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Factors Influencing the Digestibility of Solid Fats: Mammalian and Plant Lipases--Glyceride Structure and Solvent

By

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Logistical, sensory and stability constraints in the fomulation of energy-dense food packets for short-term field applications may require the use of a high proportion of solid fats. Even limited reduction in digestibility (DIG) of such fats will introduce metabolic and logistical inefficiencies and may result in gastrointestinal problems. Nonanimal assays using pancreatic lipase (PL) were used to estimate DIG. Two commercial solid fats were well digested (residual triacylgylcerols (TG) was 6-11%). As an application of this method to a specific ration item, the complete digestion of heat-resistant desert chocolate bars was demonstrated: 86% fatty acids (FA and 14% monoacylglycerols (MG). The positional isomers of distearoyloleoylglycerol (SSO and SOS) were differentially digested (TG of 36 and 71%, respectively), which is of interest because of the heightened awareness of the neutral effect of stearic acid on human cholesterol levels. The addition of 100 µL of hexane to the incubation medium (7.5 mL) greatly enhanced DIG of SOS (96%) and SSO (86%). Tristearin digestion increased four-fold with added hexane based upon FA released from an initial value of 11%.

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PREFACE

The need for stable fats in military rations that are nutritionally and physiologically adequate is well recognized. Concerns have been raised regarding the digestibility of some solid fats that were considered as candidate fats to be used in calorically dense rations for short-term military feeding. The nutritional effect of fats is commonly evaluated by animal experimentation. The closing of animal facilities at the U. S. Army Natick Research, Development and Engineering Center in 1982 provided a challenge as well as an opportunity to seriously consider nonanimal assay techniques to determine the bioavailability of fats and other nutrients.

The present study (Project No. IL16AH99BAOD00) was undertaken during the period Oct. 1989 to Jan. 1993 to develop a simple and rapid enzymatic digestion method to meet the unique reaction requirements of solid fats. In addition, the effect of various factors such as the source of lipase, the structure of the fat, and the effect of certain additives, on the in vitro digestibility of fats was examined. It is anticipated that this study would be beneficial in screening various commercial fats prior to animal and human experimentation to meet the special requirements of energy-dense foods.

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FACTORS INFLUENCING THE DIGESTIBITY OF SOLID FATS:

MAMMALIAN AND PLANT LIPASES -

GLYCERIDE STRUCTURE AND SOLVENT

INTRODUCTION

In the formulation of energy-dense foods for special military uses, whether it be by infusion into extruded porous food products or by thermal inclusion into new fabricated foods, it may be desirable to use a high proportion of semisolid and solid fats because of demanding logistical, sensory, and stability constraints under extreme environmental conditions. A case in point was the requirement during the Persian Gulf war that desert chocolate bars remain solid and not melt into a viscous, sticky mass under the prevailing high temperatures. In addition to preserving food values, it was important that such melt be avoided in order not to obstruct the gun mechanism or its sight.

Since hydrogenation is frequently used to solidify liquid fats, some amount of simple and mixed triacylglycerols (TGs), such as tristearin, distearoyl olein, dipalmitoyl olein, and palmitoyl oleoyl stearin and mixed TGs containing solid trans fatty acids (FAs) may be present. Early work of Calloway et al.[1] with fully hydrogenated corn, cottonseed, soy bean, palm oils and lard (m.p. 61-68° C) demonstrated that they were poorly digested (12-24%) by rats. The incomplete digestion (65%) by rats of cocoa butter (m.p. 36° C), which consists predominantly (50%) of a TG containing palmitic, stearic, and oleic acids and of 18% of oleoyl distearin, has been reported by Apgar et al.[2] On the other hand, normal unhydrogenated fats and oils are almost completely digested (97-99%) by rats.

Mattson[3] has demonstrated that it is the tristearin content rather than the stearic acid content or the melting point of fats that shows an inverse correlation with digestibility in rats. However, on the basis of random rearrangement of a mixture of hydrogenated linseed oil (I.V. 3) and safflower oil, it is also concluded by Mattson that distearoyl monounsaturated TGs are completely absorbed by rats. Since the safflower oil used consisted of 78% linoleic acid, this distearoyl monounsaturated TG would more likely be distearoyl monolinolein rather than distearoyl monoolein. Except for one subsequent article by Mattson et al.[4] and the early studies of Mattil and Higgins[5] on the digestibility by rats of various TGs of oleic and stearic acids, there is limited literature on the utilization of relatively high melting TGs containing stearic and oleic acids.

There is considerable concern regarding the need to minimize animal experimentation not only from the standpoint of humane treatment of animals but also to develop rapid, cost-effective and efficient screening procedures. However, it has to be recognized that nonanimal assays can provide only partial answers: for example, in these in vitro experiments, while information can be obtained upon digestibility under specified conditions, conclusions on absorbability will have to be deduced from animal experiments.

The specific objectives of this work were: a) to modify enzymatic procedures to meet the unique reaction requirements of solid and semisolid fats; b) to assess the suitability of commercially available solid fats for use in specific military ration components; c) to gain a better understanding of the relationship between TG structure and in vitro digestibility so as to recommend fats of specific structure to meet the special requirements of energy-dense foods.

METHODOLOGY

The fats were digested with pancreatic lipase (Int. Biochemicals Tech., Madison,WI) at pH 8.0 in 7.5 mL of 1M ammonium chloride-ammonium hydroxide buffer containing 0.27 M calcium chloride and 0.073 mM sodium deoxycholate for 2 h. The procedure was an adaptation of the method of Mattson and Beck.[6] A small sample quantity (25 mg) and a high ratio of enzyme to substrate (1400 USP units/25 mg) were observed to maximize the rate of the reaction and were routinely used in these studies. The reaction was stopped with an excess of 1N HCl and the mixture was extracted with chloroform. The lipid components in the extracts were resolved on TLC plates coated with silica gel GHL (Analtech, Newark, DE) using hexane-diethyl ether-acetic acid (80/20/1, v/v/v) and quantified, after charring with sulfuric acid, using a Schoeffel densitometer coupled to a Waters 930 Data Module.[7] All values are mean of four replicate determinations. Where appropriate, the standard Student's *t*-test was used to assess the significance (expressed as probability (*p*) values) of differences of the group means. Fatty acid compositions were determined in a Perkin Elmer Gas Chromatograph. The glyceride and fatty acid components, resolved by preparative TLC, were reacted with 14% boron trifluoride in methanol to convert them to methyl esters.[7,8]

The synthetic triacylglycerols were purchased from Sigma Chemical Co. (St. Louis, MO). The method of synthesis provided some assurance as to position of the fatty acids in the TG. The purity of these compounds was evaluated in three ways: 1) by TLC to ensure there were no fatty acid, mono or diacylglycerol or other contaminants; 2) by GLC to ensure that the fatty acid compositions were close to the calculated values; 3) by pancreatic

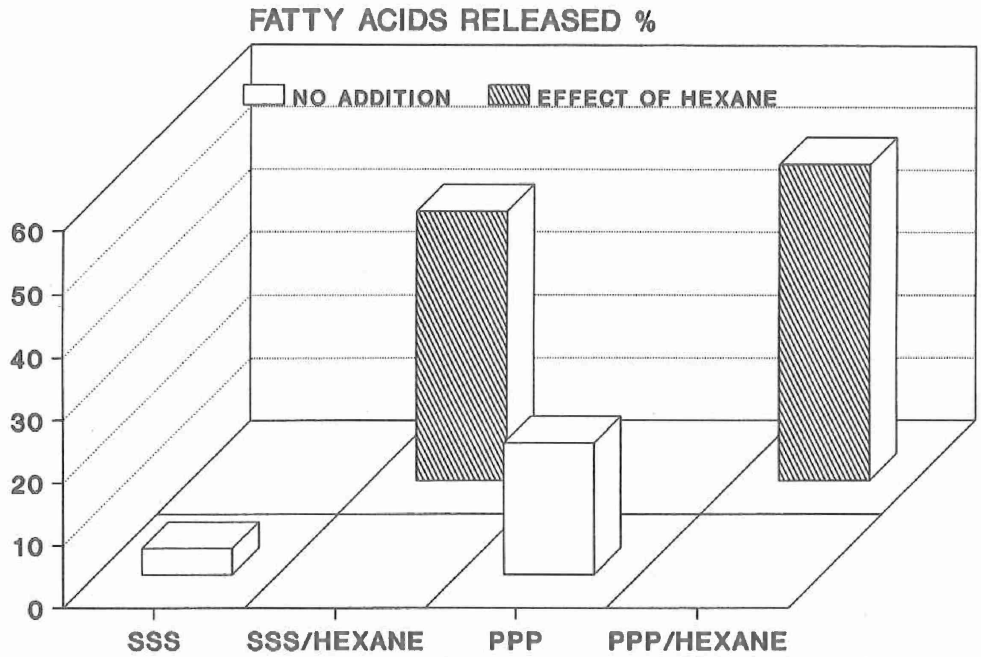

Figure 1. Lipase digestion of tristearin (SSS) and tripalmitin (PPP) with and without hexane

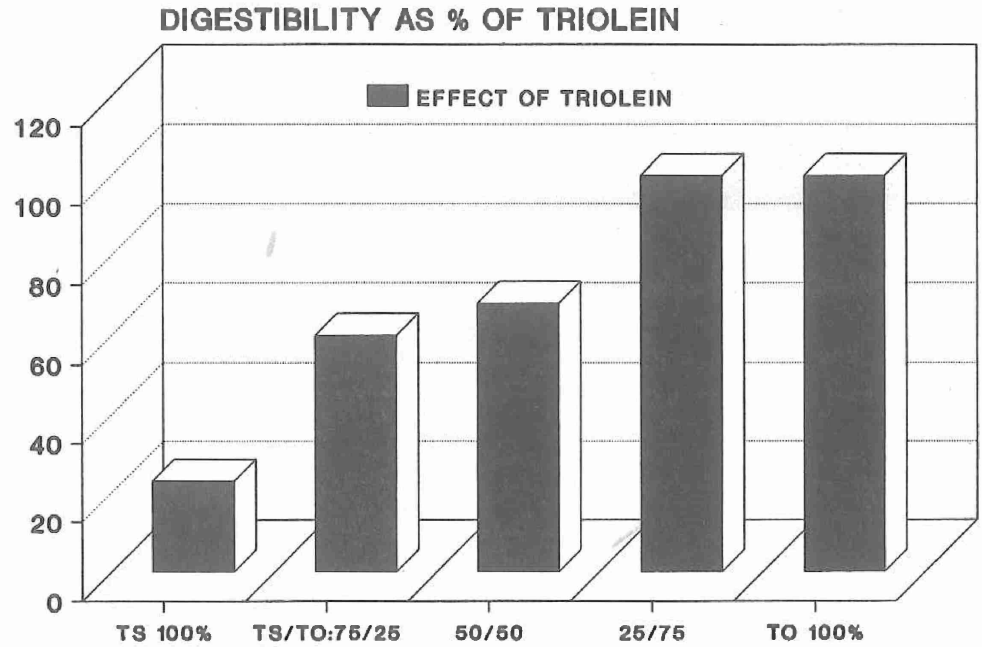

Figure 2. Digestion of tristearin/triolein mixtures by microbial lipase

lipase (PL) digestion to ensure that the predominant FA in the monoacylglycerol component was the fatty acid in the 2-position of the TG.

RESULTS AND DISCUSSION

Pancreatic lipase digestion of tristearin and tripalmitin with and without hexane

Since the purpose of this investigation was to assess the digestibility of solid fats, it was decided to tackle first the most resistant solid fats (tripalmitin and tristearin) with high m.p. (65 and 73° C, respectively). Consistent with other data in the literature, it was observed that both tristearin (TS) and tripalmitin (TP) were poorly digested by PL even under our ideal experimental conditions. Cheng et al.[9] observed that TS and TP were poorly digested (19 and 28% respectively) by rats. The low solubility of high melting point fats is generally considered to be a major reason for their poor digestibility. An attempt was made to enhance their solubility by the addition of a solvent. It has been reported that solvents miscible with water may enhance lipase action either by influencing the conformation of the enzyme or the substrate micellar structure.[10] Polar solvents, such as methanol or ethyl alcohol, were ruled out because of possible denaturing effects. Hexane was selected since it was considered safe from the standpoint of enzyme activity and because it would partially enhance the solubility of the fats. To our surprise, this solvent was very effective (Fig. 1) even when a small volume (0.1 mL) was added to the incubation medium of 7.5 mL total volume. However, it has to be conceded that this finding is primarily of theoretical interest at this time.

A comparison of the digestion of tristearin/triolein mixtures using microbial and mammalian lipases

Plant lipases are known to differ from mammalian lipases with respect to their lack of specificity of the acyl position in the TGs that are cleaved. In this experiment, a microbial lipase of fungal origin (*Rhizopus arrhizus*) was tested for its ability to digest TS and several mixtures of TS and triolein (TO). While only 20% of the TS was digested to glycerol and fatty acids (FAs), the addition of TO resulted in increased digestion of TS. At a ratio of 25/75 of TS/TO, all of TS was digested to the same extent as TO (Fig. 2).

With pancreatic lipase (PL), however, there was no beneficial effect (i.e., no increase in the digestibility of tristearin) of adding TO even when a ratio of 25/75 (TS/TO) was used (Fig 3); this finding is in agreement with the in vivo data of Mattson concerning the digestibility of TS. Whether there is an effect at lower ratios such as 10/90 or 5/95, which may be of some importance in obtaining desired fat consistencies, remains to be determined.

Pancreatic lipase digestion of cocoa butter and palmitoyloleoylstearin

Contrary to the results obtained in vivo by Apgar et al.[2], only 7% of the TGs of cocoa butter remained undigested during in vitro digestion by PL (Fig 4). There were about 6% diacylglycerols (DGs) and 3% of monoacylglycerols (MGs) in the digest. The synthetic TG, 1-palmitoyl-2-oleoyl-3-stearin (POS) was fully digested (1% TG).

Pancreatic lipase digestion of the the fats extracted from heat-resistant desert chocolate bars from two commercial sources

It was important to establish whether the fats in the heat-resistant

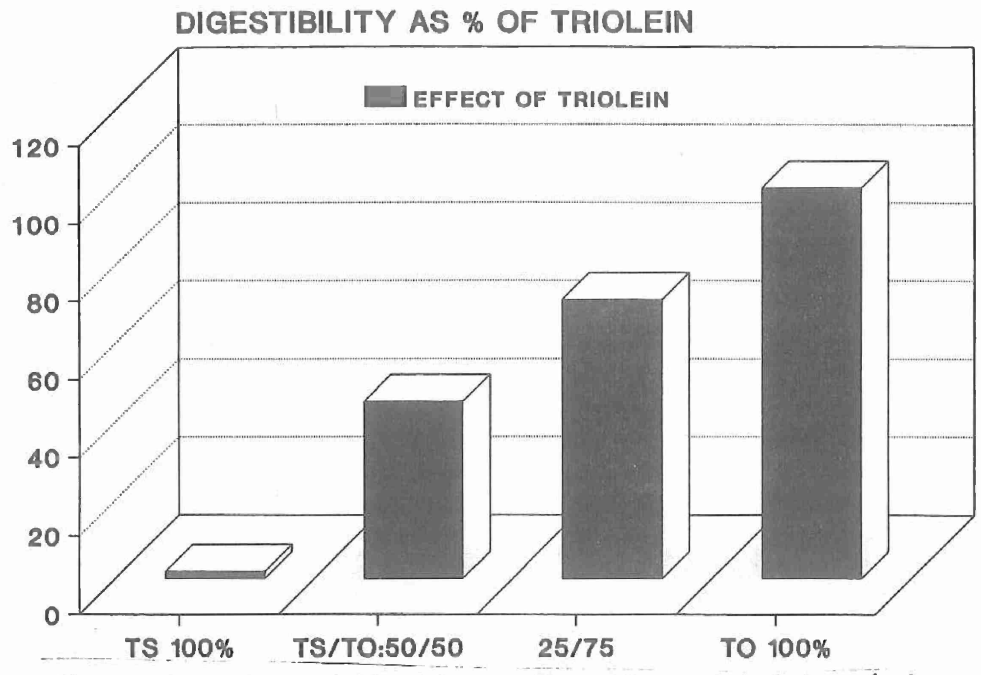

Figure 3. Pancreatic lipase digestion of tristearin/triolein mixtures

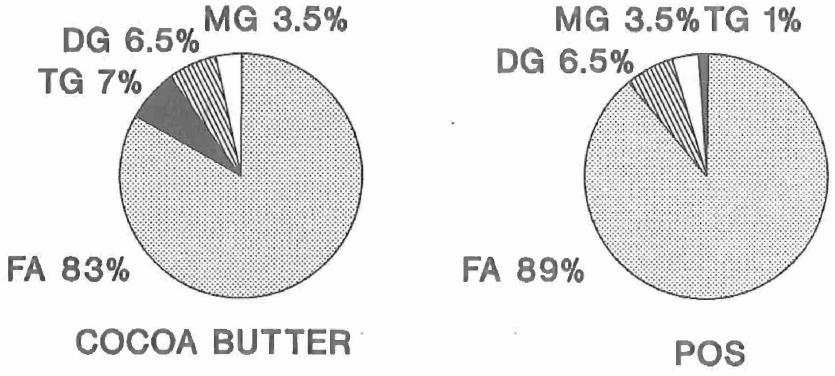

Figure 4. Pancreatic lipase digestion of cocoa butter and 1-palmitoyl-2-oleoyl-3-stearin (POS)

desert chocolate bars (HR-DB) used in the Persian Gulf war were biologically available. The fats, extracted from HR-DBs from two commercial sources, were observed (Fig. 5) to be well digested (86% FA and 14% MG). The FAs in these fats were essentially those of cocoa butter and consisted primarily of 25% palmitic, 33% stearic, 33% oleic and 3% linoleic acid.

Pancreatic lipase digestion of corn oil and a solid fat from a commercial source

Corn oil (Fig. 6) and triolein (data not shown) were fully digested with little or no remaining TG (less than 1%). On the other hand, the digestion of cocoa butter indicated there was 7% of residual TG under the in vitro conditions used here (Fig. 4). Another representative solid fat (Fig. 6), with a m.p. of 38° C,which was also tested under these conditions was well digested, but it had a residual TG of 11%.

Pancreatic lipase digestion of 1,3-dioleoyl-2-stearin and 1,2-dioleoyl -3-stearin

The PL digestion of two isomeric liquid TGs, 1,3,-dioleoyl-2-stearin (OSO) and 1,2-dioleoyl-3-stearin (OOS) showed that only 4-5% of residual TG was left after 2 h digestion (Fig. 7). The amount of undigested DG (10%) in OOS was greater ($p < 0.025$) than in OSO (5%). The fatty acid profiles of OSO and OOS indicated that their undigested MGs contained exclusively stearic and oleic acids, respectively, as would be expected.

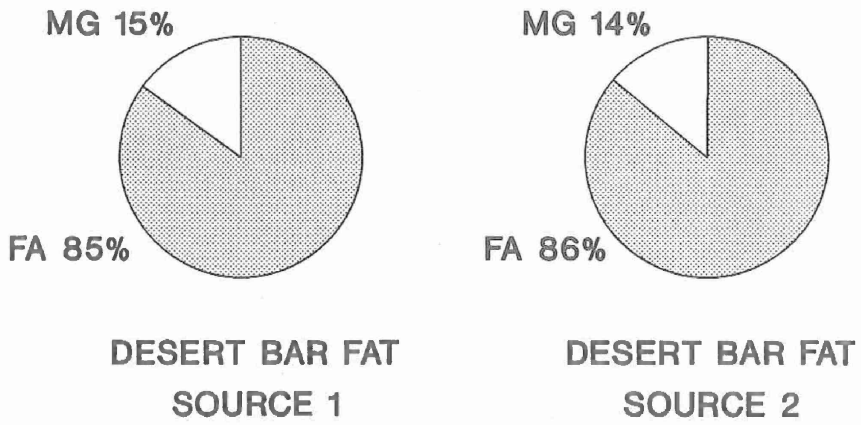

Figure 5. Pancreatic lipase digestion of the fats extracted from desert chocolate bars from two commercial sources

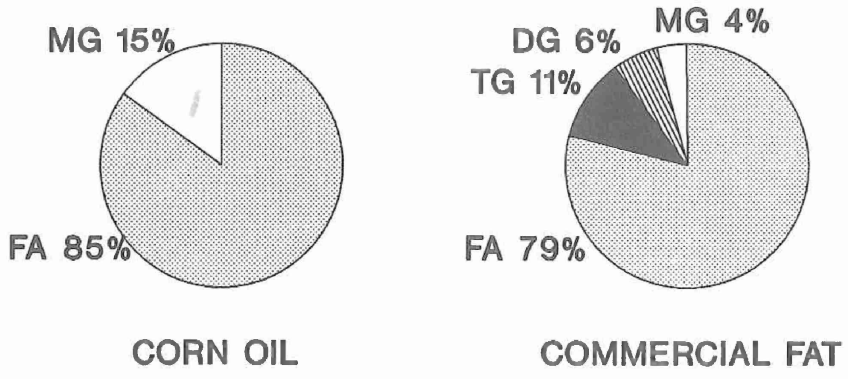

Figure 6. Pancreatic lipase digestion of corn oil and a commercial solid fat

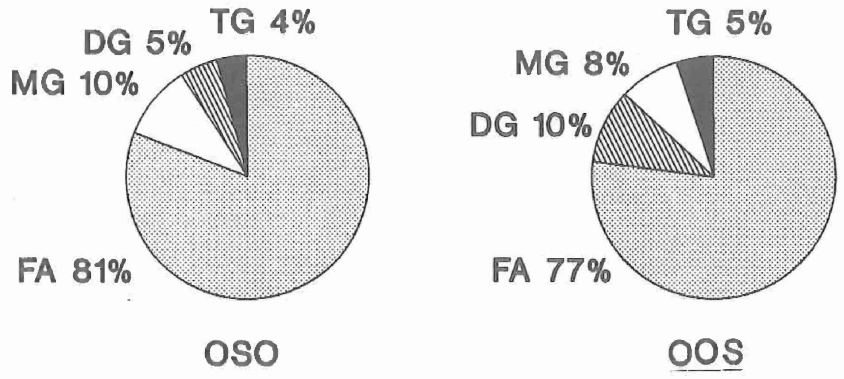

Figure 7. Pancreatic lipase digestion of 1,3-dioleoyl-2-stearin (OSO) and 1,2-dioleoyl-3-stearin (OOS)

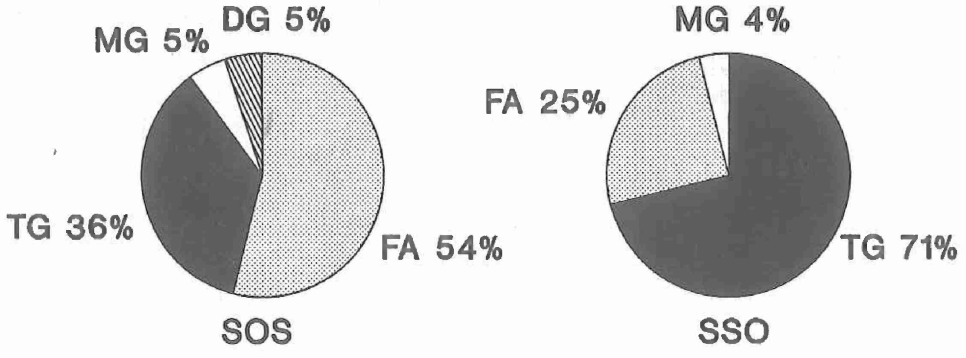

Figure 8. Pancreatic lipase digestion of 1,3-distearoyl-2-olein (SOS) and 1,2-distearoyl-3-olein (SSO)

Pancreatic lipase digestion of 1,3-distearoyl-2-olein and 1,2-distearoyl-3-olein

When we consider the digestion (Fig. 8) of the two isomeric distearoyl monooleins, (m.p. 44°C) there is a large difference ($p < 0.001$) in the residual TG, 71% in the case of 1,2-distearoyl-3-monoolein (SSO), as opposed to 36% in the case of 2-oleoyl-1,3-distearin (SOS). Mattson et al.[4] have also reported intermediate rat digestibility values for the stearate component of SSO and SOS (60 and 70%, respectively), which is to be contrasted with the digestibility value of nearly 100% for OSO. However, the differences that they observed between SOS and SSO in vivo were less dramatic than seen here in vitro. Colon bacteria are known to metabolize peptides, fibers, di- and trisaccharides. It is possible that some of the saturated fat that is undigested in the small intestine may be metabolized by colon microorganisms before it is excreted. In such an event, absorbability based upon fecal excretion would be overestimated.

The distribution of FAs in the digested products shows that stearic and oleic acids are the predominant FAs in the undigested MGs of SSO and SOS. The lack of complete digestion of the TG remnants is difficult to explain except on the basis of the time frame of the experiments. These remnants were not greatly different from their parent compounds except that they were slightly enriched in oleic acid in the case of SOS and that stearic acid was slighly more abundant in the case of SSO than would be expected. A speculative explanation is that there is some acyl exchange during lipolysis. It has been reported that some acyl wandering occurs during catalytic hydrogenation under certain conditions and provides some support for this possibility.

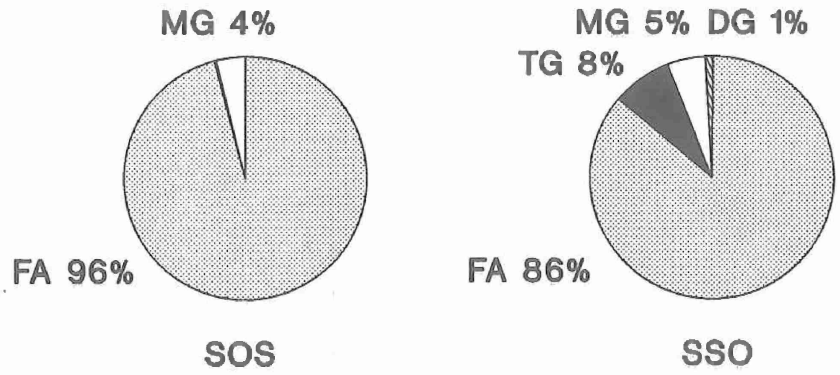

Figure 9. The effect of hexane on the digestion of isomeric distearoyloleins (SOS & SSO)

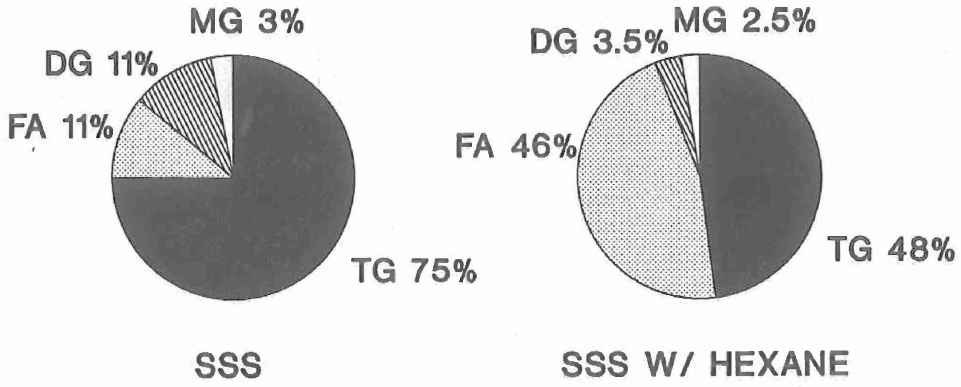

Figure 10 Confirmation of the effect of hexane on the digestibility of tristrearin: (SSS) Quantification of the digested components

The effect of hexane on the digestibility of isomeric distearoyl oleins

As mentioned earlier, our working hypothesis has been that in order for the enzyme to react with solid fats, which are poorly solubilized by deoxycholate, increase in fluidity of the substrates would aid the digestibility. It was hoped that hexane would either aid in better displaying the substrate to the enzyme or would facilitate the solubilization of the fat by deoxycholate micelles. The almost complete digestion (Fig. 9) of SOS (4% TG) and SSO (8% TG) by the addition of hexane appears to support this possibility, although other explanations such as changes in enzyme conformation cannot be ruled out.

The effect of hexane on the digestibility of a hard fat like tristearin

The remarkable increase ($p < 0.001$) in the digestibility of tristearin (SSS) by the addition of 0.1 mL of hexane to the incubation medium was confirmed in this experiment (Fig. 10). The stearic acid released increased ($p < 0.001$) dramatically (4-fold). This increase may, in part, be ascribable to the increase in fluidity of the fat, which makes it easy to interact with the enzyme. On the other hand, it is possible that the solvent merely dissolves a small amount of the fat and facilitates the continuous partitioning of it into the aqueous phase and thereby permits its solubilization into deoxycholate micelles.

CONCLUSIONS

The enzymatic assay provides a simple and rapid means of evaluating the digestibility of liquid, semisolid and solid fats. The application of this method to liquid fats, such as corn oil, triolein, OSO, and OOS, as well as to semisolid and solid fats, such as cocoa butter, commercial solid fats, the fats in heat-resistant desert chocolate bars, POS, SOS, SSO, PPP and SSS (in the range of m.p. of 38 to 73° C) is indicative of its utility and versatility. That fatty acid composition or the m.p. alone cannot predict digestibility is clearly seen from the data on SOS and SSO. However, it is not advocated that in vivo experimentation be ignored.

From the literature, it is known that 2-monoacylglycerols of stearic and other FAs are well absorbed. The absorption of the free acids, palmitic and stearic, is diminished by the presence of dietary calcium and magnesium due to the formation and excretion of insoluble soaps. The enhancement of the lipolytic reaction of PPP, SSS, SOS and SSO by the addition of a small amount of hexane is primarily of theoretical interest but it may have diverse practical ramifications (e.g., splitting of high melting fats with plant lipases, production of 2-monoacylglycerols and other specific synthetic chemical reactions using enzymes where two phase-systems are desirable). Finally, the high digestibility of SOS opens up the possibility of utilizing this mixed TG in food applications. It is recognized that stearic acid has no effect upon human plasma cholesterol levels, unlike hydrogenated coconut oil (HCO),[11] currently used in dairy products and beverage powders. Fats containing high levels of SOS can replace HCO in many foods where there is need for long-term stability without attendant deleterious consequences to health.

RECOMMENDATIONS

It is recommended that:

1. The pancreatic lipase digestibility method developed here be used as a routine screening method prior to animal and human experimentation to determine the in vitro digestibility of fats to be included in military foods.

2. Further studies be carried out with food-grade emulsifiers, such as gum acacia, lecithin, etc., to determine whether the digestibility of distearoyl stearins can be enhanced.

3. A search be instituted for other natural fats, such as shea fat, isolated from the kernels of the African plant, Butyrospermum parkii, that are abundant in SOS or SSO with the objective of including them in shelf-stable foods.

4. A simple method for synthesizing SOS and SSO be developed avoiding the use of nonGRAS chemicals or reagents.

5. Studies be conducted in collaboration with U.S. Department of Agriculture to determine the specific effect of SOS and SSO upon human cholesterol metabolism.

6. Investigate the possibility of using time-release encapsulated immobilized plant lipases as possible adjuvants in the utilization of difficult to digest solid fats.

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