by heating, they form gamma- and delta-lactones, which contribute fruity and coconut notes to the flavors of dairy products (Hammond, 1989). The removal of

such flavor compounds from milk fats along with cholesterol may occur by the application of the process studied in this thesis. Possibly such hydroxy fatty acids can react with succinic anhydride or glutaric anhydride and be concentrated from milk fat by alkali extraction. This may affect the utilization of milk fat in some applications.

Milk fats also contain beta-keto fatty acids esterified in its triglycerides (Hammond, 1989). These compounds, when released from milk fat, form alpha-ketones and contribute to the flavor of dairy products. Possibly these keto acyl groups will enolize and also react with cyclic anhydrides and be removed along with hydroxy compounds by alkali refining. This would remove and concentrate another important group of flavors.

Vegetable oils, such as soybean oil, are believed to be less stable if they have been oxidized before they are refined (Jung et al., 1989). The hydroxy and keto fatty acids resulting from heating hydroperoxy fatty acids are considered to be the factors that cause this loss of stability (Mistry and Min, 1988; Yoon et al., 1988). The reaction between succinic anhydride or glutaric anhydride and hydroxy groups may produce a way to remove these oxidation products and stabilize fats and oils that have been partly oxidized.



## MATERIALS AND METHODS

### Materials

#### Sources of materials

**Lard** Oscar Mayer brand lard was obtained from Hy-Vee Food Store in Ames, Iowa. It had been separated from pork tissue by wet rendering, and the antioxidants butylated hydroxyanisole (BHA), propyl gallate, and citric acid had been added.

**Butter** Unsalted butter produced by Mid-America Farms was purchased at Cub Foods Store in Ames, Iowa. The melted butter was fractionated by centrifugation into a top form layer, a middle butter oil layer, and bottom serum portions. The butter oil layer was pipetted out after removing the foams, and this was used for further studies.

**Tallow** Tallow was prepared from beef suet, which was bought from Hy-Vee Food Store in Ames, Iowa. The suet was rendered by heating in boiling water. The fat phase was filtered and cooled.

#### Storage of materials

All these fats were stored at 4.4°C.

## Exploration of Reaction Conditions

### Analyses with Thin-layer Chromatography

Thin-layer chromatography (TLC) was used to monitor the reaction between cholesterol and anhydrides. For analytical separations, silica gel G was used to prepare plates of 0.25 mm thickness. The developing solvent was hexane/ethyl ether/acetic acid (80:20:1,v/v/v). A 5- $\mu$ l sample was spotted on the plate with a 5- $\mu$ l disposable micropipet. As an indicator, 0.2% 2,7-dichlorofluorescein in ethanol was sprayed on the plates, and they were viewed under ultraviolet light.

### Preparation of cholesteryl hemisuccinate standard

Cholesteryl hemisuccinate standard was prepared following the method of Klein et al. (1974). Cholesterol (0.1 mol) and succinic anhydride (0.1 mol) were heated and stirred in a 250-ml flask with 100 ml dry pyridine. The reaction was heated under the reflux for 8.5 hr. The product was concentrated under reduced pressure in a Buchi Model Rotavapor-R (Brinkmann Inc., Westbury, NY) at 50°C. The residue was crystallized twice from 50 ml acetone and once from ethanol. The melting point was determined using a Thiele tube. The purified cholesteryl hemisuccinate was tested by

TLC, along with cholesterol and succinic anhydride. This purified cholesteryl hemisuccinate was used later as a standard in TLC analyses.

#### **Production of cholesteryl hemisuccinate in various solvents**

Cholesterol (1.29 mMol), and succinic anhydride (2.58 mMol) were heated and stirred in a 250-ml flask with 33 ml of various solvents. Samples of the reaction mixture were tested periodically by TLC. Dodecane (Sigma Chemical Company St. Louis, MO) was tried at 30°C, 60°C, 80°C, 120°C, 140°C and tested by TLC every 30 minutes for 5 hr. Pyridine and xylene (Sigma Chemical Company St. Louis, MO) were tried at their boiling points, 115-116°C and 137-140°C respectively, and tested by TLC every 30 min. for 4 hr and at 8 hr.

#### **The effect of the ratio of succinic anhydride/cholesterol on the production of cholesteryl hemisuccinate**

Cholesterol and succinic anhydride in molar ratios of 1:1, 1:2, 1:3, and 1:4 were tested in xylene at 134°C. Each reaction was tested by TLC at 2, 4, 6, and 8 hr.

### Removal of Cholesteryl Hemisuccinate by Alkali Refining

Cholesterol (1.29 mMol) and succinic anhydride (3.87 mMol) were heated and stirred with 33 ml xylene at 134°C for 7 hr. Various amounts of different alkalis were mixed with 5 g of reaction product in a plastic centrifuge tube using a Vortex Genie (Fisher Scientific Co. Bohemia, NY.), and then centrifuged (Beckman Model J2-21 Centrifuge, Rotor JA-17, Beckman Instruments Inc. Palo Alto, CA.) at 15,000 rpm, 20°C for half an hour. Each sample was tested by TLC to see whether there was a complete removal of cholesteryl hemisuccinate without any increase of cholesterol.

The following alkalis were tested on 5 g of the xylene solution in the weight ratios of solution:alkali indicated.

11% NaOH 1:1/3

20% Na<sub>2</sub>CO<sub>3</sub> (pH = 11.03) 1:1, 1:1/2, 1:1/3

5% Na<sub>2</sub>CO<sub>3</sub> (pH = 10.40) 1:1, 1:1/2, 1:1/3

5% NaHCO<sub>3</sub> (pH = 8.6) 1:1, 1:1/2, 1:1/3

KH<sub>2</sub>PO<sub>4</sub> 1:1, 1:1/2, 1:1/3

K<sub>2</sub>HPO<sub>4</sub> 1:1, 1:1/2, 1:1/3, 1:1/4

## Analyzing Cholesterol by Gas Chromatography (GC)

### Preparation for GC analyses

In order to do GC analysis for cholesterol, fats were saponified after removing cholesteryl hemisuccinate by extraction with 5% sodium carbonate solution. Saponification was done by a method provided by Michelson Laboratories Inc. (1989). Twenty-five milliliters of ethyl alcohol, 1.5 ml potassium hydroxide solution (potassium hydroxide:H<sub>2</sub>O = 3:2 by weight) and 2.5 g fat were refluxed for 30 min with occasional swirling. The sample then was transferred to a 250-ml separatory funnel with 50 ml of water. After cooling, 50 ml of ethyl ether was added to the separation funnel and shaken vigorously to extract the cholesterol and other unsaponifiable matter. The extraction was repeated four times. The ether extracts were combined and washed three times with 25 ml of water. Next the ether layer was washed with 20 ml of 0.5 N aqueous potassium hydroxide, followed by 25 ml of water. The washing with potassium hydroxide and water was repeated, and then the ether layer was washed with additional 25-ml portions of water until the washings did not change the color of 2% phenolphthalein indicator to pink. The washed ether layer was evaporated under nitrogen in an 80°C water bath. The residue was transferred to a vial with several portions of chloroform,

and the solvent was again evaporated to the dryness under nitrogen.

#### Preparation of internal standard

5- $\alpha$ -Cholestane was chosen as an internal standard for the GC analyses. 5- $\alpha$ -Cholestane was stable under the analytical conditions and its peak was easily separated from cholesterol's peak. It had properties similar to cholesterol. A 4.0 mg/ml 5- $\alpha$ -cholestane stock standard solution was prepared in ethyl acetate. This was stored in a freezer (-18°C). A 0.4 mg/ml 5- $\alpha$ -cholestane internal standard solution was prepared when needed by diluting the 4 mg/ml solution.

#### GC analyses

Analyses were performed on a Varian Model 3700 Gas Chromatography (Palo Alto, California) with a flame ionization detector and a direct capillary injector. The output of the electrometer was integrated with a HP 3396 A (Hewlett-Packard Co., Avondale, PA) instrument. The conditions were:

For GC: column	fused silica capillary
	30 m x 0.32 mm ID
	SPB-1, 0.25 $\mu$ m
injector temperature	300°C <i>no polar</i>

detector temperature	300°C
column temperature	150°C for 5 minutes, programed at 30°C/min to 260°C, hold for 5 min
carrier gas and flow rate	nitrogen gas, 7 ml/min
by pass nitrogen	18 ml/min
hydrogen gas flow rate	25 ml/min
air flow rate	250 ml/min
Integrator: attenuater	8
chart speed	0.7 cm/min
threshold	5

$$\text{Cholesterol (mg/ml)} = \frac{0.4 A_s}{A_i}$$

Where  $A_s$  and  $A_i$  are the average peak areas of cholesterol in the sample and internal standard, respectively. And 0.4 is the concentration of the internal standard 5- $\alpha$ -cholestane in mg/ml.

All the GC analyses were conducted in duplicate.

## Reaction on Animal Fats

### Finding suitable conditions for the lard reaction

Various reaction systems were tried. Initially, lard (50 g) and ground succinic anhydride (0.09 g) were heated and stirred in a 250-ml flask by means of a mantle with a voltage regulator and a magnetic stirrer. The reaction was run at 133°C for 7 hr. The reaction product was treated with 5% sodium carbonate solution to remove cholesteryl hemisuccinate, saponified, and extracted. The unsaponifiable residue was dissolved in 1 ml of internal standard solution and tested by GC. Peaks were identified by injecting cholesterol standard solutions and 5- $\alpha$ -cholestane under identical conditions. During the reaction, some succinic anhydride sublimed to the upper part of the reaction flask and deposited as white needle-like crystals. To minimize the loss of succinic anhydride, a piece of glass wool was used to wrap the top of the reaction flask. In order to avoid cholesterol and fatty acids oxidation during the reaction, nitrogen was conducted through the gas space at the top of the reaction flask.

A closed reaction system for lard was also tested. Lard and ground succinic anhydride were added to a 100-ml round bottom flask with a 24/40 standard taper opening. The flask was stoppered with a 24/40 standard taper stopper containing a

6 molecules



stopcock. Two metal springs were used to hold the stopper in place and protect the stopper if gas pressure developed in the flask. The flask was evacuated through the stopcock and heated in an oven at 150°C or 180°C. Every hour, the flask was taken out and shaken by hand. After 7 hr, the reaction product was extracted by 5% sodium carbonate solution, saponified, and the fat phase was tested by GC. In some instances, the gas space in the reaction flask was filled with nitrogen rather than being evacuated.

#### Fats reaction with acetic acid

Reaction on lard was run as before, except that acetic acid (2.5 g, which was the minimum amount to stop the sublimation of succinic anhydride) was added as a catalyst. Samples of 5 g were collected at 3, 5, 7, 9, 11, and 24 hr, extracted with 5% sodium carbonate solution, saponified, and examined by GC.

The same reaction using glutaric anhydride (0.09 g) instead of succinic anhydride was run, and the treatments and analyses of samples were the same as above.

Tallow and butter oil were also run under the same reactions as the lard for 8 hrs.

### Lard reaction on completely closed systems

Five grams of melted lard and 12 mg of succinic anhydride were filled into Pyrex glass tubes that were 3 cm long and 1 cm in diameter. The tubes were sealed under vacuum with a hand torch. The sealed tubes were suspended by wires in a flask of boiling xylene. Similar tubes were prepared that also contained 10 mg of acetic acid. Tubes were removed at 4, 6, and 8 hr, and alkali refined with 5% sodium carbonate solution. The refined lard was saponified and analyzed for cholesterol by GC.

Sealed tubes prepared as above also were heated in the apparatus shown in Figure 1 with refluxing xylene vapor. Samples were heated for 4, 6, and 8 hr and analyzed as before.

### Lard reaction in pretreated succinic anhydride

In order to improve the solubility of succinic anhydride in lard, succinic anhydride (0.09 g) was dissolved in 0.04 mol acetic acid at room temperature for half an hour and 140°C for 3 hr respectively. Lard was then added to these mixtures and reacted at 130°C-140°C for 7 hr, and the products were extracted with 5% sodium carbonate solution, saponified, and the unsaponifiable fractions were analyzed by GC.

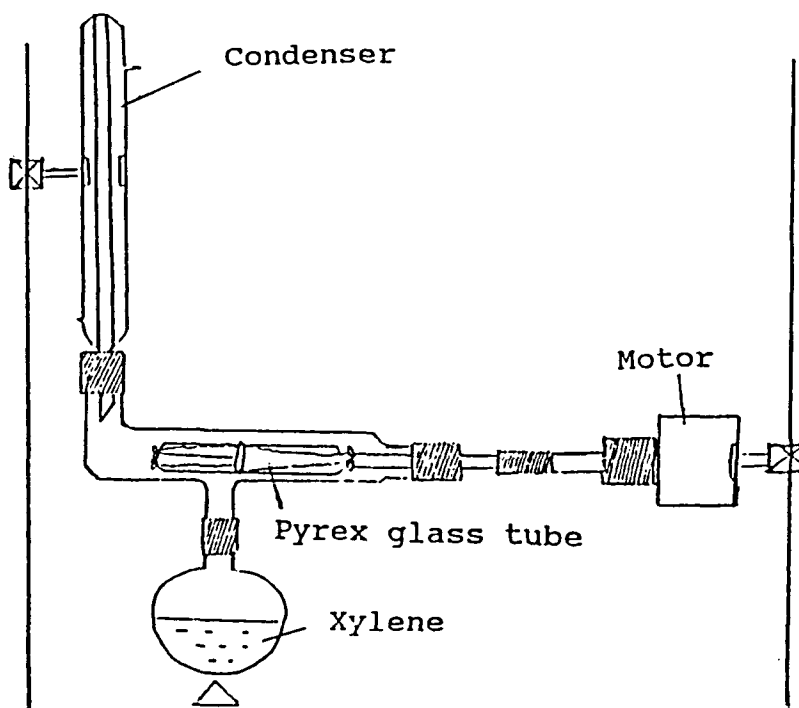


Figure 1. A specially designed apparatus for the completely closed reaction system. Pyrex glass tubes containing 5 g of melted lard, 12 mg of succinic anhydride and either 0 or 10 mg acetic acid were sealed under a water pump vacuum with a hand touch. The sealed tubes were attached to a stirrer with a sealed shaft and rotated slowly in refluxing xylene vapors for 4, 6, 8 hr. Analyses were as before

### Catalyst testing

Catalysts for the reaction between cholesterol (1.29 mMol) and succinic anhydride (3.87 mMol) were tested in refluxing xylene. The results were compared with a control without added catalyst. The disappearance of cholesterol was tested by GC. All catalysts were tested at a concentration of 0.833 mol/l. Some were tested at 2 fold and 1/2 of this amount. Samples were taken at 0.33, 0.67, 1, 2, and 4 hr. Catalysts tested were acetic acid, propionic acid, fumaric acid, succinic acid, cholesteryl hemisuccinate, monomethylsuccinate, and 4-dimethylaminopyridine (DMAP). For DMAP, 0.167 mol/l was used in each reaction at 40, 60, 80, and 140°C and samples were taken at 3, 5, and 7 hr.

### Investigation of the solvent effect on the reaction

Reactions in xylene of cholesterol and succinic anhydride were compared with those in nonane which was expected to create reaction conditions similar to those in the animal fats. Since xylene gave better results than nonane, the dielectric constant of nonane was adjusted approximately to that of xylene by adding ethyl butyrate, or propyl propionate. These solvents were chosen to maintain the boiling point in the same general range. The dielectric constant of the mixture was estimated from the following

calculation:

$$D(\text{nonane}) \times F(\text{nonane}) + D(\text{ethyl butyrate}) \times F(\text{ethyl butyrate}) = D(\text{mixture})$$

Where D is the dielectric constant and F is the volume fraction of the solvent in the mixture.

$$D(\text{nonane}) = 1.972$$

$$D(\text{xylene}) = 2.374$$

$$D(\text{ethyl butyrate}) = 5.10$$

D(propyl propionate) was unavailable and was assumed to be similar to that of ethyl butyrate.

The reactions were run at reflux with about 33 ml of solvent, 0.5 g cholesterol, and 0.379 g succinic anhydride either with and without propionic acid catalyst. Samples were taken at 0.33, 0.67, 1, 2, and 4 hr. The disappearance of cholesterol was followed by GC.

### The effects of different concentrations of catalyst on the reaction

The reaction systems with nonane (33 ml), ethyl butyrate and propyl propionate were used to study the reaction between cholesterol and acid catalysts (propionic acid). Propionic acid was used in these studies because the formation of cholesteryl esters needed to be tested and it was easier to separate cholesteryl propionate than cholesteryl acetate from

cholesterol by GC. Propionic acid at concentrations of 0.15 ml, 0.45 ml, 0.89 ml, and 1.6 ml in 33 ml solvent with 0.5 g cholesterol and 0.379 g succinic anhydride were used under reflux. Samples were taken at 0.33, 0.67, 1, 2, 4 hr. The disappearance of cholesterol was followed by GC.

**Product analyses by TLC and GC to study the reasons for the incomplete reaction in fats**

Preparative TLC on silica gel G 1 mm thickness was done to prepare sufficient material for GC analyses. The reaction product from lard was mixed with an equal amount of hexane and 160- $\mu$ l was streaked on the plate with a streaker (Applied Science, State College, PA). The bands were scraped from plates, eluted with ether, and analyzed by GC both before and after saponification. The lower two bands were mixed with 1.0 N sodium methoxide and transesterified for 1 hr. Water was added and the solution was then extracted by chloroform, and the chloroform layer was tested by GC to determine the fatty acid contents.

Monoolein (0.28 mMol) and cholesteryl hemisuccinate (0.56 mMol) were reacted in 33 ml reflux nonane for 7 hr and 5- $\mu$ l product was tested on a 0.25-mm thickness TLC plate.

## RESULTS AND DISCUSSION

### Preparation and TLC Analysis of Cholesteryl Hemisuccinate

#### Preparation of cholesteryl hemisuccinate

Cholesteryl hemisuccinate was prepared following the method in the literature (Klein et al., 1974), purified by crystallization, and dried in a desiccator. The melting point of two samples of purified cholesteryl hemisuccinate were 174-176°C and 175-177°C. These agreed with the melting point of cholesteryl hemisuccinate, 175°C, reported by Page and Rudy (1930).

#### Migration on thin-layer chromatography (TLC) plates

Cholesterol, cholesteryl hemisuccinate, and succinic anhydride were dissolved in xylene and spotted on a TLC plate. Cholesterol and cholesteryl hemisuccinate, but not succinic anhydride, could be detected under UV light after spraying with 2, 7-dichlorofluorescein. After development in hexane/ethyl ether/acetic acid, cholesterol had a  $R_f = 0.2037$ , and cholesteryl hemisuccinate had a  $R_f = 0.2515$ . Thus, cholesterol tended to migrate a little below cholesteryl hemisuccinate on TLC (Figure 2).

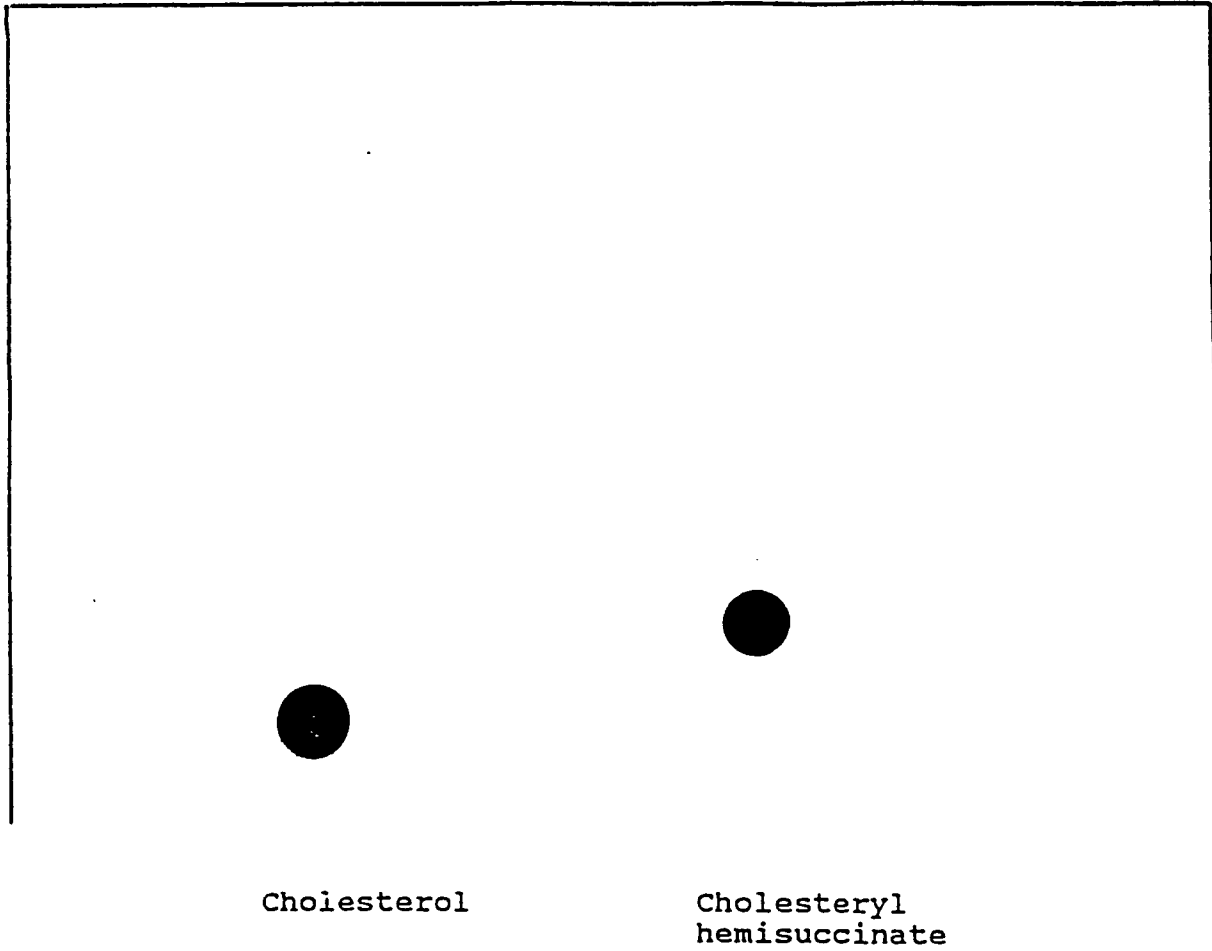


Figure 2. TLC testing of pure cholesterol and cholesteryl hemisuccinate



### **Optimization of Reaction Conditions in Model Systems**

Experiments shown that reaction temperature, solvent, and the ratio of cholesterol to succinic anhydride were critical to the production of cholesteryl hemisuccinate.

#### **Establishment of optimum reaction temperature and solvent**

The polarity of solvent was thought to affect the solubility of the reactants and reaction rate. Dodecane was first chosen as a solvent because its polarity was similar to that of nonpolar fats. Also it had a high boiling point (208-10°C), so that a wide range of reaction temperatures could be tested. Reaction of pure cholesterol and succinic anhydride in dodecane at different temperatures shown that no reaction occurred at 30°C, 60°C, and 80°C. At 120°C and 140°C, tests on TLC plates shown a significant decrease in the spot sizes of cholesterol and an increase in those of cholesteryl hemisuccinate. Further studies shown that reaction at 130°C-140°C gave the best results.

According to reports in the literature (Brasar et al., 1983), pyridine (boiling point 115-116°C) and xylene (boiling point 137-140°C) had been used as solvents in producing cholesteryl hemisuccinate, and reaction in pyridine and xylene also were tested. Xylene was a better solvent than pyridine,

because cholesterol was soluble in xylene at the room temperature and succinic anhydride at 100°C while much higher temperatures were required in pyridine. The reaction between cholesterol and succinic anhydride went well in refluxing xylene. TLC results demonstrated that after reaction for 8 hrs, in xylene much less unreacted cholesterol was left than with pyridine. Xylene was also more convenient than dodecane, since the optimum reaction temperature (130°C-140°C) was near xylene's boiling point and the use of refluxing xylene simplified temperature control.

#### Optimization of cholesterol to succinic anhydride ratio

Reactions in which the molar ratio of cholesterol to succinic anhydride varied from 1:1 to 1:4 were run in refluxing xylene and tested on TLC. The result (Figure 3) shown that after 8 hr the reaction product with one mole of cholesterol and three moles of succinic anhydride (1:3), the unreacted cholesterol was much less than with 1:1 and 1:2 ratio reactants. A 1:4 ratio did not improve the reaction yield noticeably. Therefore, a molar ratio of cholesterol and succinic anhydride of 1:3 was chosen as the optimum.

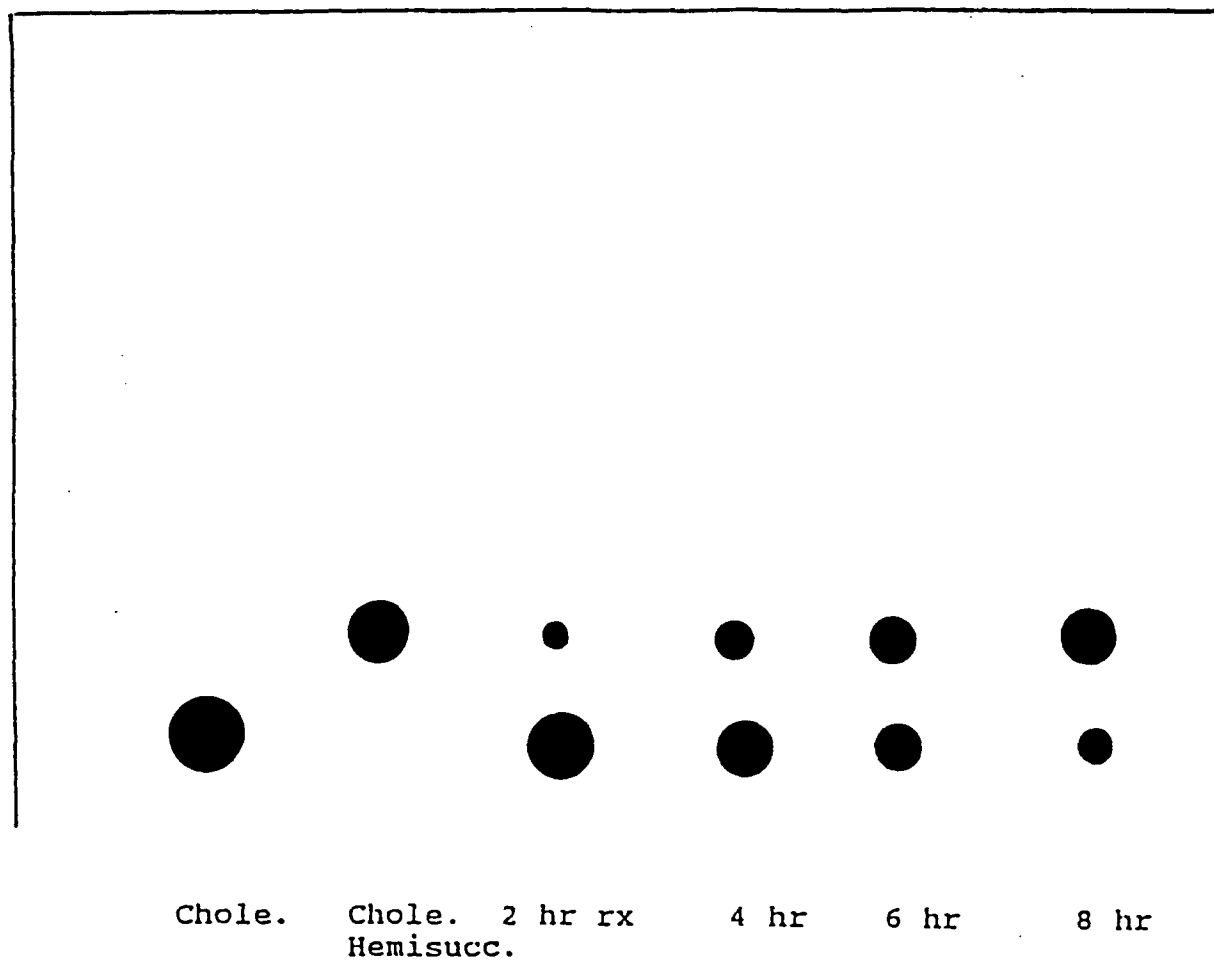


Figure 3. TLC results of reaction with cholesterol and succinic anhydride (molar ratio 1:3) in refluxing xylene for 8 hr

### Exploration of a Suitable Alkali Treatment Condition

To explore the removal of cholesteryl hemisuccinate from a reaction mixture with alkalis, cholesteryl hemisuccinate was generated by reaction of cholesterol and succinic anhydride (1:3) in xylene at 134°C for 7 hr. TLC test shown that when alkalis with too high a pH, such as 11% of NaOH (pH = 12.47), were used to extract the reaction mixture, cholesteryl hemisuccinate tended to be hydrolyzed back to cholesterol. In tests of such extractions on TLC plates, the cholesterol spots became bigger while the cholesteryl hemisuccinate spots almost disappeared. The sodium hydroxide solution was believed to catalyze the saponification of cholesteryl hemisuccinate. Extraction using lower concentrations of sodium hydroxide and various concentrations of sodium carbonate, sodium bicarbonate, carbonate-bicarbonate buffer, and sodium phosphate buffer, respectively, were tested. A 5% aqueous solution of sodium carbonate was the best alkali among those tested. When this alkali was used, TLC tests shown that cholesteryl hemisuccinate was extracted completely from the reaction mixture without any apparent increase of free cholesterol.

### Reaction with Lard

After optimizing the reaction and alkali refining conditions, this procedure was applied to fats.

Lard (50 g) and succinic anhydride (0.09 g) were used based upon the assumption of 0.12% cholesterol in lard and a 3 molar excess of succinic anhydride. They were reacted at 133°C for 7 hr, treated with 5% Na<sub>2</sub>CO<sub>3</sub> and tested on TLC. Qualitative analyses of lard samples on TLC plates did not provide as clear a picture of the extent of lard reaction as that of xylene reaction. There were components in the lard, such as mono-, di-, and tri-glyceride, that overlapped with cholesterol and cholesteryl hemisuccinate spots on the TLC plates so that cholesterol and cholesteryl hemisuccinate were not resolved but became parts of an elongated single spot.

The gas chromatographic (GC) method provided a way to monitor this reaction in lard and gave more quantitative results. In a typical reaction, 50 g of lard was reacted with succinic anhydride (0.09 g) at 133°C for 7 hr. The product was extracted with 5% sodium carbonate solution, saponified, and the unsaponifiable fraction was analyzed by GC. GC results shown that there was only about a 20% decrease in total cholesterol level in lard (Table 1).

Table 1. Amount of cholesterol in lard after reaction with succinic anhydride and extraction with 5% sodium carbonate solution

Sample	Cholesterol (mg/g lard)	% of cholesterol reduced
0 hr rx	1.0624	0
3 hr rx	0.8843	7
5 hr rx	0.9132	15
7 hr rx	0.8497	20

During this reaction succinic anhydride tended to sublime from the lard to the upper part of the reaction flask and deposited as white needle-like crystals. This loss of reagent undoubtedly affected the reaction with cholesterol and might account for there being only a 20% reduction in free cholesterol. To solve this problem, the reaction flask was wrapped to keep an even distribution of reaction temperature throughout the reaction flask, but succinic anhydride still sublimed from the lard. A few closed reaction systems were tried by sealing the reaction flask under nitrogen or vacuum and heating in an oven. By doing this, the sublimation of succinic anhydride was eliminated, but there was no improvement in the extent of the reaction. The possibility

that these sealed flasks might explode and catch fire in the oven was a concern, so it was decided to use a solvent which would reflux and wash the sublimed succinic anhydride back into the reaction. A neutral high boiling point solvent, such as xylene, could not be used, because it could be difficult to remove from the fats. A low boiling point solvent could not be used without lowering the reaction temperature. So, acetic acid was chosen, because it had an acceptable boiling point of 118°C, was safe for food, and could be washed out from the fats with aqueous alkali after the reaction.

#### Reaction in fats with acetic acid

Reaction with lard (50 g) was first accomplished by adding 0.04 mol of acetic acid, which was the minimum amount necessary to maintain reflux during the reaction, and succinic anhydride (0.09 g) at 133°C for 7 hr. The reaction mixture was treated by 5% sodium carbonate solution, saponified, and the unsaponifiable fraction was analyzed by GC. Acetic acid prevented the sublimation of succinic anhydride from the reaction and the reaction yield was increased, so that 40% of the cholesterol was removed as shown in Table 2.

Table 2. Amount of cholesterol remaining in lard after reaction with succinic anhydride in the presence of acetic acid and extraction with sodium carbonate solution

Sample	Cholesterol (mg/g lard)	% of cholesterol reduced
0 hr rx	1.0624	0
3 hr rx	0.9801	8
5 hr rx	0.8338	21
7 hr rx	0.6373	40

A similar reaction in 50 g lard with 0.09 g glutaric anhydride and 0.04 mol of acetic acid at 133°C for 7 hr gave results similar to those with succinic anhydride.

Reaction in tallow (50 g) with 0.09 g succinic anhydride and 0.04 mol of acetic acid at 133°C for 7 hr shown results similar to that of lard. However, reaction with butter oil (50 g) also with 0.09 g succinic anhydride and 0.04 mol of acetic acid at 133°C for 7 hr was not as good as that on lard and tallow (Table 3).



Table 3. Amount of cholesterol remaining in tallow and butter oil after reaction with succinic anhydride in the presence of acetic acid and extraction with sodium carbonate solution

Fat	Sample	Cholesterol (mg/g fat)	% of cholesterol reduced
Tallow	0 hr rx	0.6069	0
	7 hr rx 1	0.3509	42.1
	7 hr rx 2	0.3501	42.2
Butter oil	0 hr rx	1.3178	0
	7 hr rx	0.9346	29.1

#### Lard reaction on completely closed systems

The amount of acetic acid needed to cause reflux and avoid sublimation of succinic anhydride might be reduced if other means of avoiding sublimation could be devised. To do this, additional work with closed reaction systems were tried. Reactions both with and without catalyst (acetic acid) were run on lard and succinic anhydride using sealed glass tubes immersed in boiling xylene in the specially designed apparatus shown in Figure 1. With acetic acid present the results obtained in these experiments were similar to those obtained with acetic acid reflux. Experiments conducted on the effect

of catalyst concentration (see p. 39) eventually indicated that the concentration of acetic acid could not be reduced without slowing the reaction, so this line of experimentation was abandoned.

#### Reaction kinetics of the lard reaction

The time course of the lard reaction is shown in Figure 4. Extending the reaction time beyond 7 hr did not lead to any more reduction of the residual cholesterol level in lard. The results in Figure 4 show that the reaction started slowly. After about 1 hr, the reaction rate increased, and at about 7 hr, the reaction slowed and leveled off. The reasons for this slow-quick-slow reaction pattern were studied. The slow reaction rate at the beginning could be caused by the slow rate of solubility of succinic anhydride in fats. It also seemed possible that the reaction was catalyzed by a reaction product such as succinic acid or cholesteryl hemisuccinate. It is possible that the accumulation of these products in lard helped acetic acid to catalyze the reaction so that the reaction went faster.

In order to improve the solubility of succinic anhydride in lard, succinic anhydride (0.09 g) was first dissolved in acetic acid (0.04 mol) at room temperature for half an hour, and 140°C for 3 hr, respectively, and then lard was added to

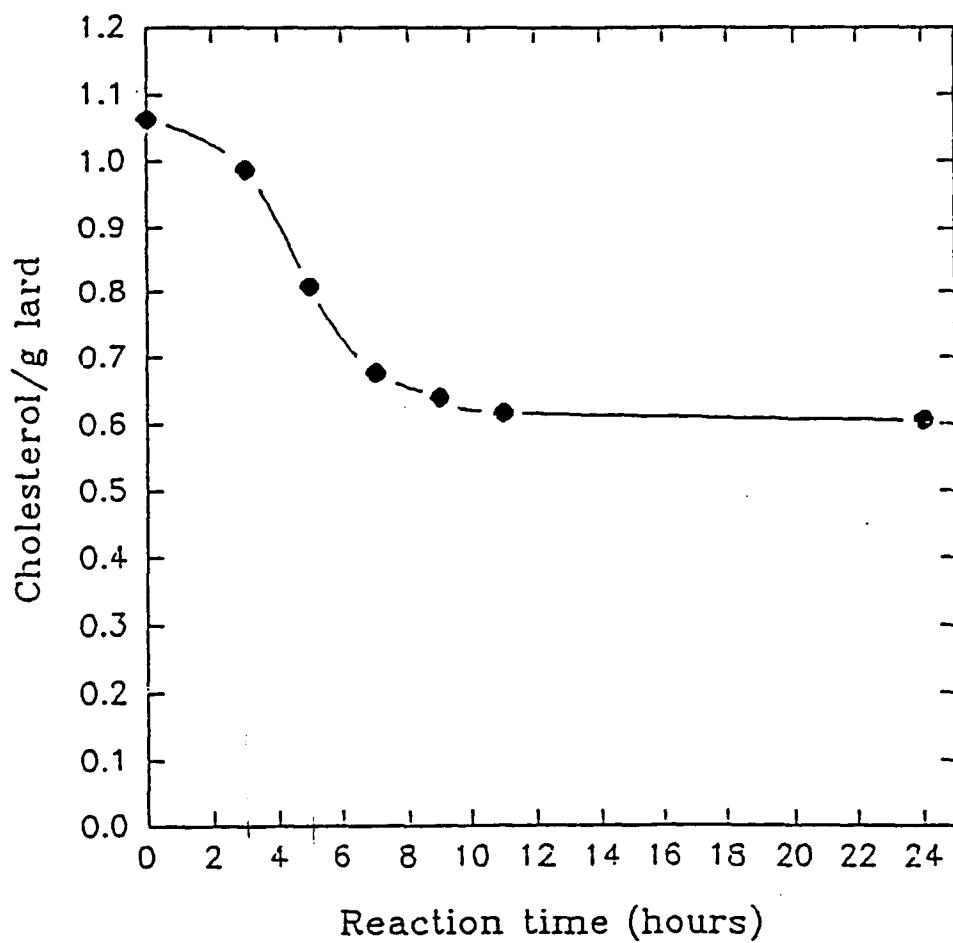


Figure. 4. The effect of reaction time on cholesterol concentration in lard reaction at 133°C with acetic acid added as a catalyst

start the reaction. The results shown there was no change in the reaction kinetics shown in Figure 4 when succinic anhydride was first dissolved in acetic acid at room temperature before the start of the reaction. Thus, the slow rate solution of succinic anhydride in the reaction mixture does not account for the kinetics in Figure 4. When succinic anhydride was dissolved in acetic acid at 140°C for 3 hr, there was no significant reduction of the cholesterol level in lard. This possibly was because a transacylation reaction between succinic anhydride and acetic acid resulted in the formation of acetic anhydride and succinic acid (Haddadin et al., 1975). Thus, cholesterol had no chance to react with succinic anhydride.

In studying the kinetics of the reaction, and from the idea that acetic acid and succinic acid (or cholesteryl hemisuccinate) may catalyze the reaction, seven catalysts were tested. They were acetic acid, propionic acid, cholesteryl hemisuccinate, fumaric acid, succinic acid, monomethylsuccinate, and 4-dimethylaminopyridine (DMAP). These were tested in xylene with cholesterol (1.29 mMol), succinic anhydride (3.87 mMol) and 0.833 mol of catalyst at 133°C for 4 hr. The cholesterol remaining was quantified by GC. Fumaric and succinic acids were not considered good catalysts, because both were difficult to dissolve in xylene

at 133°C even after a few hours reaction. Monomethylsuccinate dissolved in hot xylene very well, but crystallized out when the temperature went down. DMAP has been recommended as a catalyst for the reaction between hydroxy groups and anhydrides (Fieser and Fieser, 1972). Experiments monitored by TLC verified that DMAP was a very good catalyst in the xylene reaction system. It decreased both reaction time and temperature, but it is very toxic and difficult to remove from the reaction product. Furthermore, DMAP did not dissolve in lard very well. The experiments shown that acetic acid, propionic acid, and cholesteryl hemisuccinate could catalyze the reaction (Figures 5 and 6). When comparing these three catalysts, the results shown that acetic acid was slightly better than propionic acid, because acetic acid gave a little steeper slope (slope = -0.02007) than propionic acid (slope = -0.1790) when log cholesterol concentration versus reaction time was plotted. Cholesteryl hemisuccinate did catalyze the reaction (slope = -0.1032), but was not as good as acetic acid and propionic acid. Since cholesteryl hemisuccinate was not be produced in amounts as great as acetic acid, it does not seem to account for the reaction kinetics observed in Figure 4. Propionic acid could also be used as a catalyst for this reaction. Both acetic and propionic acids would be safe for use in foods. The price of propionic acid is higher than that

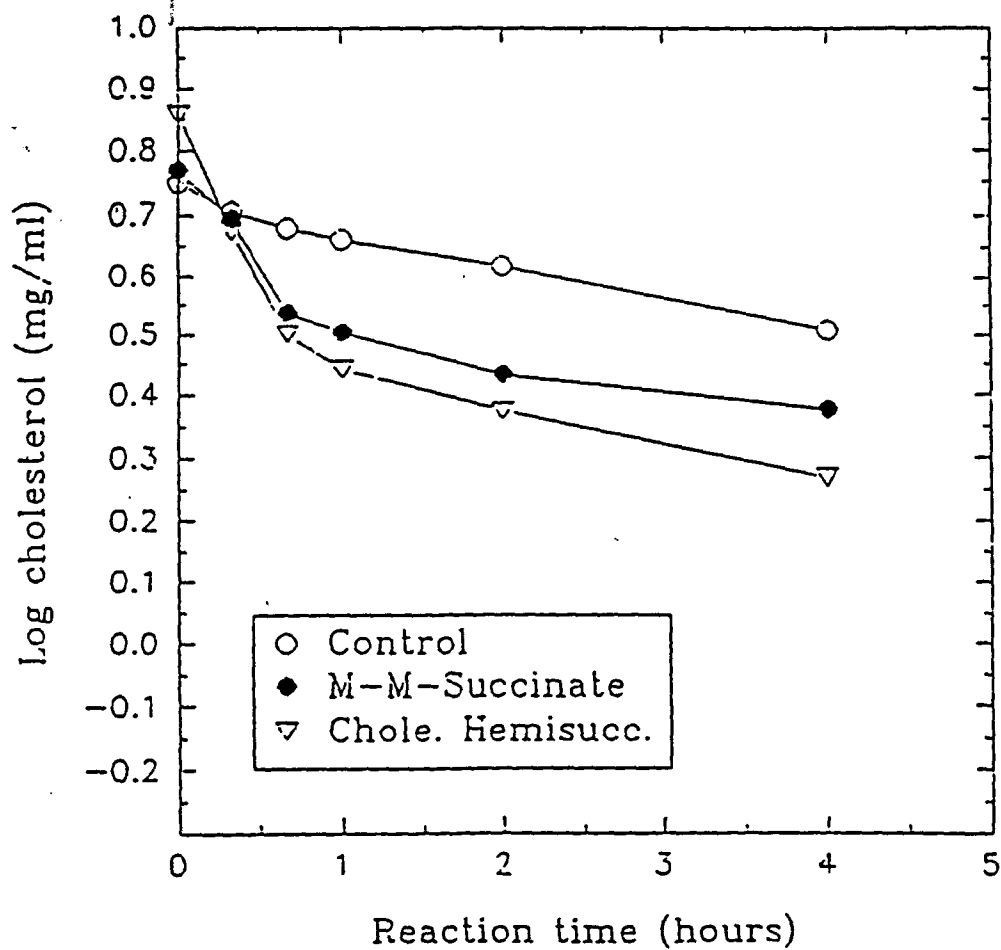


Figure 5. The effect of catalysts on the reaction kinetics in xylene with a ratio of cholesterol to succinic anhydride of 1:3 and with 0.833 mol of each catalyst. The reaction without a catalyst is the control

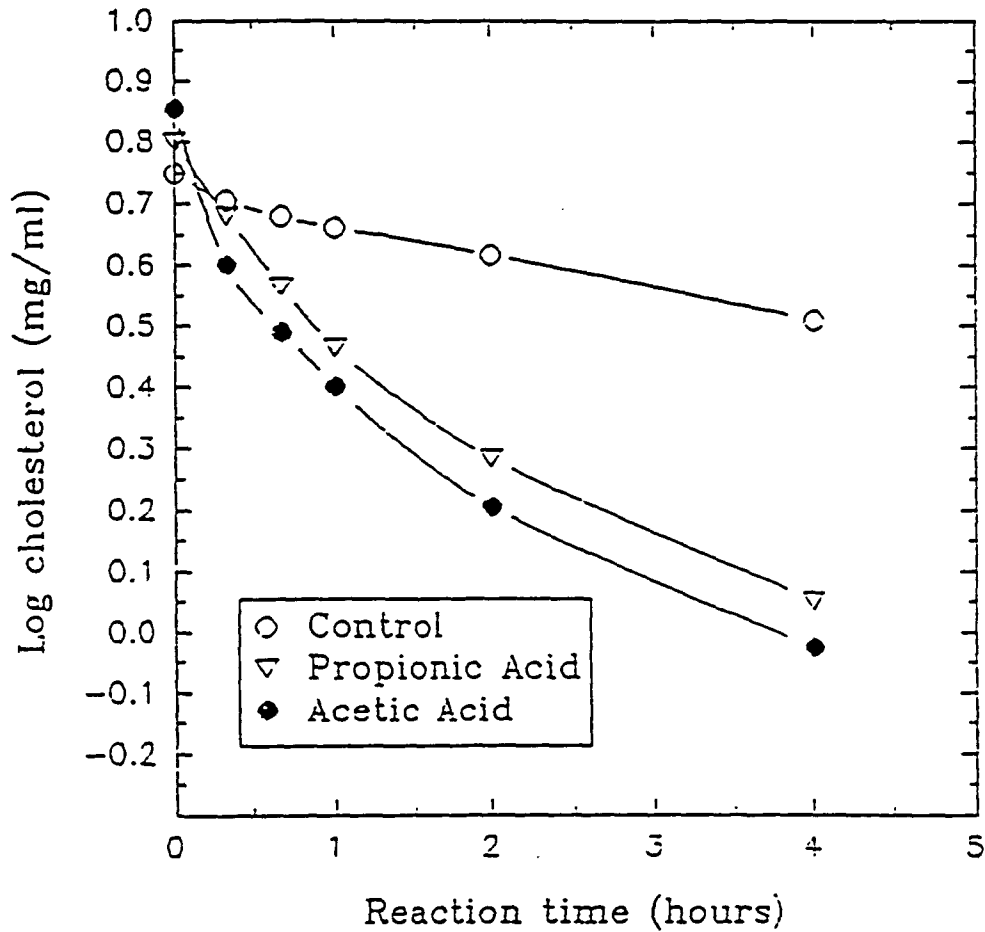


Figure 6. The effect of catalysts on the reaction kinetics in xylene with a ratio of cholesterol to succinic anhydride of 1:3 and with 0.833 mol of each catalyst. The reaction without a catalyst is the control

of acetic acid. Acetic acid is removed easily by the alkali treatment, and is more easily partitioned into the water phase and removed than propionic acid. So acetic acid is the most suitable of the seven possible catalysts tested.

It might be that stronger acids than acetic and propionic acids would catalyze the reaction even better. But if the acid is too strong, ester interchange in fats could occur and the esterification of cholesteryl hemisuccinate into glycerol could be catalyzed. Solubility problems might also be encountered for many of the stronger acids.

#### Effects of concentration of catalyst on the reaction

The effects of catalyst (as propionic acid) concentration also were studied in a nonane reaction system. Nonane was considered to have a polarity similar to that of lard. The reaction with cholesterol (1.29 mol) and succinic anhydride (3.87 mol) was run at 126°C for 4 hr in 33 ml nonane with different concentrations of propionic acid and tested by GC. Propionic acid was used instead of acetic acid to look for possible cholesteryl ester formation from the acid being used as catalyst. Propionic acid was preferred because it was easier to resolve cholesteryl propionate from cholesterol than cholesterol from cholesteryl acetate by GC. The results are shown in Figure 7. With too low a concentration, the catalyst



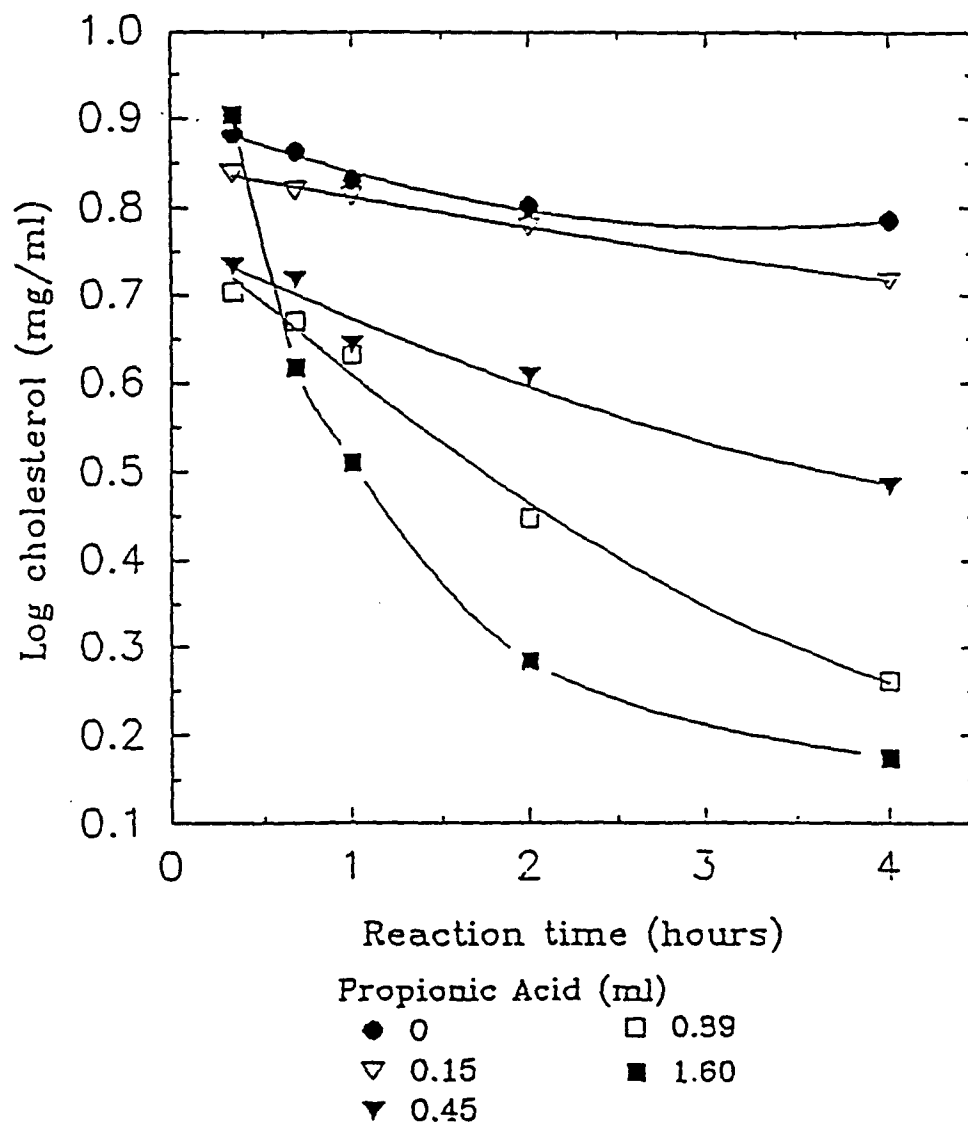


Figure 7. The effect of the concentration of the catalyst on the reaction kinetics in nonane with the ratio of cholesterol to succinic anhydride of 1:3 at 126°C

did not work well. But with a higher concentration (1.6 ml) of acid, there was about 5% cholesteryl propionate formed. This suggested that possibly cholesterol reacted with acetic acid to form cholesteryl acetate when the reaction had been conducted in fats. In a xylene reaction system, about 1% of the cholesterol was recovered as cholesteryl acetate. Possibly in a nonpolar environment, such as lard, more cholesterol reacted in this way. This might be the reason that the reaction would go completion in solvents such as xylene, but in lard only a 40% reduction of cholesterol level was achieved.

#### **Attempts to Understand the Solvent Effects**

To test the above hypotheses, pure cholesterol and succinic anhydride were reacted in nonane and xylene to study the solvent effect. It had been noticed that the reaction in xylene was much more complete than that in nonane. Nonane was considered a solvent which had a polarity similar to fat. Ethyl butyrate and propyl propionate, which had boiling points similar to xylene, were added to nonane to obtain a dielectric constant which was similar to xylene. Cholesterol (1.29 mol) and succinic anhydride (3.87 mol) were reacted in 33 ml of nonane, nonane with ethyl butyrate and propionic acid or

nonane with propyl propionate and propionic acid, for 4 hr at 133°C. The result shown that the cholesterol and succinic anhydride reaction in nonane could barely go (Figure 8). When the dielectric constant of nonane was made more like that of xylene by the addition of ethyl butyrate or propyl propionate, the better cholesterol reduction was achieved. Almost the same results were obtained in the nonane-ester reaction system as in xylene. Ethyl butyrate and propyl propionate had similar effects on the dielectric constant and reaction rates, but in Figure 8 only the results with ethyl butyrate are given. It could be concluded that lard as a nonpolar solvent has a negative effect on the reaction. This could slow the reaction compared with the rate in xylene. But in none of these reactions was there much cholesteryl propionate formed, so this theory would not account for the reaction in lard stopping at about 40% yield.

#### **Study the Reasons for Incomplete Reaction in Fats**

One reason for the reaction in lard stopping at about 40% reduction might be that much of the cholesterol in lard is present naturally as esters of long chain fatty acids. Another might be that cholesterol or cholesteryl hemisuccinate

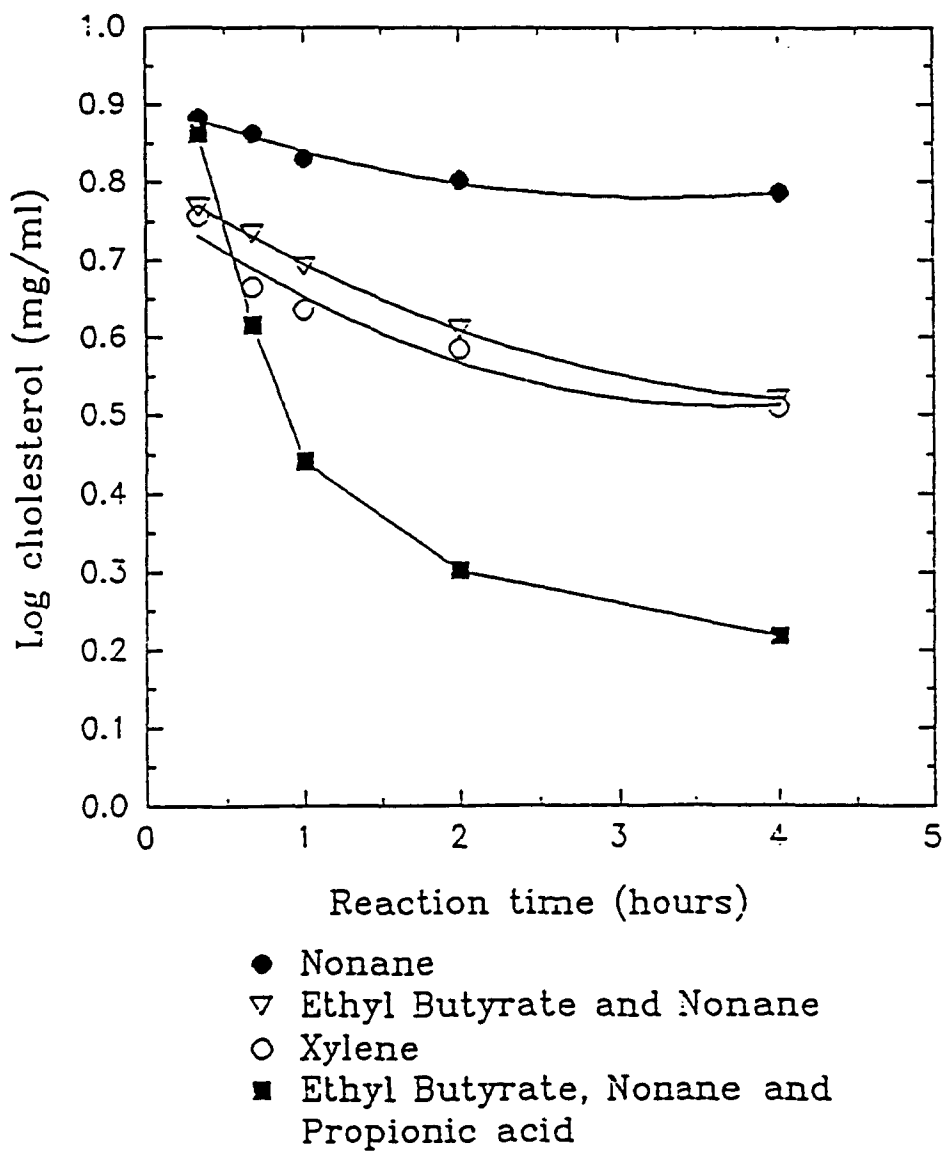


Figure 8. The effect of the solvent dielectric constant on the reaction kinetics in nonane at 126°C with the ratio of cholesterol to succinic anhydride of 1:3

reacts with other components to form nonacid cholesterol containing products, that could not be removed by alkali refining. To elucidate these possibilities, the product of a lard reaction catalyzed with acetic acid was streaked on a preparative TLC plate. The various bands were collected and analyzed by GC before and after saponification. The results shown there were only traces of free cholesterol left in lard after 7 hr of reaction. Also there was very little cholesteryl acetate (< 0.5%) or cholesteryl esters (< 1%) of long-chain fatty acids. The cholesterol was found in a band that migrated on the TLC plate like that of free cholesterol or diglyceride, but it was not free as shown by GC. The cholesterol was associated with large amount of fatty acids, possibly in the form of diglyceride. Possibly all the cholesterol had reacted with succinic anhydride, but some of the cholesteryl hemisuccinate had reacted with monoglyceride or other fat components to form a neutral product as shown in Figure 9. The migration of the cholesterol-containing product by TLC suggested that it contained a free hydroxy group.

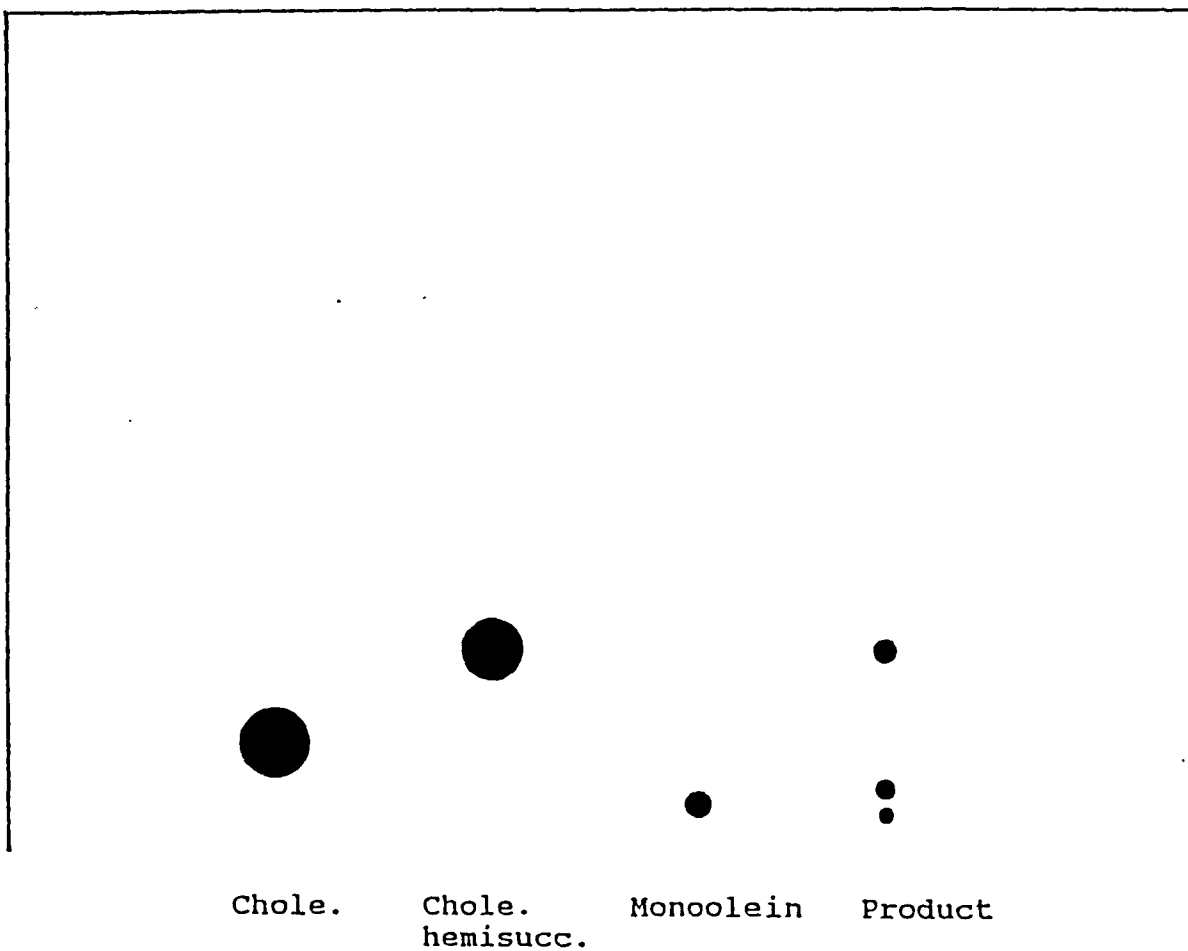


Figure 9. TLC results of reaction with 0.28 mMol monoolein and 0.56 mMol cholesteryl hemisuccinate in refluxing nonane for 7 hr

### SUMMARY AND CONCLUSION

The possibility of reacting cholesterol and succinic anhydride was explored. Thin layer chromatography shown that this reaction proceeded at 133°C in xylene to partial completion in 7 hr. A study of alkali treatments to remove cholesteryl hemisuccinate from the reaction mixture indicated that 5% sodium carbonate solution removed cholesteryl hemisuccinate completely from the reaction products. The molar ratio of cholesterol and succinic anhydride 1:3 was optimum in model systems. Gas chromatography shown that the reaction in lard resulted in only a 20% reduction of the cholesterol level of lard. Seven catalysts were tried to increase the reaction rate, and acetic acid was chosen as the best on the basis of effectiveness and costs. Model reactions in xylene with an acetic acid catalyst gave virtually complete reaction. With this catalyst, the reaction of lard and succinic anhydride (or glutaric anhydride) gave about a 40% reduction of the cholesterol content in lard. The reaction rate in lard shown a curious slow-quick-slow pattern. Reaction in tallow gave a 42% reduction of cholesterol, but the reaction in butter oil shown only about 30% reduction of cholesterol level.

The polarity of the reaction solvent affected the

reaction rate and nonpolar solvents such as nonane were not as good as xylene as a solvent. Lard's dielectric constant is more like that of nonane.

More detailed analyses of the lard reaction products by combined TLC and GC indicated that the cholesterol remaining in the lard after 7-hr reaction with succinic anhydride was not free but was combined in a neutral, fairly polar product.



## APPENDICES

Appendix 1. The effect of reaction time on cholesterol concentration in lard reaction (Figure 4)

Reaction time (hrs)	Cholesterol (mg/g lard)
0.00	1.0624
3.00	0.9874
5.00	0.8072
7.00	0.6748
9.00	0.6381
11.00	0.6160
24.00	0.6053

Appendix 2. The effect of catalysts on the reaction in xylene (Figure 5)

Reaction time (hrs)	Cholesterol (mg/ml solution)		
	Catalysts		
	Control	M-M-Succinate	Chole. hemisucc.
0.00	0.7500	0.7680	0.8579
0.33	0.7050	0.6947	0.6737
0.67	0.6800	0.5357	0.5005
1.00	0.6616	0.5033	0.4412
2.00	0.6176	0.4325	0.3738
4.00	0.5094	0.3765	0.2698

Appendix 3. The effect of catalysts on the reaction in xylene (Figure 6)

Reaction time (hrs)	Cholesterol (mg/ml solution)		
	Catalysts		
	Control	Propionic acid	Acetic acid
0.00	0.7500	0.8000	0.8553
0.33	0.7050	0.6812	0.6009
0.67	0.6801	0.5651	0.4913
1.00	0.6616	0.4641	0.3986
2.00	0.6176	0.2835	0.2046
4.00	0.5094	0.0510	-0.0264

Appendix 4. The effect of the concentration of the catalyst  
(Propionic acid) on the reaction in nonane  
(Figure 7)

Reaction time (hrs)	Cholesterol (mg/ml solution)				
	Propionic acid (ml)				
	0	0.15	0.45	0.89	1.60
0.33	0.8827	0.8381	0.7343	0.7038	0.9041
0.67	0.8624	0.8201	0.7183	0.6696	0.6176
1.00	0.8307	0.8133	0.6429	0.6318	0.5106
2.00	0.8016	0.7794	0.6067	0.4474	0.2829
4.00	0.7864	0.7173	0.4835	0.2610	0.1739

Appendix 5. The effect of the solvent dielectric constant on the reaction in nonane (Figure 8)

Reaction time (hrs)	Cholesterol (mg/ml solution)			
	Solvents			
	Nonane	Ethyl butyrate + nonane	Xylene	Ethyl butyrate + Nonane + propionic acid
0.33	0.8827	0.7685	0.7567	0.8629
0.67	0.8627	0.7337	0.6653	0.6160
1.00	0.8307	0.6920	0.6352	0.4411
2.00	0.8016	0.6097	0.5850	0.3020
4.00	0.7864	0.5208	0.5094	0.2176

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**ACKNOWLEDGMENTS**

I express my sincere thanks to my major professor, Dr. Earl G. Hammond, for his suggestion of this project, his patient help, guidance and care throughout this study and in the preparation of this manuscript.

I would also like to thank the members of my committee, Dr. Pam White and Dr. Lester A. Wilson for their interest and help during this study.

I also express my thanks to my fellow lab workers, especially Mr. Dan Duvick for the analyses of fatty acid composition, and Inmok Lee and Shantilal Parmar for their help and advice.

I would also like to express special thanks to my parents, for their loving support and encouragement throughout my study in ISU.

Finally, I wish to send my deepest love and appreciation to my husband, Ming-Huan. For his unending encouragement, support and understanding.