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Preliminary Quantitative Investigation of Postmortem Adipocere Formation

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ABSTRACT: The accurate determination of postmortem interval (PMI) using the formation of adipocere presents a significant challenge to forensic scientists interested in determining the time of death. Several attempts have been made to determine the time since the occurrence of death. However, up to date, this has been difficult because previous approaches have been mainly qualitative, focusing on the later stages of degradation processes. This work presents preliminary results of an experimental model of postmortem adipocere formation using liquid chromatography. Three pig cadavers were submerged in distilled water, chlorinated water, and saline water. Fresh specimens resulting from the degradation in the subcutaneous fat were obtained from the pigs at two-week intervals for a period of ten weeks, and were subjected to chromatographic analysis. By correlating the ratio of the disappearance of hydrolyzed fatty acids with the formation of hydroxystearic and oxostearic acids after death, a simple, quantitative analytical method was developed for the determination of PMI. Experimental observation of the chemistry of adipocere formation indicated that adipocere can be formed only a few hours after an incidence of death and this continues until the saturation of oleic acid degradation after several weeks. Different time courses were obtained for cadavers immersed in distilled, chlorinated, and saline water, respectively. This work has not in any way solved the time since death problem. But it may be an approach to the problem that has not been adequately explored.

KEYWORDS: forensic science, adipocere formation, quantitative analysis, saturation index ratio, chemistry, postmortem interval, high-performance liquid chromatography

When a person dies, a sequence of processes takes place that leads to the decomposition of the dead body. When the dead body is located in a damp environment, these complex postmortem actions present a significant challenge to forensic scientists who are interested in determining the time that death occurred. Estimating postmortem interval is a complex task that is currently plagued by interrelated factors, which include temperature, humidity, aerobic and anaerobic conditions, and the presence of microorganisms (1–4). Such factors occur simultaneously in the presence of other chemical transformations leading to the eventual disintegration of the dead body, thus making "time of death" inquiries all the more difficult. In addition, the task is further complicated when a substantial amount of adipocere formation has taken place (4,5).

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Adipocere is a waxy or greasy decomposition product of fatty acids that is formed by the hydrolysis and hydrogenation of adipose fats. The formation of adipocere in aqueous environments is manifested by the conversion of fats found in human body into palmitic and stearic (3–9) acids. Adipocere is generally considered to result from bacterial action, common in warm, damp, anaerobic environments. However, its frequency, rate of formation, factors affecting its formation, and physical characteristics are not yet clearly understood (10). Extensive studies regarding the chemical composition of adipocere demonstrated that it consists mainly of saturated fatty acids with a large amount of hydroxy fatty acids (2–10). Some scientists believed that 10-hydroxy and 10-oxo fatty acids are present in human adipocere after the original adipose tissue has been converted through bacterial activity (11). Using gas chromatography with mass spectrometry (GC/MS), Takatori and his coworkers have shown that the primary and secondary components of adipocere are the 10-hydroxyoctadecanoic and 10-hydroxyhexadecanoic acids (6,7,11). However, the need to derivatize fatty acids prior to analysis, plus the high temperature required for GC/MS techniques (12), imply that several microbial degradation products resulting from adipocere formation may be lost or inadequately reported.

Although its presence is initially observable, adipocere formation commences rapidly after death. This is rapidly accelerated or retarded depending on the circumstances surrounding the death as well as environmental factors. In general, complete transformation of all soft tissues to adipocere in the presence of water or damped environment may occur within three to six months or even longer (8). Some workers have reported an extensive adipocere formation within only 22 days (9). Most of the works on adipocere formation have focused on the later stage of the degradation processes, which range from weeks to years. The goal of our work is to elucidate the chemistry of adipocere formation at the onset of degradation, specifically within hours of the occurrence of death.

Using adipocere formation indicators such as the disappearance of oleic acid together with the associated formation of hydroxystrearic acid, we examined the presence of early signals. The knowledge gained could be used to determine possible time of death. We hereby report our preliminary results using high performance liquid chromatography to study the formation of adipocere. Results obtained were used to correlate the ratio of normal adipose tissue to hydrolyzed fatty acids for subsequent estimation of postmortem interval.

Materials and Methods

The goal was to recreate the essential conditions for the formation of adipocere including the presence of skin and fatty tissues in

an excess of moisture, controlled low temperature level (28°C), and anaerobic environment. Consequently, three different pig cadavers were submerged in distilled water, chlorinated water, and saline water. Fresh specimens resulting from the degradation in the subcutaneous fat were obtained from the pigs. Six different samples were obtained from each of these specimens, weighing approximately 50 g in 50 mL centrifuge tubes. The samples were taken at two week intervals starting from 2 h after postmortem and continuing on for ten weeks. Parameters such as color, odor, and stability as well as other physical characteristics of the samples all were noted. Samples for each given week were obtained from the same general location and these were frozen immediately before being analyzed. The Institutional Review Boards of SUNY-Binghamton and Lourdes Hospital approved the research protocol employed in this study.

Sample Preparation

Minimal sample preparation was conducted without any derivatization so as to prevent contamination of the samples. Extraction of fatty acid was carried out using solid phase extraction techniques (UCT). Briefly, frozen specimens consisting of fat, skin, and hair were allowed to thaw for several minutes in a petri dish. An aliquot sample weighing 1 g was obtained from the larger specimen which was mixed with 10 mL of phosphate buffer (pH 6.0) and was subsequently homogenized. All samples were centrifuged simultaneously for 20 min or until the solid contents of the homogenates had all settled down. Liquid homogenate was loaded into the UCT extraction columns (CECt4/2Z) after conditioning the column with a mixture of 3 mL methanol, 3 mL distilled, deionized water and 3 mL phosphate buffer in a CEREX SPE processor vacuum manifold (Varian). The column was washed with distilled water and hexane before the fatty acids was eluted by a mixture of chloroform and isopropanol at a volume ratio of 90:10. After drying under nitrogen, the samples were stored at 4°C before liquid chromatographic analysis.

Chromatographic Analysis

HPLC analysis was carried out at room temperature using a DIONEX DX 500 chromatography system together with an AD20 absorbance detector and AI 450 Chromatographic software. Chromatographic separation was achieved at about 4.6 × 150 mm Zorbax RP300-C8 column using eluent mixture of acetonitrile/water (95:5) and H_3PO_4 (0.1%) solutions as the solvent (unless otherwise stated). Peaks were obtained at 210 nm using the absorbance detector at a flow rate of 1 mL/min. The resulting chromatograms were compared to a standard mixture of oleic, palmitic, and stearic acids.

Results

This work presents preliminary results of an experimental model of postmortem adipocere formation using liquid chromatography. Our goal is to demonstrate a quantitative chromatographic analysis of fatty tissues as a means of assessing the rate of decomposition of adipocere tissue. *In vivo* fatty tissues contain only slight quantities of free fatty acids. After death, there is a rapid and considerable quantitative increase in these fat fractions. By measuring the ratios of hydroxy fatty acids and the oleic acids, a correlation was found between the ratio of normal adipose tissue and hydrolyzed fatty acid. Pig cadavers that are allowed to decay for about ten weeks can also provide an excellent basis for conducting rate study using disappearance of oleic acid to the appearance of hydroxy stearic acid. Samples were extracted as described in the Experimental Section but no prior derivatization process was carried out.

Significant changes in the physical characteristics of pig cadavers were observed after being immersed in water tanks. During the first two weeks, samples continued to maintain whitish color, with some offensive odors. After the sixth week, the samples turned grayish brown. At this stage, the skin could not be easily distinguished from the fat and the odor became very offensive. By the tenth week a final waxy-gray consistency was observed, which is a distinguished characteristic of adipocere (1,2). To fully characterize the formation of adipocere, we investigated the chemical transformation of fatty acid components at the onset of degradation process. For this purpose, high performance liquid chromatography

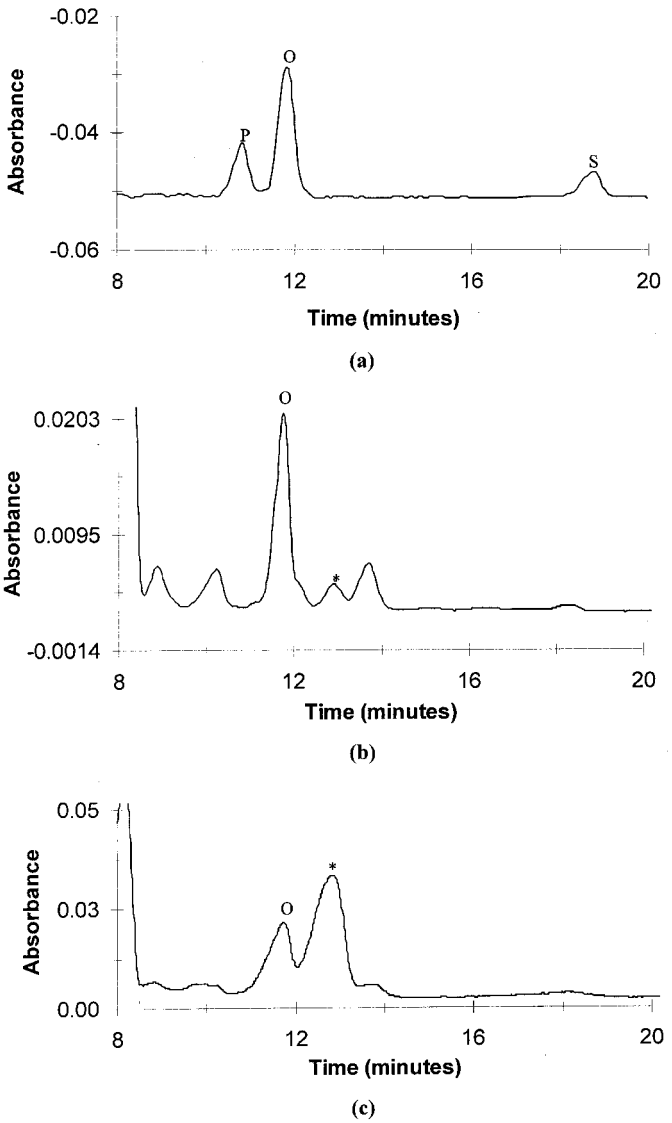
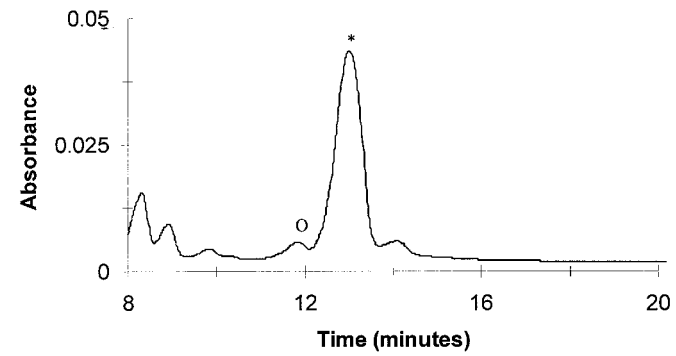


FIG. 1—*Chromatograms recorded for pig samples immersed in water showing the onset of adipocere formation* (a) *standard mixtures of fatty acids starting with palmitic acid (P), followed by oleic acid (O), and stearic acid (S);* (b) *chromatograms recorded at Week 0; (*) hydroxystearic acid* (c) *Week 2. Conditions: Zorbax RP300-C8, 4.6 × 150 mm; mobile phase: acetonitrile/H_2O (95:5): H_3PO_4 (0.1%) = 40: 60; flow rate = 1 mL/min; detection: 210 nm.*

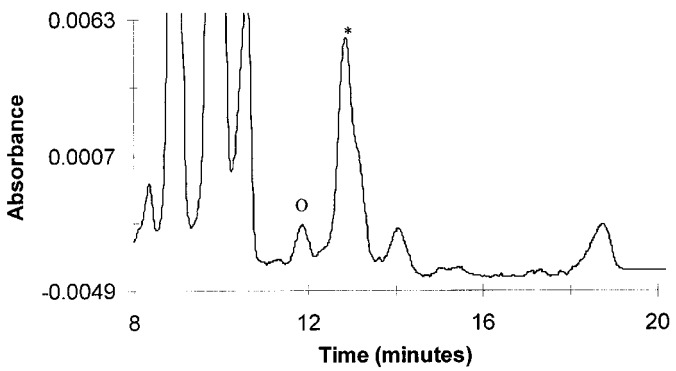
(HPLC) was used to preserve any volatile components of the degradation. The *Experimental* Section describes how samples were obtained from the pig cadavers immersed in three different tanks containing tap water, chlorinated water, and salt water. The tanks were maintained at temperature and humidity conditions in which a significant amount of decomposition bacteria were expected.

Figure 1 shows the chromatogram recorded for pig cadavers immersed in tap water. A comparison was made of the chromatograms obtained for a standard solution of fatty acid mixtures consisting of palmitic, oleic, and stearic acids, with that of pig samples immersed in tap water (Fig. 1*a*). Adipocere formation became manifested by the conversion of fats in the body into palmitic and stearic acids. Figure 1 indicates that at Time 0, i.e., approximately between 0 to 4 h after sampling, the presence of oleic acids was already evident (Fig. 1*b*) together with a mixture of hydroxystearic acid. This is consistent with previously reported results (4). During Week 2, there was a noticeable decrease in the level of oleic acid by about 41.72%. This is also in line with previous work in which microbial degradation of oleic acid was found to involve hydration or epoxidation of the double bonds (6,7,10).

We continued to monitor the degradation of oleic acid as well as the ratios of degradation products for about ten weeks. Figure 2 shows the chromatograms recorded for the same pig cadaver immersed in tap water at Weeks 8 and 10, respectively. During Week 4, the magnitude of oleic acid peak decreased to 11.85% while two new peaks identified as isomers of hydroxystrearic (HOXY) acids

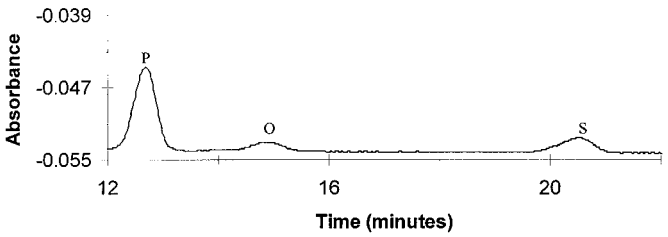


(a)

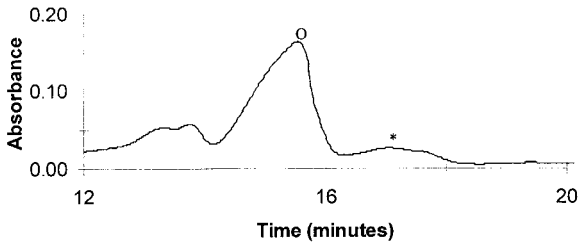


(b)

FIG. 2—*Chromatograms recorded for pig samples immersed in water showing continued fatty acid degradation of oleic acid and formation of hydroxystearic acid* (a) *Week 4,* (b) *Week 10. Other conditions as in Fig. 1.*



(a)



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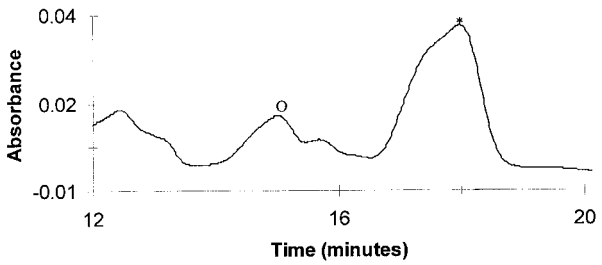

(c)

FIG. 3—*Chromatograms obtained for pig samples immersed in chlorinated water* (a) *standard fatty acids;* (a) *Week 4;* (b) *Week 10. Conditions: Zorbax RP300-C18, 4.6 × 150 mm; mobile phase: acetonitrile/H_2O (95:5): H_3PO_4 (0.1%) = 40: 60; flow rate = 1 mL/min; detection: 210 nm. AU refers to absorbance units.*

increased by a factor of 3 and 5, respectively. During Week 10, the rate of disappearance of oleic acid was peaked and this stagnated at 6.17%. In contrast, HOXY peaks increased by approximately ten. The estimated saturation index ratio recorded at Week 2 was 12.61 (i.e., hydroxystearic acid to oleic acid).

This study also includes samples immersed in chlorinated water. Figure 3 shows the chromatogram obtained when the sample was immersed in chlorinated water. As in the sample immersed in tap water, the presence of oleic acid could be observed a few hours after sampling. Moreover, we observed that, prior to the first four weeks, the hydrolysis of fatty acids was nearly nonexistent in samples that were sterilized using chlorine. This was evident from the quantitative measurement of individual fatty acid peaks. For example, during Weeks 0 to 2, the magnitude of oleic acid decreased by about 95.88% of the original size whereas during Weeks 3 to 4, a reduction of about 94.46% was obtained. This slow degradation observed in this system could be attributed to the absence and or low level of decomposing bacteria, which are capable of initiating the degradation of the fatty acids. However, immediately after Week 5, a slight increase in oleic acid decay was observed while a significant change was recorded after Week 6. At this stage, the rate of oleic acid decay was about 27.16%. In comparison with the first sample, this rate of degradation was approximately less by

about 68%. Figure 4 shows the chromatograms recorded for samples immersed in salt water. The rate of oleic acid decay in this particular sample was far less than the one recorded for Pig #1 but not as much as for Pig #2. In Weeks 2, 4, and 10, the magnitudes of oleic acid peaks were 97.52%, 96.83%, and 94.55%, respectively. At the same time, the rate of increase for hydroxystearic acid peak was less than 3% for Sample 2.

A summary of the overall rate of degradation for the major peaks recorded for the three cadavers are shown in Table 1. Figure 5 is the ratio of hydroxy fatty acid to oleic acid for samples immersed in tap water, chlorinated water, and salt water, respectively. It was observed that the hydrolysis of fats occurred most rapidly when the sample was immersed in tap water. This was followed by samples immersed in salt water, and lastly the sample immersed in chlorinated water. At Week 2, the index ratio was estimated to be 12.61, 1.26, and 1.04 for samples immersed in tap, chlorinated, and salt water, respectively, thus indicating a factor of ten between tap water sample and chlorinated sample and a factor of 12 for samples immersed in salt water. Depending on the time of death, an increase in the quantitative ratio of saturated-to-unsaturated fatty acids during adipocere formation was therefore predictable.

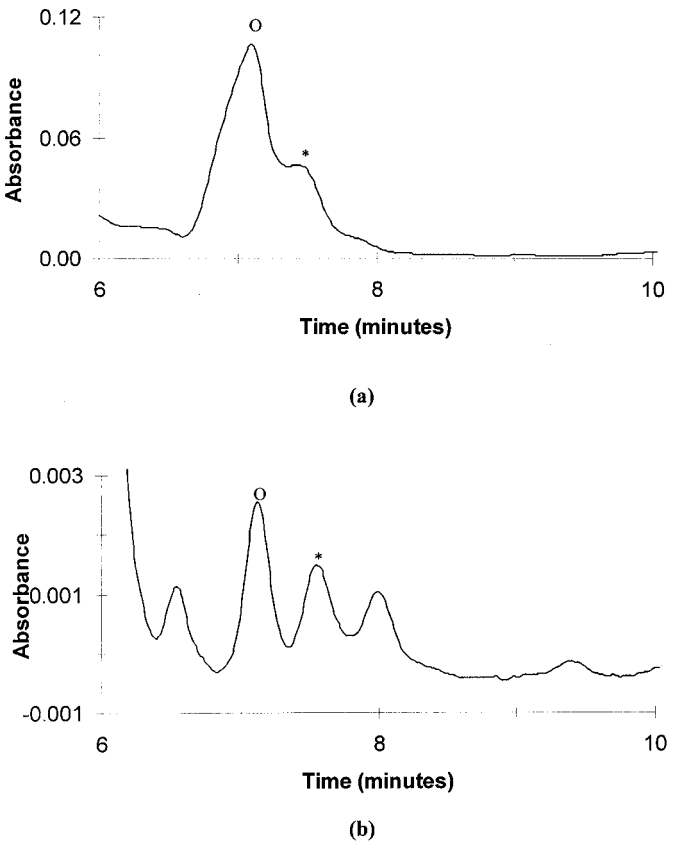

FIG. 4—*Chromatograms obtained for pig samples immersed in salt water* (a) *Week 4;* (b) *Week 10. Column: Conditions: Zorbax RP300-C8, 4.6 × 150 mm; mobile phase: acetonitrile/H_2O (95:5): H_3PO_4 (0.1%) = 40: 60; flow rate = 1 mL/min; detection: 210 nm.*

Discussion

Oleic acid (or 9-octadecenoic acid) has been shown to be a major component of adipocere formation (1–3). Some studies have shown that oleic acid could be converted to 10-hydroxystearic acid and/or 10-oxostearic acid by different microbial enzymes under aerobic and anaerobic conditions] (6,7,13–16). However, there has been no evidence about the mechanism of hydroxylation of fatty acids in the adipocere. Since a decrease in oleic acid concentration in the adipocere was observed as the hydroxy fatty acids were newly found, this suggested that oleic acid was being converted into other fatty acid derivatives as previously suggested (6,7). It appears that the mechanism of transformation of adipocere in the presence of microbes follows a similar trend that was reported for oleic acid. Using oleic acid as an indicator, the mechanism of adipocere formation should therefore follow the trend that was depicted in Fig. 6. During partial hydrogenation, some double bonds are saturated while others undergo stereomutation and/or double bond migration (11). Hydroxylation at various positions in the saturated carbon chain could result in the transformation of oleic acid into other classes of chemical products including [R-(Z)-12-hydroxycis-9-octadecanoic acid, 10-hydroxyoctadecanoic, and 10-oxo-octadecanoic acids (15,13–18).

Saturation Index and Effect of Water on Degradation of Adipocere

Results indicate that the saturation of oleic acid took place more rapidly in distilled water, and very slowly in the saline water (Table 1). This is contrary to earlier reports in which the existence of electrolytes was considered to accelerate artificial production of adipocere under the laboratory conditions (16). This discrepancy may be attributed to the difference in experimental conditions. In the present study, factors contributing to bacterial activities may include the nature of the electrolytes, ionic strength, pH, and temperature.

Chlorinated water shows a sudden increase after six weeks. It should be noted that chlorine is the most common disinfectant suitable for killing bacteria in water. The active ingredients in the chlorination process include hypochloric acid (HOC1) and hypochlorous ions (OCl^-). HOCl is approximately 100 times more effective as a disinfectant than the $OC1^-$. This is probably because the neutral HOC1 molecule can penetrate the cell membranes of microorganisms more easily than the ionic $OC1^-$. However, the ability of these chlorine derivatives to achieve a specified level of dis-

TABLE 1—*Summary of degradation trends and index ratio during adipocere formation.*

Time / Acids	Pig 1 (weeks)			Pig 2 (weeks)			Pig 3 (weeks)		
	2	4	10	2	4	10	2	4	10
Oleic Acid	41.72%	11.85%	6.17%	95.88%	94.46%	27.16%	97.52%	96.83%	94.55%
Hydroxystearic Acid	5.26	9.68	11.11	1.21	1.25	4.41	1.01	2.22	2.25
Index ratio	12.61	81.69	180.06	1.26	1.32	16.24	1.04	2.29	2.38

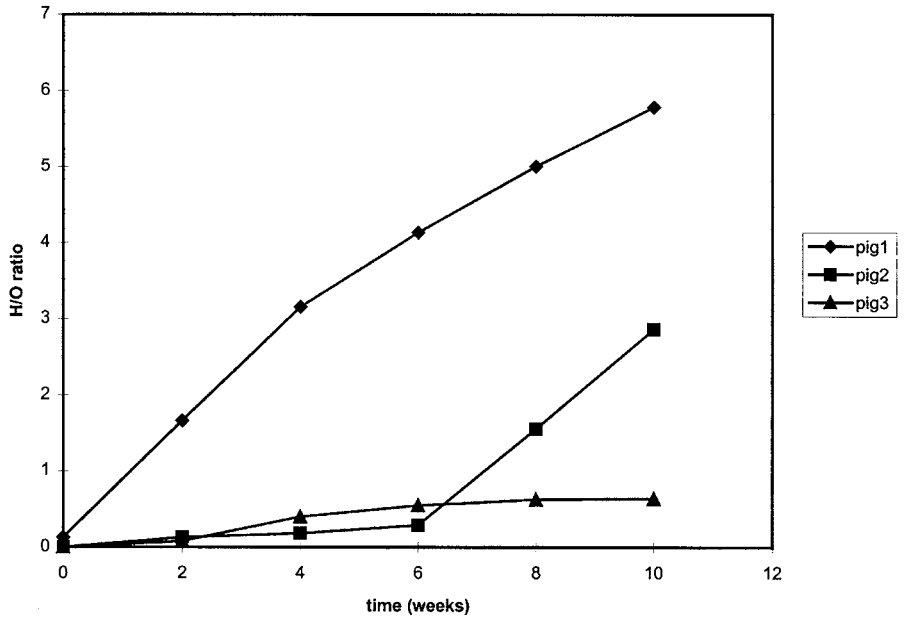

FIG. 5—*Determining the quantitative ratio of hydroxystearic acid and oleic acid for all systems investigated.*

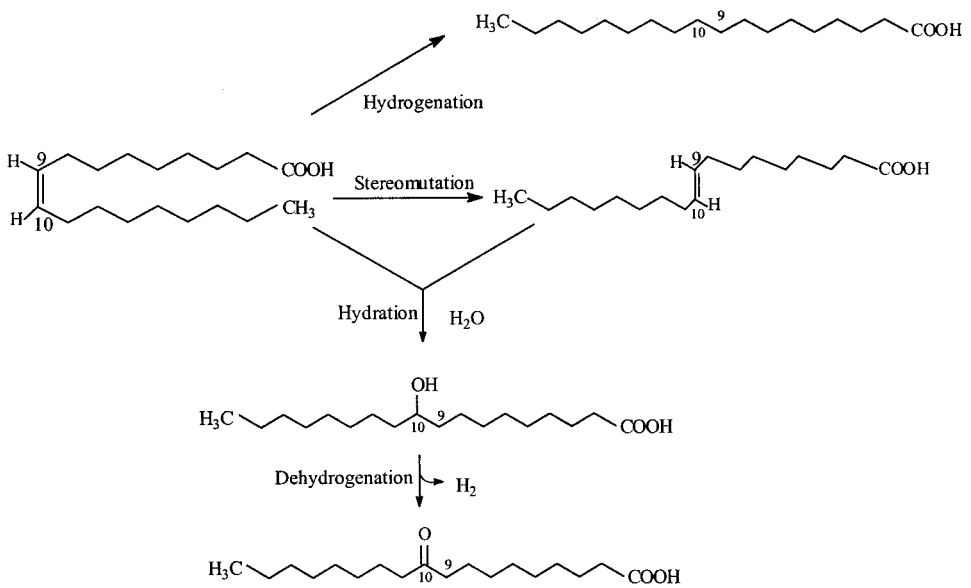

FIG. 6—*Chemistry of microbial degradation of oleic acid during adipocere formation.*

infection depends on the water, pH, and a fixed dose of chlorine. Since some of the chlorine would have been used up in destroying any of the microorganisms present as a result of the degradation process and some purely chemical reactions, the amount of chlorine in the tank is less than the total amount that was originally used. Hence this explains the delayed degradation observed in this sample.

Conclusions and Future Work

This work presents the preliminary results for the quantitative analysis of oleic acid degradation using chromatography analysis. Results obtained using pig cadavers immersed in tap water, chlorinated water, and salt water indicate that the nature of the water content may affect the rate of oleic acid degradation probably by controlling the amount of bacteria *in vivo*. We also observed that the saturation of oleic acid took place more rapidly in distilled water, but was very slow in the saline water. The difference may be attributed to the nature of the electrolytes, ionic strength, pH, and temperature contributing to the bacterial activities and hence, rate of adipocere formation. This quantitative study may represent an alternative way of measuring the fate of oleic acid during adipocere formation. This work has not in any way solved the time of death problem. But it may provide an approach to the problem that has not been adequately explored. Investigation will continue as to the influence of complexing agents, pH, and temperature on the index ratio. Future work will also involve the use of human samples to validate the ap-

plicability of this approach for determining the mechanism of adipocere formation as well as the determination of a wider range of samples beyond the visible evidence of adipocere formation.

Acknowledgments

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