USE OF STABILIZED TALLOW-NUTRIENT AGAR EMULSION MEDIUM IN STUDYING LIPOLYTIC BACTERIA

by

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INTRODUCTION

If two immiscible liquids: e.g., tallow and nutrient agar, are put together and agitated violently, it will be noted that one is dispersed in the other. If the agitation is stopped, the particles coalesce and the two liquids separate and form two distinct layers. If it is possible to reduce sufficiently the interfacial tension between the two liquids, an emulsion will be formed which may be stable.

Soap and many synthetic products are surface-active agents. Structurewise, too, they are fundamentally similar. All surface-active agents are characterized by having a molecule which carries a hydrophobic (water-hating) hydrocarbon tail and a hydrophilic (water-loving) head. When a detergent, such as Arctic Syntex A, Duponol LS flakes, Igepal CA, Igepon AP, or Igepon T, dissolves in water the surface molecules are oriented so that their cationic heads point toward the water and the hydrocarbon tails away from it. Thus, the hydrocarbon tails are in a position to attach themselves to oil or hydrocarbon materials such as dirt. In the case of soaps the attachment for the dirt is strong enough to permit it to be emulsified and carried away in the rinse water.

Just as the detergency of soap depends to a considerable extent on the oils from which it is derived and the nature of the cationic head, so also are the properties of the synthetics related to the length and type of hydrocarbon chain and by the nature of the cationic head. When the hydrophobic tail of a

detergent gets out of balance by becoming too long or too short or internally modified, detergency decreases and the material is best suited for non-detergent purposes. Thus, compounds not capable of holding hydrophobic materials in suspension become wetting agents. Unfortunately, no hard and fast rule can be laid down to distinguish between true detergents and wetting agents, for under different conditions of use a product which is normally a wetting agent might become a detergent, and vice versa.

Santomerse 3, Triton W-30, and Victawet 58-B are surface-active agents that depress surface tension, but they will not emulsify. Santomerse 3 is really a wetting agent, but some investigators considered it to be a detergent.

By systematically modifying either or both "hydro-groups", the over-all characteristics of a surface-active compound can be changed so that a detergent may be changed into an emulsifier. For example, the group of surface-active agents known as alkyl aryl sulfonates is often thought of as comprising detergents only, but various modifications give a wide range of properties, and some products exhibit low detergency and high emulsifying action.

Generally speaking, a small ring structure, such as benzene, coupled with a single long alkyl chain of 12 to 18 carbon atoms gives the best detergents; examples are Nacconol NR and Santomerse 1. A larger ring structure such as naphthalene, or a plurality of short alkyl chains tend to reduce detergency and increase the emulsifying power; examples are Oronite 5 and Nekal BX.

Aresklene 400, Tergitol 4, 7, and 08, Triton X 400, Span 80

and Tween 80 are also examples of emulsifying agents. These emulsifying compounds are employed in this thesis.

Through the experience of earlier workers one notes that several factors should be considered in making emulsions. First, it is important to choose a suitable emulsifying agent. Also, the purer the materials used in an emulsion the less stable will be the emulsions. (It has been observed that, for pure oil and water, 2 percent of oil is the maximum amount so far that has been emulsified in water.) At the present time our knowledge of the actual mechanism of colloidal solubility for emulsions is sufficient to account for the fact that, in general, pure two-phase systems seem to be stable only over low concentration ranges. A more concentrated emulsion demands the presence of a third substance which is capable of forming an absorbed film at the oil/water boundary.

To prevent the dispersed phase from coming in contact with itself and thus collecting as a separate liquid, it is advantageous to add the emulsifier to a small part of the phase in which it is soluble, thus producing a concentrated solution, then adding all of the second phase (the dispersed phase) with constant vigorous agitation.

Another factor which should be discussed has to do with inorganic salts which are sometimes present in the water used. If "hard" water is used, calcium and magnesium salts are present. These and other compounds are detrimental to the stability of the emulsion. In fact, some emulsions are sensitive to electrolytes.

In cases like this it is best to use distilled water for making up such solutions.

Agitation plays an important part in the preparation of emulsions. It should be emphasized that the greater the agitation for a given set of conditions, the better the emulsion produced and the smaller the amount of emulsifying agent required. This is due to the fact that increased agitation breaks up the dispersed phase into smaller particles.

Such factors as pH, temperature, and the amounts of ingredients to be used in preparing the emulsion should also be considered.

The method for detecting lipase production by bacteria was apparently originated by Eijkmann (1901). This technique consists in covering the bottom of a Petri dish with sterile tallow, chilling, and pouring over this a thin layer of liquid, cooled agar. Upon this medium bacteria are planted. Any diffusion of lipase from the bacterial colonies becomes evident by a formation of white, opaque zones in the tallow adjacent to the colonies. Apart from the importance of the fat-hydrolyzing enzymes in nature, they are industrially important because of their action in rendering butter, milk, tallow, and other fatty products rancid, and also they are of interest in medicine because of their action upon fats in the intestinal canal.

This research was initiated in an effort to produce a stabilized emulsion of tallow and nutrient agar by the use of a surface-active agent, which would be neither toxic to the bacteria nor

inhibitive to lipase activity. Such a preparation might be a good medium both for demonstrating lipolytic bacteria and for making plate counts. The Eijkmann technique does not provide a satisfactory method for making counts of lipolytic bacteria.

REVIEW OF THE LITERATURE

The past 15 years have witnessed many rapid developments in the types and number of surface-active agents available, and as a result new markets and uses have been cultivated. Their uses have even extended into the fields of medicine and bacteriology, and it is the relation of surface-active agents to the latter that is of particular interest here.

It has been known for a long time that ordinary soaps have certain disinfectant properties. The development of surface-active germicides is relatively new. Hartmann and Kaegi (1928) reported that their synthesized surface-active cations were germicidal.

The use of surface-active agents for some sterilizing procedures, in place of chlorine, was demonstrated by Scales and Kemp (1938). They pointed out that wetting agents spread much more easily over a greasy surface and had the advantage of being non-corrosive to metals.

The cleansing and sterilizing of ureteral catheters with a 12½ percent solution of Tergitol 08 was suggested by Winer and LaCava (1940). They found that such a solution was powerful

enough to kill the tubercle bacillus.

Surface-active agents have also been used, by Guteras and Shapiro (1940), for sanitizing eating utensils. They concluded that anionic agents are superior to both cationic and non-ionic as detergents. However, the cationic agents were found to be superior as germicides.

The germicidal superiority of some surface-active agents to sodium hypochlorite, when used in the acid range, was demonstrated by Scales and Kemp (1941). A 30 minute exposure to a surface-active agent at 65° C. and at a pH 4.0 destroyed Bacillus subtilus* spores.

Petroff and Schain (1940) carried out phenol coefficient tests with a long list of wetting agents alone, and in conjunction with standard bactericides such as phenol, chloramine T, azochloramid, and merthiolate, and found that the bactericides killed in much higher dilutions when combined with dilutions of wetting agents, as high as 1 to 5000 and 1 to 10,000. In the treatment of empyemas they used dilutions of azochloramid 1 to 3,300 mixed with 1 to 2000 Tergitol 4 for irritations and found the results encouraging in the treatment of mixed infections of staphylococcus, streptococcus, and Pseudomonas pyocyaneus.

The extra-ordinarily high germicidal power of dodecylamine hydrochloride was observed by Domagk (1935). A 1 to 10,000 dilution of this compound killed pathogenic Micrococcus pyogenes var. aureus and Salmonella typhosa organisms.

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*All nomenclature is in conformance with Bergey's Manual of Determinative Bacteriology, 6th. Edition.

The influence of surface-active agents on the growth of acid-fast bacteria was studied by Larson, Cantwell, and Hartzell (1919), Larson (1921-22), Larson and Montank (1922-23), Cooper (1946), and Alexander and Soltys (1946). They all reported that the chemical nature of the compound was of minor importance in comparison with its effect upon surface properties as measured by surface tension. In general as the surface tension is progressively reduced below 40 dynes/cm, bacterial growth is increasingly inhibited, until below 28 to 30 dynes/cm no growth at all is observed.

As to the amount of surface-active agent necessary to kill bacteria, Valko (1946) concluded that bacteria, with combining weights similar to those to protein, are killed with 1/50 to 1/100 their weight of suitable cationic detergents and, at most, 1/10 to 1/15 their weight of anionic detergents. It was estimated that the killing of Micrococcus pyogenes var. aureus occurs with about 1/20 as much detergent as the cells are able to absorb.

Gershenfield and Milanick (1941) reported that the bacteriostatic action of surface-active agents depended upon the following factors: (1) whether the compound was anionic or cationic; (2) the specific nature of the organisms, whether Gram-positive or Gram-negative; and (3) the hydrogen ion concentration of the environment. The anionic depressants exhibited their greatest efficiency in the acid range; therefore, the lower the pH, the greater the efficiency. The cationic depressants exhibited their greatest efficiency in the alkaline range, and the higher the pH the greater the efficiency.

The nature of the bactericidal action of surface-active agents was studied by Baker, Harrison, and Miller (1941). These authors proposed a two-fold action as a working hypothesis of the bactericidal mechanism. First, a disorganization of the cell membrane by virtue of the surface-activity of the agents; and, secondly, a denaturation of proteins essential to metabolism and growth.

Probably the initial process in the action of surface-active ions on bacteria is their reversible absorption by, or in combination with, the bacteria. McCalla (1940) demonstrated the ability of bacteria to enter into ion exchange.

It was reported, by Valko and Dubois (1944), that for a brief period of about five minutes after treatment with a cationic agent, the killing of bacteria was reversed by lauryl sulfate, an anionic agent. Their interpretation of this effect is that the oppositely charged agents can interact and accordingly reverse the absorption of cationic surface-active agents upon negatively charged groups within the cell. The absorption of the cationic agents would otherwise rapidly have caused the death of the cells.

The susceptibility of Gram-negative bacteria to anionic detergents was reported by Miller, Abrams, Dorfman, and Klein (1942) to be increased by protamines. This increased susceptibility was possibly due to the interaction with a natural inhibitory substance, similar to the phospholipids.

Glassman (1948) supported the hypothesis of Baker, Harrison, and Miller (1941) of a possible disruption of some cellular membrane component (a mechanism reminiscent of that associated with red blood cell hemolysis) with a consequent increase in permeability.

With this mechanism, intracellular constituents such as enzymes, ions, coenzymes, and metabolic intermediates would be released to the surrounding medium by the lytic action of the surface-active agents. This dilution of the intracellular contents would reduce to a low level the metabolic activity observed with no effect to be expected upon addition of more surface-active agents until the concentration was high enough to interfere by denaturation and inactivation of the enzymes present.

Ever since the introduction of synthetic surface-active agents, several observations of their hemolytic properties have been recorded. Hess and Sullivan (1943) and Ponder (1947) found that the hemolytic property of an homologous series of anionic compounds varies with the carbon chain length. A chain length of 14 carbon atoms gave optimal efficiency in the sulfonated alcohols, and a 1 to 100,000 dilution was lytic within a few minutes. It was also noticed by Ponder, that at sub-lytic concentrations, surface-active agents still produced changes as evidenced by the alteration of the erythrocyte shape from discoidal to spheroidal. These disk-to-sphere transformations may be produced at surface-active agent concentrations only 1/10 of that necessary for hemolysis. Ponder believed that surface-active agents form complexes with the lipid, lipoprotein, and protein components of the erythrocyte ultrastructure as a stage in the hemolytic process.

Similar ionic surface-active compounds were found by Glassman (1948) to have widely different hemolytic activities, and certain non-ionic compounds were found to be non-hemolytic at concentrations

which demonstrate surface tension depressing properties equivalent to those of anionic and cationic compounds which are intensely hemolytic.

It was reported by Gale and Taylor (1946) that whenever the nature and concentration of the surface-active agents were adequate to be bactericidal a leakage of nitrogen and phosphorus compounds from the cells was observed. Determinations of the release of lysine from Streptococcus faecalis showed that surface-active agents (tyrocidin, CTAB, and Aerosol OT) liberated lysine proportionate to the concentration of the surface-active agent used, up to a level that was sufficient to liberate the total lysine of the bacterial cells. Non-surface-active bactericides (penicillin, acriflavin, and "sulfa" drugs) did not exhibit this effect.

Precipitation and denaturation of proteins by surface-active agents was demonstrated again and again by many workers. Precipitation according to Putnam and Neurath (1943) depended upon such factors as the length of the paraffinic carbon chain of the surface-active agent, the pH, the mass ratio of the surface-active agent to proteins, temperature, and the ionic strength.

Under constant conditions of temperature, acidity, and concentration, McMeekin (1942) found that the relative effectiveness of alkyl sulfate and sulfonate, as precipitating agents for proteins, increased in the homologous series until a maximum of precipitate per mole is reached. Further addition of methylene groups to the molecule was without effect.

With surface-active agents of appropriate length carbon chain, Kuhn and Bielig (1940) reported that proteins were precipitated by surface-active cationic compounds only if the former were present as anions.

The effect of pH as a factor influencing the precipitation of proteins by surface-active agents was studied by Schmidt (1942) and Putnam and Neurath (1944). The mass ratio of the surface-active agent to protein, which is another decisive factor, was previously studied by Putnam and Neurath (1943).

Even though Baker, Harrison and Miller (1941) and Kuhn and Bielig (1940) considered the ability of surface-active agents (at concentrations of the same order and magnitude as were bactericidal) to denature proteins as a possible mechanism of their antibacterial activity, this view was challenged by Hotchkiss (1946) on the basis of the quantitative differences in the mass ratio of protein to surface-active agent required for denaturation of ordinary proteins as compared to bactericidal effects.

As for the effects of surface-active agents on enzymes, it has long been known that surface-active agents inhibit the proteolytic activity of trypsin. This was recently confirmed by Peck (1942) using crystalline trypsin and pure soap.

Marron and Moreland (1939) found very little effect of Alphasol LA, an anionic detergent, on urease; but using a cationic surface-active agent, Kuhn and Bielig (1940) reported that catalase is precipitated and completely inactivated in weakly alkaline medium by 0.33 percent of the agent.

It was demonstrated by Freeman, Burrill, and Ivy (1945) that complete inhibition of enzymatic activity of lipase, amylase, and pepsin could be effected by an anionic surface-active agent (an alkyl-aryl sulfonate, not further identified), while partial inhibition was obtained with phosphatase and trypsin. It is interesting to note that complete inhibitions require solutions of only 0.001M or less surface-active agent.

Some surface-active agents are also known to support growth. Williams, Broquist, and Snell (1947) showed that media with the addition of certain water soluble emulsifying agents and surface tension depressants (such as Tween 40), which are inactive by themselves, render oleic acid non-toxic and greatly extend the pH range over which Lactobacillus bulgaricus activity is observed. The use of Tween 40, which is an excellent non-toxic source for oleic acid, nullified the need of biotin in culture media used for lactic acid bacteria.

Utilizing a modified Kirchner medium, Dubos and David (1946) demonstrated that the addition of Tween 80, up to an optimal level of 0.1 percent, greatly enhanced the rate and abundance of growth of tubercle bacilli. The inhibitory effect against small inocula has been found to be due to small amounts of unesterified oleic acid present in the original Tween 80 or formed as a hydrolytic product by the organisms. Extraction of this unesterified fatty acid or its removal from the field of action by "complex" formation with such substances as serum albumin, allows successful growth from inocula of two or three cells.

Dubos, Davis, Middlebrook, and Pierce (1946) reported that the tubercle bacilli grown in a submerged liquid culture in the presence of Tween 80 retained their morphologic and staining characteristics. This has been true even in cultures maintained for over a year with repeated transfers in liquid media containing this product. Return of these cultures at any time to standard media causes reversion to a granular type of growth.

Since the beginning of the twentieth century, a variety of media has been suggested for demonstrating the presence of fat-splitting microorganisms. In most instances, the proposed media employed one of the following means for detecting changes in the fat: (a) Zones of precipitated fatty acids adjacent to the bacterial colonies, Eijkmann (1901); (b) indicators such as litmus and brom phenol blue for noting changes in acidity, Buchanan (1921), Waksman and Daviston (1926), Sayer, Rahn, and Farrand (1908), and Jensen and Grettie (1933); (c) production of colored soaps thru the use of copper sulfate and other metallic salts uniting with free fatty acids, Carnot and Mauban (1918), and Berry (1933); or (d) specific indicators such as Nile-blue sulfate, for free fatty acids, Turner (1929), Hussong (1932), Smith (1907), and Eisenberg (1941). An alcoholic solution of Spirit blue for free fatty acids was used by Starr (1941).

Jenson and Grettie (1937) made a comprehensive study of the above media, except Eijkmann's medium, prior to 1937 and though unable to find one entirely satisfactory, they reported that Turner's medium in which Nile-blue sulfate was used, was superior

to all other media investigated by them.

Considerable controversy has arisen regarding the kind of fat which should be utilized in preparing the fat emulsion employed in the medium. Turner (1929) employed cotton seed oil. Jenson and Grettie (1937) who recommended and used coconut oil thought that cotton seed oil, because of its high oxygen content, inhibited the growth of many microorganisms. On the other hand, Hammer and Collins (1934) stated that butter fat, beef tallow, cotton seed oil and many other natural fats may be used with equally satisfactory results.

EXPERIMENTAL MATERIALS

It was the purpose of this research to devise a better technique for both demonstrating lipolytic bacteria and for making plate counts of total as well as lipolytic bacteria in various materials.

TALLOW

Fresh beef fat was secured from the Animal Husbandry Department, Kansas State College, ground, rendered, strained, placed into small flasks, and autoclaved at fifteen pounds pressure for a period of thirty minutes.

SURFACE-ACTIVE AGENTS

The surface-active agents were secured from Dr. T. H. Lord. Tables 1 and 2 list the trade name, manufacturer, class and formulae, uses, remarks, and approximate original concentration of the agents used in this investigation.

CULTURES

Ten of the lipolytic organisms were isolated from dairy products, soils, and chicken and cow manure. These samples were diluted with water and streaked on Eijkmann's fat plates. After five days of incubation, those colonies with white zones adjacent to them were transferred to nutrient agar slants. The remaining lipolytic cultures were secured from fat plates exposed to air by classes in General Microbiology and Agricultural Microbiology at Kansas State College.

Table 1. The anionic surface-agents employed.

Trade name	Manufacturer	Class and formula	Principal uses	Approx. conc. percent	Remarks
Arctic Syntex A	Colgate-Palmolive Peet	Sulfonated boro fatty acid.	Detergent	85	Detergency excellent. Not recommended with alkali or soaps. Not stable to strong acid or alkali.
Aersol OT	American Cyanamid	Dioctyl ester of sodium sulfosccinic acid.	Wetting Dispersing	100	Wax like, hydroscopic, water soluble to form gel at 50%. Good wetting agent but not stable.
Alkanol B	E. I. DuPont de Nemours	Alkyl naphthalene sodium sulfonate.	Wetting Dispersing	*	Used in textiles and metal cleaning.
Aresklene 400	Monosanto Chemical	Dibutyl phenylphenol sodium disulfonate.	Wetting Emulsifying	100	Dry hydroscopic powder, decomposes above 160° C. Water and polar solubility good.
Duponol LS Flakes	E. I. DuPont de Nemours	Sodium salt of "Ocenol" sulfate.	Detergent	*	Scouring agent.
Duponol WA Paste and Flakes	E. I. DuPont de Nemours	Sodium salt of crude lauryl.	Detergent Wetting Dispersing	*	Lathers well in cold water.
Igepon AP	General Dyestuff	Sodium sulfonate compound of the oleic acid ester of an aliphatic compound.	Detergent	*	Stable to acid, alkali, and hard water.
Igepon T	General Dyestuff	Sodium salt of an amide of oleic acid and methyl taurine.	Detergent	*	A tauride derivative
Santomerse 3	Monsanto Chemical	Alkyl aryl sodium sulfonate.	Detergent Wetting	100	Water soluble to the extent of 15%.

Table 1 (concl.)

Trade name	Manufacturer	Class and formula	Principal uses	Approx. conc. percent	Remarks
Tergitol 4	Carbide and Carbon Chemical	Sodium sulfate derivative of 7-ethyl-2-methyl-undecanol-4.	Wetting Emulsifying	25	Used for emulsion work where salt content is between 1 and 10% of acid content of 3-5%.
Tergitol 7	Carbide and Carbon Chemical	Sodium sulfate derivative of 3,9,-diethyl tridecanol-6.	Wetting Emulsifying	25	Used where the electrolyte is below 1%. Textiles-hypochlorite bleaching or emulsifier.
Tergitol 08	Carbide and Carbon Chemical	Sodium sulfate derivative of 2-ethyl hexanol-1.	Wetting Emulsifying	38	Very stable to high electrolyte. Mercerizing penetrant.
Triton W 30	Rohm and Haas	Sodium salt of alkylated aryl ether sulphate.	Wetting Penetrating	18	Contains 18% isopropanol. Anionic liquid leveling agent for waxes, polishes, textiles, and rubber compounding.
Victawet 58-B	Victor Chemical	Phosphorated capryl alcohol.	Wetting	70	$Na_5 R_5 (P_3O_{10})_2$: R = Capryl

*In those cases where it was impossible to ascertain the percentage of active material, it was assumed that the commercial samples were 100% active.

Table 2. The non-ionic surface-active agents employed.

Trade name	Manufacturer	Class and formula	Principal uses	Approx. conc. percent	Remarks
Carbowax 1500 dioleate	Glyco Products	Oleic acid ester of a polymerized poly-ethylene glycol.	Dispersing	*	An oily wax used as a dispersing agent for therapeutics.
Igepal CA	General Dyestuff	Polymerized ethylene oxide condensation product.	Detergent	100	Unusual property is that of losing its wetting ability when dried on fabric.
Polyethylene glycol 400 monooleate	Glyco Products	Oleic acid ester of a polymerized poly-ethylene glycol.	Dispersing	*	
Span 80	Atlas Powder	Sorbitan monooleate.	Emulsifying	97-100	More soluble than Span 40 or 60. w/O emulsifier.
Tween 80	Atlas Powder	Polyoxyethylene sorbitan monooleate.	Emulsifying	97-100	Oily liquid. Resembles Tween 20 in solubility.

*In those cases where it was impossible to ascertain the percentage of active material, it was assumed that the commercial samples were 100% active.

EXPERIMENT I

Determination of the Toxicity of Surface-Active Agents on Lipolytic Organisms

It was important that the surface-active agents employed in this work be non-toxic to the test organisms in the final concentration necessary to make a tallow-nutrient agar emulsion. Therefore, the toxicity of each surface-active agent had to be known. By subjecting the test organisms to a basic medium containing various concentrations of surface-active agent, it was possible to determine the smallest amount of agent that was toxic to the different cultures.

Procedure. Standard tryptone broth, which consisted of:

Bacto-tryptone	10.0 gm
Bacto-beef extract	3.0 gm
Sodium chloride	5.0 gm
Distilled water	1000.0 ml

adjusted to pH 7.0, served as the basic medium. Ten-fold dilutions of the agents from one to one hundred (1-100) up to one to one million (1-1,000,000) in terms of the final medium, were used in this experiment. The broth was tubed and autoclaved at 15 pounds pressure for 20 minutes. Ten tryptone broth cultures of lipolytic stock cultures were prepared and incubated for 24 hours at 37° C. Each series of agent-tryptone broth, ranging from 1-100 to 1-1,000,000 agent to broth, was inoculated with a

different lipolytic culture by placing one drop of 24-hour nutrient broth culture in each tube. The tubes were then incubated at 37° C. for a period of 48 hours. After the time allotted for incubation, the effects on the growth of the lipolytic strains were studied. The appearance of growth constituted a positive reaction. In certain tubes, where the medium was turbid due to the surface-active agent, methylene blue stains were prepared to determine growth. The presence of enormous numbers of bacteria was considered as evidence of growth. All other reactions were considered negative.

Results. Tables 3 and 4 present the results of the toxicity test.

Table 3. The lowest dilution of anionic surface-active agents in tryptone broth allowing growth of test cultures.

Agent	Minimum dilution
Aerosol OT	1-100,000*
Alkanol B	1-10,000
Aresklene 400	1-10,000
Arctic Syntex A	1-10,000
Duponol LS Flakes	1-1,000
Duponol WA Paste and Flakes	1-10,000
Igepal CA	1-100
Igepon AP	1-1000
Igepon T	1-100
Santomerse 3	1-100,000
Tergitol 4	1-10,000
Tergitol 7	1-100,000
Tergitol 08	1-100
Triton W-30	1-100,000
Victawet 58-B	1-10,000

*Minimum dilution in which all ten of the lipolytic organisms tested grew.

Table 4. The lowest dilution of non-ionic surface-active agents in tryptone broth allowing growth of test cultures.

Agent	Minimum dilution
Carbowax 1500 Di-Oleate	1-100*
Igepal CA	1-100
Polyethylene Glycol 400 Monooleate	1-100
Span 80	1-100
Tween 80	1-100

*Minimum dilution in which all ten of the lipolytic organisms tested grew.

EXPERIMENT II

Testing of Ability of Surface-Active Agents to Produce Emulsions of Tallow

When one uses surface-active agents he must bear in mind that not all surface-active agents possess the same ability to lower surface tension. Some may be producers of good emulsions, while others may not prove to be as satisfactory. This experiment proposed to determine which of the agents would show potentialities as emulsifiers. A simple and quick way to estimate the emulsifying ability of any group of surface-active agents is to use a direct and visual method. Although this technique lacks

precision, it is accurate enough to provide the necessary information for later experiments.

Procedure. Ten-fold dilutions of anionic and non-ionic surface-active agents and water, ranging from 1-100 to 1-1,000,000 were prepared. Then 0.3 ml of tallow was added to 100 ml quantities of each dilution. These mixtures were heated to approximately 70° C., violently shaken by machine (at a rate of 275 to 285 oscillations per minute) for 2 minutes, and placed side by side on a table. By direct, visual comparison an evaluation of the emulsifying ability of these agents was determined by comparison with a control composed of only water and tallow emulsion. Plus signs were used to indicate the degree of emulsification; the larger the number of plus signs, the greater the capacity to produce an emulsion. A single plus sign indicates the emulsifying power of the surface-active agent at that dilution to be practically non-existent.

Results. The results of this experiment are presented in Tables 5 and 6 which lists the surface-active agents used in this experiment and indicates their potention emulsifying power at various concentrations.

Table 5. The potential emulsifying power of anionic surface-active agents at various concentrations.*

Surface-active agents	Concentration of the agents				
	0.01	0.001	0.0001	0.00001	0.000001
Arctic Syntex A	4	3	1	1	1
Aerosol OT	4	3	2	1	1
Alkanol B	2	1	1	1	1
Aresklene 400	4	2	1	1	1
Duponol LS Flakes	4	2	1	1	1
Duponol WA Paste and Flakes	4	2	1	1	1
Igepal CA	4	3	1	1	1
Igepon AP	3	2	1	1	1
Igepon T	4	3	1	1	1
Santomerse 3	4	2	1	1	1
Tergitol 4	3	2	1	1	1
Tergitol 7	4	3	2	1	1
Tergitol 08	4	3	1	1	1
Triton W-30	2	2	1	1	1
Victawet 58-B	3	2	1	1	1

*The emulsifying power is indicated in relative degrees, 4 indicating the greatest degree and one the least degree.

Table 6. The potential emulsifying power of non-ionic surface-active agents at various concentrations.*

Surface-active agents	Concentration of the agents				
	0.01	0.001	0.0001	0.0000.	0.000001
Carbowax 1500 Di-Oleate	4	4	2	1	1
Igepal CA	4	3	1	1	1
Polyethylene Glycol 400 Monooleate	4	3	2	1	1
Span 80	4	3	1	1	1
Tween 80	4	3	1	1	1

*The emulsifying power is indicated in relative degrees, 4 indicating the greatest degree and one the least degree.

EXPERIMENT III

Comparison of Methods for Producing a Suitable Emulsion

There are several types of emulsions depending on which of the phases is dispersed. Thus, if the tallow phase is dispersed as droplets in the nutrient agar phase, we have a tallow in nutrient agar, or a T/NA emulsion. In the T/NA type it will be noted that the tallow phase is suspended in nutrient agar or the nutrient agar completely surrounds the tallow in the continuous phase. A

good emulsion should last for twenty-four hours without separating or "creaming". Creaming is evidenced by an increase of the tallow phase in one portion of the whole emulsion at the expense of the tallow phase in the remaining portion. Creaming is not a complete breaking down of the emulsion, but it is the beginning.

Agitation plays a very important part in the production of emulsions, and it is essential when mixing both phases. It is necessary to produce very small particles so that they can be dispersed. Violent agitation accomplishes this easily.

The stability of the T/NA emulsion is greatly increased by the presence of nutrient agar which acts as a protective colloid, forming a protective coating on the dispersed fat particles. The nutrient agar changes to a gel at about 40° C. and thereby renders the emulsion permanent. Even with the use of surface-active agents and the protective colloid action of the nutrient agar, it is necessary to develop a technique to be used in producing a suitable emulsion.

Procedure. Part 1. To a warm dilution bottle containing one milliliter of Tween 80, 3.5 ml of melted tallow was slowly added. The bottle was violently shaken by hand during the addition of the tallow to insure a thorough mixing of the two phases. Melted nutrient agar (pH 7) was added to the mixture and the dilution bottle was thoroughly shaken again by hand. The final mixture was poured into "Osterizer" jar, sealed and blended by mechanical mixing for 10 minutes. The blended mixture was poured back into a clean dilution bottle, and the medium was sterilized for 20 minutes at

15 pounds steam pressure. The medium was allowed to cool to about 45° C. and then poured, in approximately 10 ml quantities, into chilled Petri dishes.

Part 2. The same procedure was repeated, except that most of the materials and equipment were sterilized before the emulsification was carried out. The surface-active agent was not sterilized because of the effect of heat and its lack of ability to support bacterial growth in its concentrated form.

Results. As a result of the procedure followed in Part 1., the emulsion after sterilizing was not stable. This lack of stability was considered to be due to the temperature of sterilization. There is an optimum temperature for the production and storage of an emulsion and raising this value has a tendency to break the emulsion.

By following the procedure outlined in Part 2., a good emulsion was formed that gave promising results when submitted to the action of lipolytic organisms.

EXPERIMENT IV

Determining the Optimum Amount of Tallow and Concentration of Surface-Active Agent Necessary to Demonstrate Lipolysis by Bacteria

It was shown in the earlier experiments that various concentrations of surface-active agents in nutrient agar would allow bacterial growth and that lipolysis might occur in a wide range of tallow concentrations. The next point to consider was the

optimum ratio between the surface-active agent, the tallow, and the nutrient agar that would give the best results when acted upon by lipolytic bacteria. It has been shown that surface-active agents at certain concentrations do inhibit bacterial growth. The goal of this experiment was to use the least amount of surface-active agent with the proper amount of tallow and still have an emulsion that would yield good results with lipolytic bacteria.

Procedure. Part 1. One ml of Tween 80 was placed in each of 10 sterile, warm dilution bottles. One ml of melted, sterile tallow was placed in the first, 2.0 ml in the second, etc., increasing the amount by one ml until the tenth dilution bottle contained 10 ml of tallow. The surface-active agent and the tallow were thoroughly mixed. To each bottle, 99 ml of sterile nutrient agar was added. The mixtures were blended in an "Osterizer" blender and the emulsions poured back into dilution bottles. (The small necks of these bottles aid in preventing the foam from being added to the Petri dishes when the plates are poured.) Ten ml quantities of the emulsion were then poured into chilled Petri dishes. After the plates of media hardened, they were streaked with lipolytic organisms and incubated at 37° C. for 3 to 5 days. The plates were checked at various intervals to determine the effect of the different amounts of tallow on the lipolytic action of the bacteria. The effect of the various amounts of tallow on the emulsions was noted after the plates were poured.

Results. It was found that in a 1-100 dilution of agent to nutrient agar, 4 ml of tallow produced a satisfactory emulsion

and demonstrated sharply the lipolytic activity of the organisms tested. It was also noted that the turbidity of the emulsion increased with increasing amounts of tallow—a factor which was taken into consideration in later experiments.

Procedure. Part 2. In this part of the experiment dilutions of 1-100, 1-500, and 1-1000 Tween 80 to nutrient agar were prepared by adding 4 ml of sterile tallow to the surface-active agent and then adding the sterile nutrient agar in amounts necessary to make the above dilutions. The mixtures were blended, poured back into dilution bottles, and subsequently poured in 10 ml quantities into chilled Petri dishes. When the media had hardened they were streaked with lipolytic organisms.

Results. Tween 80 in all three concentrations (1-100, 1-500, 1-1000) produced satisfactory emulsions and demonstrated lipolytic activity. However, the 1-1000 dilution not only demonstrated lipolysis but also showed a higher rate of lipolysis. Lipolytic activity was shown by the appearance of a zone of white precipitate around the colony. This zone increased in size as the lipolytic enzymes produced by the bacteria hydrolyzed more of the tallow. Non-lipolytic bacteria did not produce such a zone. This was proven by streaking Escherichia coli (ATCC 8677) on Tween 80 stablized tallow-nutrient agar emulsion medium and incubating the plates at 37° C. for 3 days.

EXPERIMENT V

Selecting the Surface-Active Agent to be Used in Stabilizing the Tallow-Nutrient Agar Medium

Surface-active agents that possessed good surface tension depressing ability and still were non-toxic at either 1-100 or 1-1000 dilutions were selected from Experiments I and II. The following were chosen: Carbowax 1500 Di-Oleate, Duponol LS Flakes, Igepal CA, Igepon T, Polyethylene Glycol 400 Monooleate, Span 80, and Tergitol 08. The purpose of this experiment was to determine which of the above agents, in a 1-1000* dilution, would produce a satisfactory emulsion with 4 ml of tallow and at the same time demonstrate the best lipolysis when the emulsion was acted upon by lipolytic organisms.

Procedure. The technique for producing the emulsion was the same as previously described (Experiment IV; Part 1.) except that 0.1 ml of the surface-active agent, with which 4 ml of sterile tallow were mixed, was added to approximately 99 ml of sterile nutrient agar. Petri plates were poured with the tallow emulsion medium, streaked with lipolytic cultures, and incubated at 37° C. for 5 days.

Results. When the plates were examined after 2 days' incubation, it was noticed that a good demonstration of lipolysis occurred almost equally as well with media containing Polyethylene

*The 1-1000 dilution refers to the dilution of surface-active agent to nutrient agar.

Glycol 400 Monooleate, Span 80, or Tergitol 08. Lipolysis was slight in Igepon T medium but did not occur in media containing Carbowax 1500 Di-Oleate, Duponol LS Flakes, or Igepal CA. The emulsions produced by Carbowax 1500 Di-Oleate and Duponol LS Flakes were unsatisfactory.

Examination of the plates after 4 and 5 days' incubation indicated that lipolysis was more pronounced in the presence of Polyethylene Glycol 400 Monooleate, Span 80, Tergitol 08, and Igepon T. However, the lipolysis existing in Igepon T medium was not sufficient to warrant consideration.

EXPERIMENT VI

The Effect of Lowering the Concentration of the Tallow in the Tallow-Nutrient Agar Medium

It was noticed that emulsions prepared with 4 ml of tallow were very turbid and thus prevented recognition of sub-surface bacterial colonies when the medium was used for making plate counts. To minimize the inevitable turbidity in the tallow-nutrient agar emulsion medium, this experiment was devised using lower concentrations of tallow in preparing the emulsion medium.

Procedure. Emulsions were prepared, as previously described (Experiment V), using the surface-active agents Polyethylene Glycol 400 Monooleate, Span 80, and Tergitol 08 except that various concentrations of tallow (1 ml, 2 ml, and 3 ml) were used. Plates

were poured with the media, streaked with lipolytic organisms, and incubated at 37° C. for 5 days.

Results. After two days' incubation it was noticed that lipolysis was detected as expected. The turbidity of the media decreased with decreasing amounts of tallow in the media. The greater ease in detecting lipolysis in two days was a definite advantage over the technique used by Eijkmann. Demonstrating lipolysis by Eijkmann's method required approximately 4 to 5 days.

EXPERIMENT VII

Plate Count of Fat Splitters on Nutrient Agar Versus Tallow-Nutrient Agar Emulsion Medium

It has been shown that a fat-agar medium can be produced and used to determine lipolytic bacteria; but, can it be used to determine accurately the number of fat-splitting bacteria in materials? This experiment proposed to answer that question.

Procedure. A tube of nutrient broth was inoculated with lipolytic stock culture Number 15. It was incubated at 37° C. for 48 hours. Since the fat media containing Polyethylene Glycol 400 Monooleate, Span 80, or Tergitol 08 as the dispersing agent all produced very noticeable lipolytic zones around each bacterial colony; plate counts were made on all of these fat emulsion media and then compared to the count received using nutrient agar.

Ten-fold dilutions of lipolytic culture, ranging from 1-100 to 1-1,000,000 were prepared. Standard methods for the examination of milk were observed in making the dilutions. When the plates were poured, they were set on cold, fire bricks to hasten gel formation instead of using chilled plates. The plates were incubated at 37° C. for 3 days.

Results. Upon comparison of the plate count on nutrient agar to those received using the tallow-nutrient agar emulsion medium with Polyethylene Glycol 400 Monooleate and Tergitol 08 as dispersing agents, its results were similar.

Table 7 presents the results obtained.

Table 7. Comparison of plate counts using nutrient agar and tallow-nutrient agar emulsion media.

Medium	Plate count
Nutrient agar	85,000,000
Polyethylene Glycol 400 Monooleate tallow-nutrient agar medium	78,000,000
Tergitol 08 tallow-nutrient agar medium	72,000,000
Span 80 tallow-nutrient agar medium	43,000,000

EXPERIMENT VIII

Plate Counts on Various Materials Using Tallow-Nutrient Agar Emulsion Medium

Plate counts were made on various materials thought to contain lipolytic organisms. Standard methods for the examination of milk were used in making the dilutions. The results are indicated in Table 8.

Table 8. Plate counts of total aerobic and lipolytic bacteria in various materials.

Materials	Total aerobic plate count per gram	Lipolytic plate count per gram
Soil 1 (sandy)	7,600,000	2,400,000
Soil 2 (clay)	4,760,000	640,000
Soil 3 (under a pine tree)	8,800,000	700,000
Manure (goat)	170,000,000	50,000,000
Manure (horse)	590,000,000	80,000,000
Manure (cow)	640,000,000	60,000,000
Manure (chicken)	800,000,000	200,000,000
Butter	47,000	300
Peanut butter	100	100
French dressing	49,000	32,000
Wesson oil	1,000	500
Bacon fat	500	100

Table 8. (concl.)

Materials	Total aerobic plate count per gram	Lipolytic plate count per gram
Hamburger	1,200,000	200,000
Frozen fillet sole	220,000	66,000
Dried beef	16,000	12,000
Salami	11,000,000	8,000,000
Swiss cheese	1,800,000	50,000
Butter icing	3,600	900
Chocolate	160,000	53,000
Old cream	1,500,000	1,000,000
Coconut	98,000	92,000
Fat and vegetable shortening	6,000	1,000
Pie crust dough	350,000	320,000
Mayonnaise	500,000	200,000
Curried chicken	170,000	7,000

CONCLUSIONS

The main objective of this work was to devise a tallow-nutrient agar emulsion medium, using a synthetic surface-active agent, for demonstrating lipolytic bacteria and for making plate counts.

Before a surface-active agent could be employed to stabilize the tallow-nutrient agar emulsion medium, its toxicity towards lipolytic bacteria had to be known. It was found that Igepal CA, Igepon T, Tergitol 08, Carbowax 1500 Di-Oleate, Polyethylene Glycol 400 Monooleate, Span 80, and Tween 80 were non-toxic in a concentration of 1 to 100 (surface-active agent to tryptone broth). Duponol LS Flakes and Igepon AP supported growth of lipolytic bacteria when used in a concentration of 1 to 1000, but not in 1 to 100 concentration.

The results after testing the ability of surface-active agents to produce an emulsion of 0.3 ml of tallow in 100 ml of water showed that Arctic Syntex A, Aerosol OT, Igepal CA, Igepon T, Tergitol 08, Carbowax 1500 Di-Oleate, Polyethylene Glycol 400 Monooleate, Span 80, and Tween 80 had high emulsifying power.

When producing a tallow-nutrient agar emulsion, best results were obtained if the surface-active agents were thoroughly mixed with the sterile tallow in a warm bottle before adding the sterile nutrient agar and blending with an "Osterizer" for 10 minutes. Sterilization of the medium after blending leads to

an unstable emulsion. This is considered to be due to the temperature of sterilization.

Many surface-active agents are toxic to bacteria when present in high concentrations. It has been shown that a concentration of 1 to 1000 is the least amount of agent that can be used with 4 ml of tallow and still have an emulsion that will yield good results with lipolytic bacteria. This medium was too turbid, however, to be used for making plate counts. Decreasing the amount of tallow used to one ml makes the medium more transparent and satisfactory.

Of all the surface-active agents that proved to be non-toxic to lipolytic bacteria in either 1 to 100 or 1 to 1000 concentrations (surface-active agent to tryptone broth) and that proved to have high emulsifying power of tallow in water, the emulsion media produced by Polyethylene Glycol 400 Monooleate, Span 80, and Tergitol 08, when used in a 1 to 1000 concentration, gave the best results when streaked with lipolytic stock cultures and incubated at 37° C. for 2 days.

Comparison of the plate counts, on a lipolytic stock culture, received using tallow-nutrient agar emulsion medium, with Polyethylene Glycol 400 Monooleate, Span 80, and Tergitol 08 as dispersing agents, to the count received using plain nutrient agar showed that only the media containing Polyethylene Glycol 400 Monooleate and Tergitol 08 were comparable.

The tallow-nutrient agar emulsion medium was prepared as follows: One-tenth ml of Tergitol 08 was placed in a warm,

sterile, dilution bottle with one ml of sterile, melted tallow. The bottle was violently agitated by hand to insure thorough mixing. Approximately 99 ml of sterile, melted nutrient agar, adjusted to pH 7, is added and the mixture is placed in a sterile "Osterizer" jar, sealed, and blended mechanically for 10 minutes. The emulsion is transferred to another sterile, dilution bottle and allowed to cool to approximately 45° C. Ten ml quantities are poured into sterile, Petri dishes set on cool fire bricks. If the medium is to be used for "streak" plates the Petri dishes may be cooled beforehand and thus eliminate the use of fire bricks.

The medium is satisfactory for both "pour" and "streak" plates. The medium is easy to prepare and does not require the presence of an indicator to detect lipolytic bacteria. The presence of fat splitters is indicated by a white zone adjacent to the bacterial colonies. This zone is thought to be due to the presence of insoluble free fatty acids. Non-lipolytic bacteria do not show the presence of such a zone.

SUMMARY

1. The non-ionic surface-active agents in a 1 to 100 concentration were not toxic to the lipolytic organisms tested.

2. The ability of surface-active agents to produce emulsions of tallow varied. The non-ionic surface-active agents, employed in this work were good emulsifiers of tallow. A

1 to 1000 dilution of surface-active agent should be used in the medium.

3. Agitation plays a very important part in the production of emulsions, and it is essential when mixing both phases. The best tallow-nutrient agar emulsions were obtained by first thoroughly mixing the surface-active agent with the tallow.

4. High temperatures of sterilization produced an unstable tallow-nutrient agar emulsion.

5. Decreasing the amount of tallow used in the tallow-nutrient agar emulsion decreased the turbidity of the medium.

6. Lipolysis was detected in the medium by the presence of a white zone adjacent to the bacterial colony, while non-lipolytic bacteria did not produce such a zone. Polyethylene Glycol 400 Monooleate, Span 80, and Tergitol 08 equally demonstrated lipolysis by the test organisms after 2 days' incubation at 37° C.

7. Polyethylene Glycol 400 Monooleate and Tergitol 08 stabilized tallow-nutrient agar medium gave similar plate counts on a pure culture of lipolytic organisms when compared to the count using plain nutrient agar.

8. Tergitol 08, an anionic surface-active agent, did not inhibit lipolytic activity of the organisms tested.

9. The tallow-nutrient agar medium stabilized with Tergitol 08 is satisfactory for both "pour" and "streak" plates.

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USE OF STABILIZED TALLOW-NUTRIENT AGAR EMULSION MEDIUM IN STUDYING LIPOLYTIC BACTERIA

by

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It was important that the surface-active agents employed in this work be non-toxic to the test organisms in the final concentration necessary to make a tallow-nutrient agar emulsion medium. Therefore, the toxicity of each surface-active agent had to be known. By subjecting the test organisms to tryptone broth containing concentrations of surface-active agent varying from 1-100 to 1-1,000,000, it was possible to determine the smallest amount of agent that was toxic to the different cultures. It was found that Igepal CA, Igepon T, Tergitol 08, Carbowax 1500 Di-oleate, Polyethylene Glycol Monooleate, Span 80, and Tween 80 were non-toxic in a concentration of 1-100. Duponol L.S. Flakes, and Igepon AP supported growth in a concentration of 1-1000, but not in 1 to 100 concentration.

Some surface-active agents are good producers of emulsions, while others may not prove to be as satisfactory. To test the emulsifying power of the agents employed in this work, ten-fold dilutions of the agents and water, ranging from 1-100 to 1-1,000,000 were prepared. Then 0.3 ml of tallow was added to 100 ml quantities of each dilution. These mixtures were heated to approximately 70° C., violently shaken by machine for 2 minutes, and placed side by side on a table. By direct visual comparison with a control composed of only water and tallow emulsion, it was found that Arctic Syntex A, Aerosol OT, Igepal CA, Igepon T, Tergitol 08, Carbowax 1500 Di-oleate, Polyethylene Glycol 400 Monooleate, Span 80, and Tween 80 had high emulsifying power.

When producing a tallow-nutrient agar emulsion, best results

were obtained when the surface-active agent was thoroughly mixed with the sterile tallow in a warm dilution bottle before adding the sterile nutrient agar and blending with an "Osterizer" for 10 minutes. Sterilization of the medium after blending leads to an unstable emulsion. This was considered to be due to the high temperature of sterilization.

The next point considered was the optimum relationship between the surface-active agent, tallow and nutrient agar that would give the best results when acted upon by lipolytic bacteria.

One ml of Tween 80 was placed in each of 10 sterile, warm dilution bottles. One ml of melted sterile tallow was placed in the first, 2.0 ml in the second, etc., increasing the amount by one ml until the tenth dilution bottle contained 10 ml of tallow. The surface-active agent and the tallow were thoroughly mixed. To each bottle, 99 ml of sterile nutrient agar was added. The mixtures were blended and the emulsions poured back into sterile dilution bottles. Ten ml quantities of the emulsions were then poured into chilled Petri dishes. After the plates of media hardened, they were streaked with lipolytic organisms and incubated at 37° C. for 3-5 days.

It was determined that the use of 4 ml of tallow in the medium produced a satisfactory emulsion and demonstrated sharply the lipolytic activity of the organisms tested.

After preparing emulsions containing Tween 80 in concentrations of 1-100, 1-500, and 1-1000 (agent to nutrient agar) and 4 ml of tallow, streaking with lipolytic organisms, incubating at 37° C.

for 3-5 days, results showed that Tween 80 in all three concentrations produced satisfactory emulsions and demonstrated lipolytic activity. However, lipolysis was first detected in the 1-1000 dilution.

Lipolytic activity is shown by the appearance of a white zone adjacent to the bacterial colony. This zone increased in size as the lipolytic enzymes produced by the bacteria hydrolyzed more of the tallow.

Surface-active agents that possessed good surface tension depressing ability and still non-toxic at either 1-100 or 1-1000 dilution were selected and emulsions prepared. Plates were poured, streaked with lipolytic organisms, and incubated at 37° C.

When the plates were examined in 2 days, it was noticed that a good demonstration of lipolysis occurred almost equally as well with media containing Polyethylene Glycol 400 Monooleate, Span 80, or Tergitol 08. Lipolysis was slight in Igepon T medium but did not occur in media containing Carbowax 1500 Di-oleate, Duponol L.S. Flakes, or Igepal CA. The emulsions produced by Carbowax 1500 Di-oleate and Duponol L.S. Flakes were unsatisfactory.

Examination of the plates after 4 and 5 days' incubation indicated that lipolysis was more pronounced in the presence of Polyethylene Glycol 400 Monooleate, Span 80, Tergitol 08, and Igepon T. However, the lipolysis existing in Igepon T medium was not sufficient to warrant consideration.

It was noticed that emulsions prepared with 4 ml of tallow were very turbid and thus prevented recognition of sub-surface

bacterial colonies when the medium was used for making plate counts. To minimize the inevitable turbidity in the tallow-nutrient agar emulsion medium, emulsions were prepared, using the surface-active agents Polyethylene Glycol 400 Monooleate, Span 80, and Tergitol 08 in a concentration of 1-1000 and various concentrations of tallow (1 ml, 2 ml, and 3 ml). Plates were poured with the media streaked with lipolytic organisms and incubated at 37° C. for 2 to 3 days.

After two days' incubation it was noticed that lipolysis was detected as expected. The turbidity of the media decreased with decreasing amounts of tallow in the media.

To prove that the tallow-nutrient agar emulsion medium could be used for "pour" plates, plate counts of a 48-hour broth culture of lipolytic organisms were made on nutrient agar and on the emulsion media containing Polyethylene Glycol 400 Monooleate, Span 80, and Tergitol 08 as the dispersing agent.

The plate counts on the emulsion media were compared to the count received on nutrient agar, and it was found that only the counts received with media containing Polyethylene Glycol 400 Monooleate and Tergitol 08 were similar to the count on nutrient agar.

As a final experiment total "aerobic" and "lipolytic" bacterial plate counts were made on 25 various materials thought to contain lipolytic bacteria. The tallow-nutrient agar medium used was stabilized with Tergitol 08 and the results were satisfactory.

Non-lipolytic bacteria did not show the presence of a white

zone adjacent to the bacterial colony when grown on the emulsion medium. This fact was proved by streaking Escherichia coli (ATCC 8677) on Tergitol 08 stabilized tallow-nutrient agar medium and incubating the plates at 37° C. for 3 days.

The stabilized tallow-nutrient agar emulsion medium is prepared as follows: One-tenth ml of Tergitol 08 is placed in a warm, sterile dilution bottle with one ml of sterile, melted tallow. The bottle is violently agitated by hand to insure thorough mixing. Approximately 99 ml of sterile melted nutrient agar, adjusted to pH 7, is added, and the mixture is placed in a sterile "Osterizer" jar, sealed, and blended mechanically for 10 minutes. The emulsion is transferred to another sterile, dilution bottle and allowed to cool in a water bath to approximately 45° C. Ten ml quantities are poured into sterile Petri dishes set on cool fire bricks. If the medium is to be used for "streak" plates the Petri dishes may be cooled beforehand and thus eliminate the use of fire bricks.